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**PREPARATION OF CHEMICALLY MODIFIED
BEAD CELLULOSE RESINS AND THEIR
APPLICATION TO PROTEIN PURIFICATION**

A thesis presented in partial fulfilment of the requirements for the degree of
Doctor of Philosophy in Biochemistry at Massey University

Simon Christopher Burton

1995

ERRATA

- Page 39 : the legend for Table 2.7 (line 7) should refer to Table 2.6, not 2.7.
- Page 55 : insert "+ 1 ml ethanol" (in paragraph 5), to dissolve ligands.
- Page 56 : line 5 of paragraph 2 should read "0.7-1.0 ml/g".
- Page 61 : the activation level should be "0.093 mMoles/g" in line 1, paragraph 2.
- Page 72 : swap titration figures of AGE cysteamine (rows 6 and 7).
- Page 118 : the figure caption should be "...PPA (59%)".
- Page 120 : delete "for", line 7, paragraph 1.
- Page 129 : "mMoles/g dry" should read "0.043 mMoles/g", line 1, paragraph 1.
- Page 139 : change the legend labels for AMP to "(D)" and APIMID to "(C)".
- Page 153 : change Figure 6.10 to Figure 6.11, lines 5 and 9, paragraph 2.
- Page 161 : change "Figure 5.1" to "Figure 5.3", paragraph 1, line 4.
- Page 164 : delete "and eluted", paragraph 1, line 4.
- Page 169 : the legend's second sentence should read "pH 5.2 and 7.5 respectively".
- Page 171 : the percentage should be 50-70% paragraph 2, line 3.
- Page 171 : change "Figure 7.6" to "Figure 7.5", paragraphs 1 and 2.
- Page 180 : the vinyl pyridine figure should be "\$7.60".

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ABSTRACT

A bead cellulose matrix, Perloza™, was chemically modified to prepare inexpensive resins for chromatography. Conventional and novel resins were produced. Adsorption and elution methods suitable for industrial chromatography were developed. An agarose matrix, Sepharose™, was used for comparison.

Matrix activation with carbonyldiimidazole (CDI) was optimised for Sepharose and Perloza. Improved, reliable performance was obtained using column solvent exchange, with an imidazole tracer. Substitution efficiency of 75-98% was obtained for aminoacyl ligands/spacer arms by minimising water content. The aqueous carboxymethylation level obtained for Perloza was 0.3-0.4 mMoles/g dry. This was increased to 1.3-2 mMoles/g dry, using 75-80% DMSO solvated Perloza. Epichlorohydrin and bisepoxide activation levels (+/- organic solvents) were low.

Etherification of Perloza with allyl bromide or allyl glycidyl ether resulted in high **allylation** levels (> 1.50 mMoles/g), even in aqueous media. Matrix allyl groups were reacted with bromine water or aqueous N-bromosuccinimide, to produce (predominantly) bromohydroxypropyl groups. Subsequent attachment of amine and thiol ligands, by nucleophilic **substitution**, was simple and efficient.

Allyl matrices were also used for free radical **addition** of sulphite and various thiols (mercaptoethanol, mercaptoacids, glutathione). Efficient addition was found without thermal or chemical catalysis. Addition of mercaptoacetic acid followed by carboxylate titration was the preferred measure of (allyl) activation level. Addition of several other thiols occurred at 60°C.

The usefulness of allyl chemistries was exemplified by preparation of **ion exchange** resins. Their physical and chromatographic properties compared favourably with commercial resins. They combined good laboratory performance with high flow rates and simple, cheap preparation suited to large scale use.

Mixed mode resins were prepared from CDI and allyl matrices. These contained charged (secondary amine or carboxylate) and hydrophobic (alkyl spacer arm and/or ligand) groups. The milk clotting enzyme **chymosin**, was adsorbed to these resins at high and low ionic strength. Near homogeneous chymosin was eluted by a pH change, which induced electrostatic repulsion. Alkyl carboxylate resins were preferred. They were simple to prepare, use and regenerate, despite the use of crude broths.

The presence of charged groups could cause non-specific adsorption, interference with target protein adsorption and greater fouling. Weak acid and base hydrophobic groups (e.g. pyridyl) were attached to matrices and titration confirmed that uncharged and charged forms were obtained in a pH range (5-9) suitable for protein chromatography. At low ligand density, the salt promoted hydrophobic adsorption properties of these resins (uncharged form) were similar to those of Phenyl Sepharose. At higher ligand density, retention was longer, eventually leading to adsorption independent of ionic strength. Complete elution was obtained by pH adjustment (to the partially ionised resin form). Chymosin was strongly adsorbed to uncharged pyridyl (**hydrophobic ionisable**) resins and rapidly eluted by a small pH change. High ligand density (strong adsorption) is favourable for large scale use because the ionic strength of feedstreams does not need to be adjusted prior to loading.

Strong adsorption to mixed mode and weakly ionisable resins was also found for **amylase**. Rapid elution (and significant purification) was again obtained by a small pH change. **Subtilisin** was adsorbed likewise by most hydrophobic ionisable resins and recovered efficiently at pH 5.2. However, subtilisin adsorption to mixed mode resins was comparatively weak, possibly reflecting the weaker hydrophobicity of subtilisin compared to amylase.

The adsorption of **catalase** on Phenyl Sepharose and (low ligand density) pyridyl Perloza was equivalent, at pH 7.5. Catalase was eluted by a pH change from the Perloza resin, whereas elution from Phenyl Sepharose required addition of ethylene glycol. This indicated that pyridyl Perloza resins would be useful for chromatography of very hydrophobic proteins.

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LIST OF ABBREVIATIONS

AB	allyl bromide
ACA	aminocaproic acid
AEBS	aminoethylbenzenesulphonamide
AGE	allyl glycidyl ether
AMB	aminomethylbenzimidazole
AMP	(aminomethyl)pyridine (2, 3 and 4; ortho, meta and para)
APIMID	aminopropylimidazole
APM	aminopropylmorpholine
APP	aminophenylpropanediol
APS	ammonium persulphate
AUFS	full scale absorbance
AVA	aminovaleric acid
BD	butadiene diepoxide
BDE	butanediol diglycidyl ether
BHP	bromohydroxypropyl
β -lac	β -lactoglobulin
BME	mercaptoethanol
BP	benzoyl peroxide
BSA	bovine serum albumin
CD	carboxydecyl
CDI	carbonyldiimidazole
CHPA	chlorohydroxyphenylacetic acid
CM	carboxymethyl
CMC	cyclohexylmorpholinoethylcarbodiimide
CNBr	cyanogen bromide
CPAD	10-carboxypropionylaminodecyl
CYS	cysteine
DAH	diaminohexane
DCC	dicyclohexylcarbodiimide
DEA	diethylamine
DEAE	diethylaminoethyl
DEAPA	diethylaminopropylamine
DMF	dimethylformamide
DMSO	dimethylsulphoxide
DVS	divinylsulphone
ECH	epichlorohydrin

EDC	ethyl dimethylaminopropylcarbodiimide
EEDQ	ethoxycarbonylethoxydihydroquinoline
GSH	glutathione
HETP	height equivalent to a theoretical plate
HIC	hydrophobic interaction chromatography
HIS	histidine
HPA	hydroxyphenylacetic acid
HPLC	high performance liquid chromatography
IDA	iminodiacetic acid
IEC	ion exchange chromatography
IgG	immunoglobulin G
IMAC	immobilised metal ion affinity chromatography
IS	ionic strength
LCC	liquid column chromatography
MAA	mercaptoacetic acid
MB	mercaptobenzimidazole
MBA	mercaptobutyric acid
MEP	mercaptoethylpyridine
MET	methionine
MHA	mercaptohexanoic acid
MIM	mercaptomethylimidazole (methimazole)
MP	(mercapto)pyridine (2 and 4; ortho and para)
MPA	mercaptopropionic acid
MSA	mercaptosuccinic acid
NBS	N-bromosuccinimide
NHBA	nitrohydroxybenzoic acid
PAGE	polyacrylamide gel electrophoresis
PALOL	phenylalaninol
PBA	phenylbutylamine
PEA	phenylethylamine
PHE	phenylalanine
PPA	phenylpropanolamine
Q	quaternary amino
QFF	Q Sepharose Fast Flow
RPC	reversed phase chromatography
SDS	sodium dodecyl sulphate
SEC	size exclusion chromatography
SHP	sulphohydroxypropyl

SP	sulphopropyl
SPPS	solid phase peptide synthesis
TBI	tetrabutylammonium iodide
TEOH	tetraethylammonium hydroxide
TMA	trimethylamine
tosyl	toluenesulphonyl
tresyl	trifluoroethanesulphonyl
TRP	tryptophan
WGAT	wheat germ aspartyl transcarbamoylase
XL	crosslinked

CHAPTER 1 INTRODUCTION

The incentive for research in protein chromatography

Chromatography is used in laboratories and industry for protein purification and/or analysis. It is used for separation of proteins from crude mixtures. It can also provide quantitative (total amount and purity) and qualitative (broad description of a protein, especially surface properties, on the basis of interactions) information. Several forms of protein chromatography are well established (Table 1.1). These techniques differ in their mode of interaction, specificity, capacity, cost and effect on protein stability.

Nevertheless the variety of proteins in nature is immense and new purification challenges continue to arise, especially in the growing biotechnology industry. In biotechnology downstream processing typically accounts for the majority of manufacturing costs (Dwyer, 1984; Asenjo, 1991) and protein chromatography is a significant component of this. Therefore new and improved separation methods are valuable for both laboratory and industrial applications. Methods with new or improved specificity, high capacity, low cost and maximum recovery of active protein will be particularly valuable. Few realistic resin options exist for large scale operations with small margins. The use of chromatography in such operations requires inexpensive but effective resins and simple separation methods. Simple methods of resin preparation would also be an advantage for laboratory users because of the cost and narrow range of commercial products.

Basic principles of chromatography

The principles of chromatography have been reported in detail (Yost et al., 1980; Horvath and Melander, 1983). Chromatography literally means colour writing. The name arises from its first reported use for separation of plant pigments by Tswett (1903). Chromatography is the separation of components of a mixture between two phases, one **mobile** the other **stationary**. Separation occurs by repeated sorption and desorption processes during passage of the mobile phase through the stationary phase. Components separate according to their distribution coefficients. The more strongly a molecule is adsorbed, the more it distributes to the solid phase and the slower it moves with respect to the mobile phase. The mobile phase can be a liquid or a gas. The stationary phase is a solid or a liquid and can be in the form of a **layer** or a **column**. The final displacement of a solute from the stationary phase is called **elution**. A solute may elute with an unchanged mobile phase (**isocratic** elution). Alternatively a steady or sudden change in the mobile phase may be required (**gradient** or **stepwise** elution respectively).

Table 1.1 : Established methods of protein chromatography

Chromatographic method	Basis of separation	Reference
size exclusion	molecular size sorting	Hagel, 1989
ion exchange	interaction between charged surfaces	Scopes, 1987
bioaffinity	specific biological recognition	Turkova, 1993
hydrophobic interaction	entropy driven attraction of hydrophobic surfaces	Ochoa, 1978
reversed phase	protein polarity	Hancock, 1984
thiophilic	electron donor acceptor interactions	Porath et al., 1985
dye ligand	a range of interactions including ionic, hydrophobic and structural	Scopes, 1987
immobilised metal affinity	coordination between chelated metal and surface His, Cys and Trp residues	Kagedal, 1989
hydroxyapatite	mixed ionic interactions with calcium and phosphate ions	Scopes, 1987
covalent	reversible covalent binding to Cys residues	Ryden and Carlsson, 1989

For preparative protein separations a liquid mobile phase is used and a solid stationary phase (resin), typically consisting of small particles packed in a column. This is called **liquid column chromatography (LCC)**.

LCC theory

LCC has been described in terms of theoretical plates by analogy to the plate term for levels of fractional distillation (Yost et al., 1980). In chromatography the plates are not real but represent an estimate of the number of partition events the solute undergoes during column passage. Resolution (of components) is proportional to plate number. The smaller the height of a plate, the more plates there are for a given column length. Therefore the term "**height equivalent to a theoretical plate**" (HETP) is used to describe the efficiency of a column. Sharp elution peaks are ideal for LCC because they equate to high resolution of sample components and small sample volumes. Some degree of band (peak) spreading is inevitable due to longitudinal diffusion and slow partitioning equilibria. Column performance is maximised (and peak spreading minimised) by a low HETP and a high plate number.

Peak width is also affected by connecting tube "dead" volume, mobile phase elution conditions and flow rate. Dead volume should be minimised and elution conditions used which minimise sample tailing. Slow flow rates exacerbate longitudinal diffusion effects and hence band spreading. Rapid flow rates restrict solute interaction with the solid phase, reducing separation potential. In between these extremes is an optimum flow rate which equates to maximum column efficiency. The optimum flow rate is related to the solid phase particle size (Scopes, 1987). The influence of flow rate is demonstrated by a plot of flow rate versus HETP. The resulting curve (Figure 1.1), called a van Deemter curve (original description by van Deemter, 1956), derives from the equation :

$$H = Av^{-1} + Bv + C$$

H = HETP, v = velocity, A is a diffusion factor, B is a measure of non-equilibration and C is a "column quality factor" (Scopes, 1987).

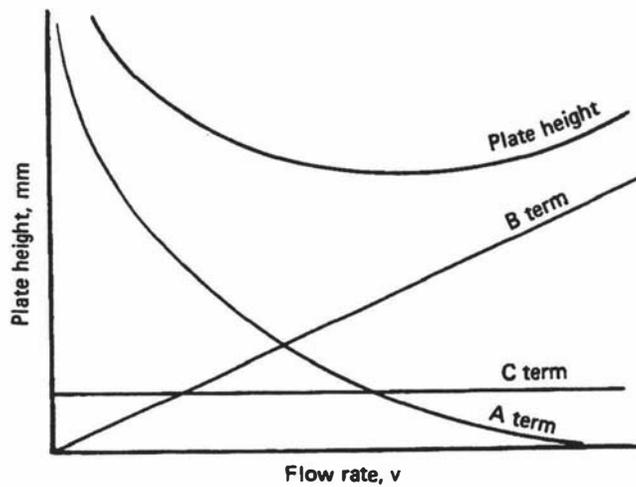
Classification of LCC

LCC has three basic forms : **size exclusion (SEC)**, **partition** and **adsorption**. **SEC** is a separation based on the size of the solute (protein) and the porosity of the **matrix**. Larger molecules pass through a smaller effective column volume and therefore elute from the column earlier. Protein capacities are small and operation times are long making this an expensive technique for large scale use. **Partition chromatography** is a

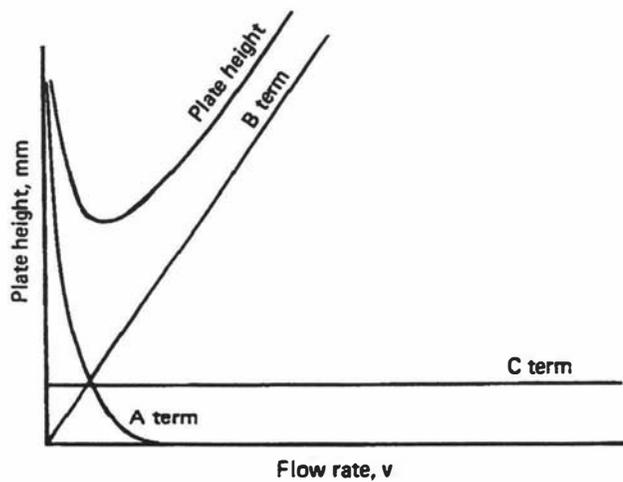
Figure 1.1 : The effect of flow rate on plate height (Scopes, 1987)

These diagrams demonstrate the narrower flow limits for optimal plate height of protein chromatography. This is due primarily to rapid reduction of equilibration efficiency for macromolecules with increasing flow rate. This effect can be lessened by use of non-porous resins or very small particles which allow rapid equilibrium. Materials of these types are therefore used for high resolution separations.

(A) Low molecular weight solutes



(B) High molecular weight solutes such as proteins



separation according to solute distribution between the mobile phase and the stationary phase (liquid coating of a solid backbone). It is usually used for analytical work.

Adsorption chromatography is a physical interaction between a solute and the matrix which retards or stops movement of the solute through the column. The sample is subsequently eluted by a change in the mobile phase. This is the most commonly used form of chromatography for medium to large scale protein purification. All the methods listed in Table 1.1, except SEC and reversed phase chromatography (RPC), are forms of adsorption chromatography. RPC can involve partition and adsorption effects. SEC is often complicated by non-specific adsorption.

A further distinction is made based on operating pressure. Traditionally LCC separations were at atmospheric pressure (**gravity flow**). Therefore performance was inherently variable, depending on the flow properties of the matrix, column shape and size, tubing diameter and height of operation (inlet to outlet). These variations can be compensated for by using low pressure pumps to standardise flow rates.

A significant improvement in performance has resulted from the introduction of high quality matrices and their use in high pressure automated systems. This is called **high performance liquid chromatography (HPLC)** and gives far superior resolution and reproducibility. Optimal separation (resolution) is obtained using small matrix particles (fast partition equilibria) and high flow rates (to minimise longitudinal diffusion). HPLC has been extensively used for analytical and laboratory scale protein purification (Hancock, 1984). Industrial preparative applications have been more limited, because of the expense of columns and poor recoveries using RPC, but it has been used for "polishing" of high value products (Dwyer, 1984).

Protein chromatography

Most chromatographic theory has been developed for isocratic elution of low molecular weight solutes. Proteins are large solutes, usually eluted by a stepwise or gradient change. Matrix exclusion effects are more important and the strength of interaction is variable (due to heterogeneity of matrix and protein binding sites). Protein adsorption can be described using an **apparent dissociation constant** (the average strength of interaction between the protein and the matrix). The dissociation constant determines whether a protein is unretarded, retarded or adsorbed. It also determines the efficiency of elution. Flow rate and matrix properties (size, shape and porosity of the particles) determine the rate and extent of equilibration and the shape of elution peaks.

The dissociation constant (K_p) is defined by the equation : $K_p = m.p / q$

where m is the concentration of effective binding sites, p is the concentration of protein in free solution and q is the concentration of adsorbed protein (Scopes, 1987). The m term does not include sterically restricted sites. The proportion of total protein (P) adsorbed at any instant is the partition coefficient (α).

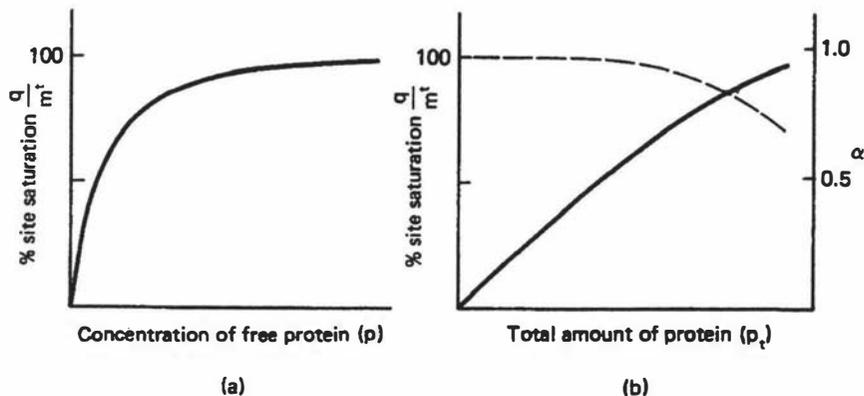
$$\alpha = q / P$$

Total concentration of protein	$(P) = p + q$
Total concentration of effective binding sites	$(M) = m + q$

From these equations a quadratic is derived : $P\alpha^2 - \alpha(M + P + K_p) + M = 0$

Solutions to the quadratic show that a K_p of 0.1 mM or less is required for useful adsorption ($\alpha \geq 0.8$). The α value is increased and adsorption favoured by increased accessible ligand density. An increase in total protein concentration increases the percentage binding site saturation and hence total adsorption. However adsorption efficiency (α) is reduced as the column approaches 100 % saturation (Figure 1.2).

Figure 1.2 : The effect of protein concentration on column efficiency (Scopes, 1987)



(a) The amount of protein adsorbed versus free protein concentration

(b) The protein adsorbed (solid line) and the partition coefficient α (dashed line) versus the total protein added.

Therefore laboratory and analytical columns are run at 20% or less of capacity for best performance (Scopes, 1987). The partition coefficient also influences the shape of elution peaks. Protein concentration is lower at the front and the rear of a band moving through a column. When protein concentration is lower, the α value is higher and this protein is retarded relative to the main band. This causes the front edge to be "pushed back" against the main band (sharpened). However the trailing edge is spread out causing peak tailing. Tailing can be compensated for by using a gradient elution. The continuous change of environment reduces α steadily and this counteracts the effect of decreasing protein concentration. If stepwise elution is used, a more dramatic change is required to produce a low α value for best efficiency.

Large scale materials and applications

Preparative protein separation

The preceding sections have focussed on high resolution techniques used for analytical chromatography. The same methods can be applied to preparative separation but are expensive and complicated to scale up. Narayanan (1994) listed high capacity, high mass transfer coefficient (rapid equilibration) and high flow rate as the most important characteristics of a resin for process scale operations, while cost was another important consideration. The choice of particle size is dependent on good throughput (flow rate) although resolution may be reduced (Golker, 1990). Larger particle resins (50 -500 μm diameter) have higher flow rates (Janson and Hedman, 1982), reducing the pressure requirement, and are much cheaper than high performance resins. Their flow properties (lower pressure drop) are more suitable for use in large columns.

Simple adsorption and step elution procedures are much easier to scale up than gradient elution (Asenjo and Patrick, 1990) although resolution may again be poorer. Stirred batch adsorption processes may be chosen (rather than column adsorption) for simplicity. Separation time, reagent consumption and product concentration become much more important factors when evaluating the desirability of a purification scheme. Large scale protein separation has been reviewed by Asenjo and Patrick (1990) and Wheelwright (1991). Large scale chromatography has been reviewed by Janson and Hedman (1982), Jones (1991), Jungbauer (1993) and Narayanan (1994). However most of these reviews focus on medium to high performance separations rather than economic separation of low value proteins.

Downstream processing

Downstream processing is the series of steps followed to obtain a final, purified product from an original, unprocessed source. Ho (1990) divided the steps involved into four : isolation, refolding, bulk purification and high resolution followed by concentration and formulation. Particulate matter is removed during isolation and chromatographic methods are not used before the third step. Chromatography already plays a significant part in downstream processing of valuable molecules, e.g. therapeutics (Janson and Hedman, 1982; Wheelwright, 1991). Major criteria of process selection are purity and the satisfaction of regulatory requirements. The value of many of these products is such that expensive and elaborate processing is economic. The research and development phase is restricted because of the importance of being first to market. Once a process is chosen and validated there is great resistance to change.

Therefore many downstream processes in place today are expensive, complex and of low efficiency. There has been little incentive to change from validated resins and methods despite the economic advantages possible with other resins, methods or adsorption designs. Few of these processes are truly large scale because the product is sold by the microgram rather than the kilogram. Process economics will become critical if the premium for these products is eroded by competition or legislation. Therefore techniques, including chromatography, developed for economic processing of lower value products such as industrial enzymes, are likely to have increasing application in the future.

The chromatographic methods used for high value products are designed to maximise purity rather than operation simplicity, capacity and cost. While a wide range of materials and methodologies are available for medium scale, high value processes, the alternatives are limited for large scale chromatography of low value products. Indeed the economics of a chromatography step in such processes, where product purity is a less overriding concern, are marginal or unfavourable. To be economic it may be necessary to use chromatography at an earlier stage (isolation) of downstream processing than is normally the case. Such use will require innovations of design, resins and methods.

If resins are to be used early in downstream processing, fouling is likely to be significant. therefore it is important that the resin can be regenerated and that it is chemically and physically stable to regeneration procedures. Most resins used in polishing steps are unsuitable for earlier use because of fragility, particularly of the ligand or its attachment chemistry, and prohibitive cost. In other cases complete removal of contaminants is difficult despite use of extreme regeneration conditions. Adequate regeneration is

important because the more cycles a resin can be used for, the less the contribution of the resin to overall process cost. Many existing ligands and attachment chemistries perform poorly on these criteria and there is a requirement for alternatives.

Preferred separation methods minimise pre-processing requirements and maximise simplicity of binding and elution. Therefore it is advantageous to use methods which do not require adjustment of ionic strength or pH prior to adsorption. Low ionic strength is a requirement of ion exchange adsorption and high ionic strength is typically required for HIC. Step elution methods, high capacity, high recovery and product concentration are other preferred process characteristics. Existing methods do not satisfy these criteria well except for some pseudo affinity examples using immobilised metal ion affinity chromatography (IMAC) or dye ligand chromatography.

A recent innovation for recovery of recombinant proteins is expression of the target protein as a fusion with a "purification tag". Examples include polyarginine (ion exchange chromatography), polycysteine (covalent chromatography), polyphenylalanine (hydrophobic interaction chromatography), polyhistidine (IMAC) and a variety of affinity tags. These methods were reviewed by Sassenfeld (1990).

Chromatographic/Adsorption design

The two traditional methods are stirred batch and axial bed adsorption. The former is simpler to operate and scale up, but is a lower resolution technique (Scopes, 1987). It is compatible with crude solutions containing particulate material such as cell debris. Therefore it has been favoured for large, relatively crude processes. Axial bed chromatography gives better separation and is therefore generally preferred, especially for the later stages of a purification. However, it requires removal of all particulate matter from samples before loading. It also requires careful packing of the bed to ensure even flow distribution and optimise the separation. Large axial beds can suffer severe flow restriction and particle deformation due to back pressure if soft gel matrices are used (Janson and Hedman, 1982; Asenjo and Patrick, 1990).

The standard axial bed chromatography design has recently been supplemented by new bed designs and media. Fluidised bed systems operate by batch-like adsorption to a "randomly suspended" resin using upward flow (Burns and Graves, 1985). Flow is reversed for washing and elution (from a settled bed). Greater separation is achieved by adsorption to an evenly suspended resin. In this case the upward flow does not disturb the "expanded bed" structure and this structure is retained when flow is reversed and the bed settles. The advantage of using an expanded or fluidised bed is that it allows free

passage of particulate matter, which would plug a fixed bed. The latter design is especially promising because it can be used earlier in downstream processing, bypassing clarification/filtration steps, while retaining the adsorption performance of a fixed bed (Chase and Draeger, 1992). New "densified" matrices (e.g. Pharmacia's Streamline™ quartz "doped" agarose beads) have allowed formation of stable expanded beds at much higher flow rates than can be used for conventional matrices. One-step separation of a recombinant protein without clarification has been reported (Hansson et al., 1994) using Streamline™.

Another innovation is radial flow chromatography. This is a packed bed procedure but flow is at right angles to the (vertical) column axis. The horizontal flow alleviates pressure and flow problems associated with axial flow. Therefore it is claimed to have processing time advantages for large scale use (Saxena, 1994). Other methods have replaced fine resin particles with large beads (Grandics et al., 1994), single unit precast gels (Frechet et al., 1994) or with membrane stacks (Langlotz and Kroner, 1992; Heath and Belfort, 1992). The latter have mass transfer advantages over particles because diffusion is not required for contact between the "chromatographic" surface and the target compound and solutions can be applied without clarification.

Matrices

There is considerable variation between commercial resins, including chemical structure and particle size, shape, porosity and rigidity. The solute equilibration rate and significance of non specific interactions vary with the choice of matrix. Matrix volume may change when the mobile phase is altered and ligand distribution may be heterogeneous. Because high resolution is required for laboratory separations, preferred matrices are typically those of small particle diameter. However the applicability of these resins to large scale use is limited by cost and pressure requirements. The properties of an ideal low pressure matrix (Roe, 1989; Haggarty et al., 1990) are:

- mechanical stability/rigidity (stirred batch or column modes)
- volume stability in aqueous, organic and mixed environments
- sufficient chemically reactive groups, usually hydroxyl
- surface hydrophilicity and minimal non-specific interactions
- uniform spherical particles with narrow particle size range
- sufficient porosity to allow rapid equilibration over an extensive surface area
- stability to conditions of ligand coupling, chromatography and sanitation
- low cost, high capacity and plentiful supply

Compromises cannot be avoided. Measures to increase particle rigidity (e.g. crosslinking) reduce porosity and capacity. Columns of larger diameter particles have lower back pressures and higher flow rates but column equilibration is less efficient resulting in larger elution volumes and poorer resolution. Many matrices perform well for most properties but are limited by high cost for large scale use. A new preferred characteristic is high density and hence settling rate, to allow optimal performance in expanded bed adsorption procedures.

The most extensively used matrix for research is crosslinked Sepharose™, a beaded agarose (Ochoa, 1978; Jones, 1991). Highly crosslinked beads have reasonable flow rates and can be scaled up to a moderate size, but are costly (e.g. the catalogue price of Sepharose Fast Flow™ ion exchange resins is US\$500/litre). Two other natural polymers, dextran and cellulose, have been used less because of poor rigidity (dextran) and irregular particle shape and low porosity (cellulose). Many well performed synthetic polymer matrices are available but these are expensive. Superior beaded cellulose matrices have been described (Determann, 1969; Peska et al., 1976; Kuga, 1980; Amicon, 1984; Schwidop et al., 1990) which are useful for protein chromatography. One of these beaded celluloses, **Perloza™**, was chosen for use in these studies.

Perloza bead cellulose

Perloza is a spherical (beaded) regenerated cellulose produced by a "Thermal-Sol-Gel-Transition" process (Stamberg, 1988). Cellulose xanthate solution is dispersed in an immiscible organic solvent, shaped into a sphere by droplet formation, and solidified by an increase in temperature. Decomposition of xanthate groups occurs during solidification and is completed by alkaline hydrolysis. The resulting cellulose beads are hydrophilic, spherical particles with minimal levels of ionic groups. Porosity is excellent (molecular weight cut-off values up to 500,000 Da for dextrans). Several grades of differing particle size and porosity are commercially available (underivatized prices between US\$ 20 and 60 /litre). Perloza also has excellent mechanical rigidity (Stamberg, 1988). Resins prepared in our laboratory, from a fine grade (80-100 µm) of Perloza have been found to have similar properties of adsorption, elution, resolution and capacity to Sepharose equivalents. The properties of Perloza and its derivatives have been described extensively by Peska et al. (1976), Stamberg et al. (1982), Gemeiner et al. (1988), Stamberg (1988) and Gemeiner et al. (1989).

Hydrophobic interaction chromatography

Three of the separation methods listed in Table 1.1 are grouped together because adsorption is enhanced by addition of salts to the mobile phase. Salt promoted adsorption has been reviewed by Porath (1986 and 1990). For immobilised metal affinity chromatography (IMAC) the main purpose of salt addition is to prevent non-specific ionic interactions. Some salts also promote adsorption while others can weaken binding causing elution (Kagedal, 1989). Thiophilic chromatography contains many elements of **hydrophobic interaction chromatography (HIC)** and can be considered as a specialised form of HIC. Protein adsorption to both forms is enhanced by high concentrations of suitable salts. Most proteins are not retained at low ionic strength and therefore can be eluted by a decreasing salt gradient (or step).

HIC is a separation based on interactions between proteins and a hydrophobic matrix. Binding strength is determined by mobile phase components, the native ability of the protein to interact with hydrophobic surfaces and matrix hydrophobicity (Ochoa, 1978). Native hydrophobicity is largely due to the presence of non-polar amino acid residues on the protein surface. These were once thought to exist exclusively in the protein interior but surface locations are now known to be a common feature (Klotz, 1970). Their location in relation to other non-polar residues is important. Lysozyme retention was modified by amino acid substitutions on the surface opposite the catalytic cleft but not elsewhere (Fausnaugh and Regnier, 1986). Internal groups can also have an influence, the degree of which depends upon structural flexibility. Proteins with numerous disulfide bridges are unlikely to have a significant contribution from internal groups (Mann, 1984).

Mobile phase effects

Adsorption capacity and efficiency is enhanced by increasing salt molarity according to the Hoffmeister series (Nishikawa and Bailon, 1975; Pahlman et al., 1977). This series lists common salts in order of salting out potential for proteins :

phosphate > sulphate > chloride > nitrate > thiocyanate.

Phosphates and sulphates are structure forming (**lyotropic**) salts. They increase the surface tension of water and decrease the number of water molecules available to interact with a protein. This promotes intermolecular and intramolecular hydrophobic bonding. Therefore lyotropic salts have been used in high concentrations to precipitate proteins. At lower concentrations they promote HIC adsorption. Chloride salts can also be used to promote adsorption but have a weaker effect than lyotropic salts.

Thiocyanate is an example of a structure breaking (**chaotropic**) salt. Chaotropic salts are used to dissolve protein precipitates. They weaken the forces maintaining internal protein structure and hence can cause denaturation. They reduce the surface tension of water, which reduces the energy requirement for protein solvation. This reduces the strength of the protein/matrix interaction and thus these salts have been used in HIC for elution of strongly adsorbed proteins (Neurath et al., 1975). However there is the associated risk of denaturation and activity loss. Polarity reducing agents (Laas, 1975; Strop et al., 1978), denaturation agents (Bigelis and Umbarger, 1975) and detergents (Caldwell et al., 1976) have also been used to elute proteins by weakening hydrophobic interactions. These agents, especially detergents, may cause protein denaturation.

Mobile phase effects on HIC have been reviewed by Arakawa and Narhi (1991).

The influence of the matrix on HIC

Unmodified hydrophobic polymers (e.g. polystyrene) can be used for HIC. The hydrophobicity of these matrices depends upon the monomer(s) used and is not readily modified after manufacture. Alternatively hydrophilic matrices, with covalently attached hydrophobic ligands, can be used. Ligands used contain an alkyl chain of varying length or an aromatic ring. The hydrophobicity of these materials can be altered by choice of :

ligand type (Ochoa, 1978; Rosengren et al., 1975)

ligand density (Rosengren et al., 1975; Jennissen and Heilmeyer, 1975)

attachment chemistry (Hjerten et al., 1974; Demiroglou and Jennissen, 1990)

base matrix (Ochoa, 1978)

Advantages of HIC

- (1) The binding mechanism of HIC is distinct from other methods (Table 1.1).
- (2) High capacities and good resolution can be obtained (Asenjo et al., 1991).
- (3) Sample preparation is simple because HIC is salt promoted. Typical sample preparation requires salt addition but not dialysis or dilution.
- (4) The salts used to promote adsorption stabilise proteins (Scopes, 1987).
- (5) HIC can be used as an alternative to ammonium sulphate precipitation (Scopes, 1987) and should be better suited to fractionation of complex mixtures.

Disadvantages of HIC

- (1) The separation mechanism is similar to that of salt precipitation (salting out) although additional specificity may be imparted by the ligand. The broad basis of interaction can result in poor specificity and capacity.
- (2) Resolution is often poor compared to other techniques because the α value changes slowly when using a decreasing salt gradient (Scopes, 1987). As a result peak tailing can be marked and elution volumes large.
- (3) When binding is strong, harsh elution conditions may be required increasing the potential for protein denaturation (Ochoa, 1978). Strong binding may also result in protein unfolding which may compromise function (Wu et al., 1986).
- (4) After use column restoration can be prolonged and expensive for strongly hydrophobic matrices.
- (5) For soft gel matrices, medium to large ligand substitutions can change basic matrix properties of swollen volume and flow rate. This also increases matrix exclusion effects (Laas, 1975) which will reduce the capacity for large proteins.
- (6) Reagent consumption (salts) will be significant for large scale use.

Potential solutions

Recombinant proteins can be expressed with a hydrophobic "purification tag" (Sassenfeld, 1990). Otherwise solutions to the problems described must come from modifications of the **mobile phase** or the **matrix**. The type and concentration of buffer salts, polarity reducing agents, denaturing agents and/or detergents can be changed. Affinity elution agents have proved successful in some cases (Yon, 1977). Temperature and pH can be manipulated to control the performance of HIC columns. A decrease in temperature weakens hydrophobic interactions, while the effect of pH changes varies from one protein to another (Ochoa, 1978). A change in pH will also affect the matrix if ionisable groups are present. Ideally the target protein can be recovered by step elution, using inexpensive load and elution buffers.

Variations of the stationary phase are potentially very useful for improving HIC performance. Ideally standard ligands which cover a range of hydrophobicities should be available (Ochoa, 1978; Hofstee, 1975; Hofstee and Otilio, 1978a). Ligands with varying alkyl chain lengths have been investigated but very few aromatic ligand variations have been used. Ligands with different substituents of the aromatic ring should have subtle variations of hydrophobicity. Also different protein specificities may exist between aromatic and aliphatic ligands (Hofstee, 1979; Hofstee and Otilio, 1978b).

An increase in ligand density can increase total protein adsorption but may reduce specific capacity by allowing a wider range of proteins to bind. The optimal ligand density will vary from one purification requirement to another. High ligand density can alter the porosity of soft matrices but this can be avoided by use of a more rigid matrix.

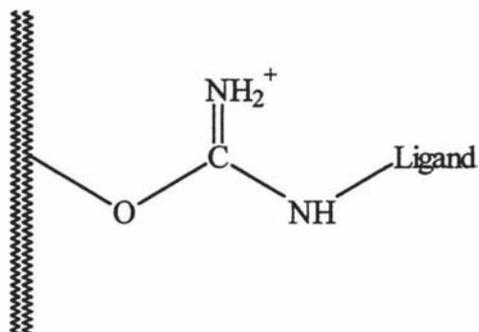
A variety of activation chemistries have been used for ligand attachment but many are unsuitable for HIC because charged groups are introduced and/or the linkage is susceptible to hydrolysis. These include the first HIC resins, prepared by the **cyanogen bromide** (CNBr) method (Er-el et al., 1972). For neutral HIC the linkage should be uncharged at pHs used for protein separation. If the chemistry involves charged intermediates these should be completely substituted or "blocked". If a spacer arm is included it should also be uncharged. The spacer arm can also make a significant contribution to binding, similar to affinity chromatography examples (O'Carra et al., 1973). Attachment by strong, neutral, covalent bonds (**ether**, Hjerten et al., 1974; **thioether**, Demiroglou and Jennissen, 1990) is preferred because these can withstand vigorous cleaning procedures..

Mixed mode HIC resins

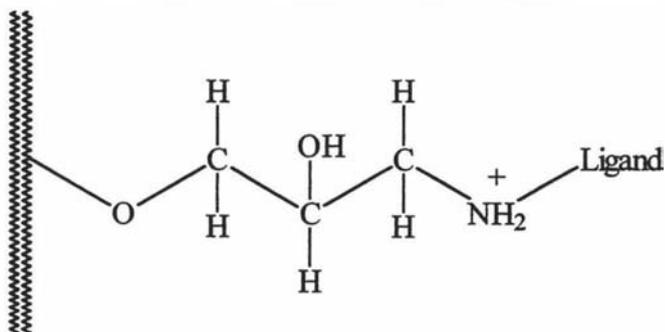
HIC performance may be improved by deliberate inclusion of charged groups when the ligand is attached. Matrices of this type are called mixed mode because they can interact with a protein by more than one mode. They can be produced in a constant 1:1 ligand to charged group ratio, if the chemistry used produces a charged linkage (Figure 1.3 a,b). Alternatively they may be produced by incomplete attachment of the ligand to matrix carboxyl or amino groups, analogous to resins described by Bischoff and McLaughlin (1984) for nucleic acid separation (Figure 1.3c). This approach allows variation (and control) of the charge to ligand ratio. Carbodiimide mediated condensation reactions could be used to attach other ionisable moieties (e.g. imidazole) to the carboxyl or amino groups. The strength of binding (compared to neutral adsorbents) may be increased or decreased depending on pH and ionic strength. As a result elution is more easily

Figure 1.3 : Activation methods for introduction of charged groups.

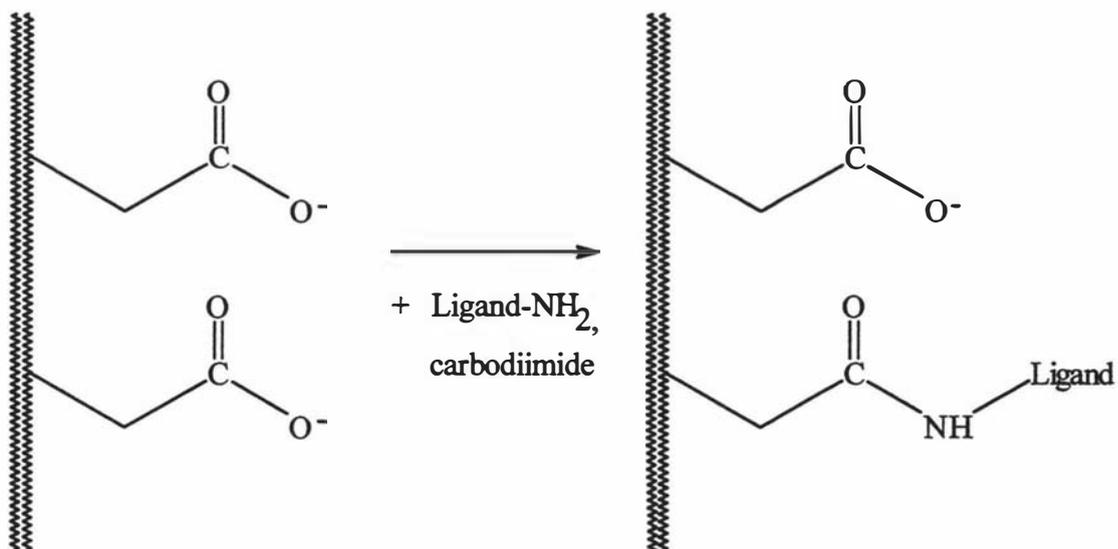
A : Amine ligand attachment to a CNBr activated matrix



B : Amine ligand attachment to an epichlorohydrin activated matrix



C : The result of incomplete carbodiimide reaction between an amine ligand and a carboxyl matrix



manipulated by mobile phase changes. This may be essential for the elution of undenatured protein (Yon and Simmonds, 1975). It may also allow use of resins containing high ligand densities and therefore having high protein capacities. A combination of interactions could allow very high capacities to be obtained without using large amounts of ammonium sulphate or similar salts. Zhuo et al. (1993) described adsorption of a recombinant protein phosphatase to a neutral HIC resin at low and high ionic strengths, but elution required inclusion of 50% ethylene glycol. If similar strong binding occurred to a mixed mode resin, elution might be obtained by a simple pH change (causing electrostatic repulsion). The presence of charged groups should also facilitate matrix cleaning and counteract any tendency of hydrophobic shrinkage of the matrix, allowing optimal flow rates and capacity.

Examples of mixed mode HIC

Substituted diaminoalkanes attached to CNBr activated Sepharose have been used for protein chromatography by Yon and coworkers. These matrices had CNBr derived isourea linkages (cationic) and terminal carboxyl groups (anionic). At neutral pH their overall charge was negative and the level of negative charge could be increased by raising the pH. The hydrophobic component was an eight or ten carbon alkane chain. The rationale for their use was that they could provide finer discrimination than conventional HIC matrices and reduce protein denaturation (Yon, 1972). Proteins which interacted strongly could be loaded at pH values which resulted in charge repulsion (to weaken binding). Proteins with weaker hydrophobic interactions were loaded at their isoelectric pH. Electrostatic attraction did not contribute to binding. Electrostatic repulsion was used to narrow the specificity of binding and assist elution. The resulting moderate binding strength translated to optimal purification and recovery.

Wheat germ aspartyl transcarbamoylase (WGAT) was separated using these principles (Yon and Simmonds, 1975). The enzyme bound strongly to dodecyl Sepharose (no terminal carboxyl group) but could not be eluted by pH, ionic strength or polarity adjustments. It was presumed that strong binding resulted in extensive denaturation. In contrast 10 carboxypropionylaminodecyl Sepharose (CPAD) also bound strongly but with lower capacity. Enzyme activity was readily recovered (up to 100%) by various elution methods. WGAT did not bind to pyromellitylaminodecyl Sepharose, a matrix with three carboxyl groups at the end of each alkyl chain (Yon, 1974). For this matrix electrostatic repulsion was presumed to outweigh hydrophobic attraction.

These matrices were also used for the fractionation of erythrocyte membrane proteins (Simmonds and Yon, 1976 and 1977). The principle mode of binding was hydrophobic attraction. This was demonstrated by the effect of varying the alkyl chain length and inclusion of a detergent (SDS) in the mobile phase. Electrostatic repulsion was again used for elution. The weakest adsorbing protein, glycophorin, was effectively purified (up to 90% purity). However **dodecyl** Sepharose gave the best glycophorin separation.

CPAD and **10 carboxydecyl** (CD) Sepharose proved amenable to biospecific elution of a variety of enzymes (Yon, 1977 and 1981; Kyprianou and Yon, 1982; Beadle et al., 1984). WGAT was purified 8000 fold in a single chromatographic step on 10 CD Sepharose by addition of its substrate carbamoyl phosphate (Yon, 1981).

Aromatic and aliphatic amines linked to CNBr activated Sepharose were used by Hofstee (1973a/b/c and 1975) for protein chromatography and immobilisation. Ligands of weaker hydrophobicity than CPAD and CD, and without terminal carboxyl groups, were used. These matrices had a positive charge (isourea group) at neutral pH. Because the ligands were less hydrophobic, binding of most proteins required a combination of electrostatic and hydrophobic interactions. Proteins were eluted by weakening one or both of these interactions but electrostatic repulsion effects were not used for elution. Chymotrypsin bound more strongly and a combination of high ionic strength and a polarity reducing agent was required to elute it from **phenylbutylamine** (PBA) Sepharose. Chymotrypsin was bound at low ionic strength despite electrostatic repulsion. Chymotrypsin was found to have a lower affinity for alkylamine ligands of similar hydrophobicity, implying a special affinity for PBA (Hofstee, 1973a/b). Negatively charged proteins bound strongly to alkylamine matrices. The effects of increasing alkyl chain length were investigated. A minimum carbon chain length of four was required for protein binding. Proteins could be eluted from **butylamine** matrices with 1 M NaCl. For **octylamine** matrices ethylene glycol was required for elution of bovine serum albumin (BSA) and lactoglobulin. A variety of enzymes were reversibly immobilised to butylamine and octylamine matrices (Hofstee, 1973c) and octylamine matrices bound all enzymes more tightly.

Aliphatic and aromatic amines attached to CNBr Sepharose were compared with their uncharged hydrazide equivalents by Jost et al. (1974). The presence of charged groups was essential for adsorption of ovalbumin and lactalbumin. BSA bound to both charged and uncharged matrices but was only readily desorbed (by 1 M NaCl) from charged matrices. Charged PBA matrices were used by Kasche et al. (1990) to facilitate elution (by electrostatic repulsion) of penicillin amidase. Charged alkylamine matrices were used

for endotoxin removal (Hou and Zaniewski, 1991). Charge was essential for high capacity when long chain (decyl) ligands were used.

A different approach was used by Sasaki et al. (1979 and 1982). An Amberlite ion exchange matrix (a hydrophobic polystyrene backbone with attached carboxyl groups) was used. Some proteins were retained by hydrophobic interactions within a narrow pH range (4-4.5). It was claimed that the matrix was uncharged in this pH range. An increase in pH resulted in elution, apparently due to electrostatic repulsion and/or weakened hydrophobic interactions.

Histidine and **histamine** ligands attached to epoxy activated matrices have been used for chromatography of various proteins, especially immunoglobulins (Amourache and Vijayalakshmi, 1984; Kanoun et al., 1986; El-Kak and Vijayalakshmi, 1991; El-Kak et al., 1992; Wu et al., 1992; Haupt and Vijayalakshmi, 1993; Hu and Do, 1993). These matrices had a mixture of ionisable groups and an imidazole group which can form electrostatic and hydrophobic interactions. Hu and Do demonstrated elution by a decreasing ammonium sulphate gradient for alcohol dehydrogenase. Otherwise proteins were bound at low ionic strength and eluted by an increase in ionic strength (NaCl), suggesting electrostatic interactions were predominant. The presence of a negative charge on histidyl Sepharose was shown to weaken immunoglobulin (IgG) binding and reduce purification efficiency (El-Kak and Vijayalakshmi, 1991). Immobilised histamine has also been used for pyrogen adsorption (Minobe et al., 1983).

Ngo and Khatter (1990) used resins containing substituted pyridyl groups (hydrophobic) and tertiary amine linkages for separation of IgG's. IgG was bound at low and high ionic strength whereas BSA only bound at low ionic strength. IgG was eluted by a pH reduction (usually to pH 3.5). The chromatographic behaviour was compared to that of **Protein A** and **Protein G** affinity matrices. It could also be argued that a combination of hydrophobic and ionic interactions resulted in tight adsorption while electrostatic repulsion caused elution. Chromatography was at pH 7.4, close to the isoelectric point of IgG. BSA will be oppositely charged to the resin at this pH, explaining binding at low ionic strength. Hydrophobic interaction of IgG should be favoured near its isoelectric point. Furuya (1983) used an aromatic ligand attached to Sepharose through a charged linkage (CNBr) and an aliphatic (six carbon) spacer arm for IgG separation. It was shown that IgG binding involved electrostatic and hydrophobic interactions.

The requirement for further investigation of mixed mode chromatography

Mixed mode chromatography has been described for a wide range of applications. It has proved useful, particularly for immunoglobulin fractionation. However complex chemistries and ligands were used in most cases and a variety of functional groups were attached to the resins. The resins would be impractical and expensive to prepare and their operation and interpretation of results could be complicated. For example, one of the resins used by Vijayalakshmi and coworkers was prepared by carbodiimide condensation of histidine with a resin. This method allows formation of oligo-HIS, in solution or attached to the resin, rather than the single HIS attachment described.

Furthermore, most work has used expensive matrices such as Sepharose and Eupergit C. Mixed mode chromatography has not been described on Perloza. The focus of the literature has been on specific separations rather than general principles and applications. The importance of adsorption independent of ionic strength has been largely ignored although it would be a considerable advantage for industrial applications. Therefore there is an opportunity to develop new mixed mode resins suitable for industrial use. These would be based on Perloza bead cellulose, using cheap, durable, uncomplicated chemistries and ligands and simple robust separation methods.

Research aims

The aims of this project are :

Development of cheap, efficient chemical methods for Perloza activation and ligand attachment, to complement the advantages of this matrix.

Use of these methods to prepare standard resin types. Preparation of uncharged and mixed mode HIC resins using novel aromatic ligands.

Measurement of physical and chromatographic properties of these resins and comparison with commercial resins.

Development of simple separation methods, suitable for scaling to industrial use.

Application of new resins and methods to purification of recombinant enzymes, especially chymosin and subtilisin. The development of one-step, high capacity purification schemes which require minimal preparation of crude enzymes.

CHAPTER 2 STANDARD ACTIVATION CHEMISTRIES

INTRODUCTION

Resins used for adsorption chromatography of proteins are composed of a polymeric matrix and covalently attached functional groups (ligands). For example covalent attachment of sulphonic acid groups to a neutral matrix produces a strong cation exchange resin. In principle, the full variety of adsorption resins can be produced, using a variety of ligands, without changing matrix or linkage chemistry. Adsorption is ideally due to interactions between a protein and ligand groups. In practice, proteins may also interact with the matrix and/or the covalent linkages by which the ligands are immobilised. The ideal linkage should usually be neutral and hydrophilic and always chemically stable. Chemical modification should not result in deterioration of any of the preferred matrix properties described in Chapter 1. Uncontrolled crosslinking and/or significant alteration of swollen volume should be avoided. The simplest and most economical method of ligand attachment is a direct reaction between the matrix and ligand but multiple step procedures are more common.

The preparation of ion exchange (IEC) resins for protein chromatography was first described by Sober and Peterson (1954). The most significant of these, carboxymethyl (CM) and diethylaminoethyl (DEAE) celluloses, were produced by one step, alkaline etherification of cellulose with halide reagents. The ether linkage is desirable because it is neutral, not significantly hydrophobic and is chemically stable. Preparation of HIC resins by one step etherification was described by Hjerten et al. (1974) using hydrophobic epoxide reagents.

Similar methods could not be applied to synthesis of affinity resins because suitable halide/epoxide affinity ligands were unavailable or impractical. Therefore affinity resin preparation has been a two or more step process. Matrices are modified to more reactive forms (activation) followed by attachment of suitable, especially amine, ligands. Descriptions of commonly used activation methods are available in many texts : Reactifs IBF (1983), Carlsson et al. (1989), Hermanson et al. (1992) and Turkova (1993). Two step methods have also been used to prepare supports for IEC (Peska et al., 1976), HIC (Er-el et al., 1972), IMAC (Porath et al., 1975a), thiophilic chromatography (Porath et al., 1985) and multiple step methods have been employed for enzyme immobilisation (Hermanson et al., 1992). They have also been used to functionalise matrices with thiol groups for covalent chromatography (Brocklehurst et al., 1973) or further reaction (Carlsson et al., 1989), and amine groups for further reaction (Matsumoto et al., 1980).

A wide variety of activation methods have been described for hydroxyl group containing matrices such as polysaccharides. All methods suffer from one or more disadvantages and only a few are practical for large scale use. The use of cyanogen bromide (CNBr) was described by Axen et al. (1967) for covalent immobilisation of peptides and proteins. It has been used widely for attachment of amine ligands. This method introduces a variety of reactive groups onto polysaccharides. Amines are thus attached through a variety of linkages including isourea, and these linkages are susceptible to hydrolysis, especially in acidic ($\text{pH} < 5$) or basic ($\text{pH} > 9$) media. This limits the possibilities for resin regeneration and sanitation. The isourea groups are charged below $\text{pH} 10$ (the range normally used for protein chromatography). The reagent is also highly toxic. It has not been used in these studies because of its toxicity, the low chemical stability of the ligand attachment and the introduction of charged groups (causing non-specific interactions). Despite these drawbacks it remains in widespread use.

Other methods for attaching amines eliminate or reduce the problems associated with the CNBr method by attachment through a neutral urethane linkage. Attachment of amine ligands to agarose activated by carbonyldiimidazole (CDI) was described by Bethell et al. (1979). The stability of the urethane linkage was superior to that of CNBr but leakage still occurred at pH extremes (Bethell et al., 1981b). Amine ligands have also been attached by urethane linkages using chloroformate reagents (Wilchek and Miron, 1982) and disuccinimidylcarbonate (Wilchek and Miron, 1985). The sensitivity of the urethane linkage to strong acid or alkali limits the options for resin regeneration and sanitation. All these reagents are expensive. They are moisture sensitive, necessitating anhydrous storage, and reactions must be carried out in anhydrous solvents such as acetone or dioxan. Costly solvent exchange to replace water is necessary for a water swollen matrix such as bead cellulose which is sensitive to vacuum drying (Gemeiner et al., 1989). The large requirement for organic solvents is a serious handicap (cost, hazard and disposal) for industrial scale use.

Amine ligands can also be attached to matrix carboxyl groups by a condensation reaction to form an amide bond. The requirements of such reactions are attachment of carboxyl groups to the matrix followed by their activation to a form which reacts readily with an amine. Carboxyl groups can be attached by carboxymethylation (Peterson and Sober, 1956) or by reaction of aminoalkyl carboxylic acids to activated (e.g. CDI) matrices. Reagents which have been used to activate carboxyl groups are water soluble carbodiimides (Cuatrecasas, 1970) and ethoxycarbonylethoxydihydroquinoline (EEDQ) (Boschetti et al., 1978). Activation and amine attachment has typically been carried out in a single step. A carbodiimide (dicyclohexylcarbodiimide, DCC) has also been used in

conjunction with N-hydroxysuccinimide to produce an active ester. This was then isolated and reacted with an amine in a second step (Cuatrecasas and Parikh, 1972). DCC requires organic solvents and water soluble carbodiimides are expensive. EEDQ is less expensive and can be solvated with ethanol water mixtures, which suggests its use could be economic. The amide bond stability is only moderate in the strong alkaline conditions preferred for resin sanitation. Incomplete reaction or hydrolysis of amides results in free carboxyl groups which confer ion exchange properties on the resin.

Stronger chemical linkages are obtained by use of sulphonyl chloride reagents : toluenesulphonyl (tosyl) or trifluoroethanesulphonyl (tresyl) chloride. Tosyl chloride is inexpensive and thiol or amine ligands can be attached to the activated matrix by strong thioether or secondary amine bonds (Nilsson and Mosbach, 1980). Tresyl chloride is more reactive (Nilsson and Mosbach, 1981) but extremely expensive. Another method was described by Ngo (1986) for attaching thiol and amine ligands through thioether and amine bonds. However the fluoromethylpyridinium toluenesulphonate reagent used is expensive. All these reagents are moisture sensitive and require anhydrous reaction conditions for activation. This is expected to restrict their industrial application.

A less costly method for amine attachment is by reductive amination of matrix aldehyde groups. The most common method for introducing aldehyde groups is periodate oxidation (Sanderson and Wilson, 1971). Neither activation nor reductive amination require organic solvent use, reagents are moderately priced and the amine linkage is stable. However oxidation can adversely affect matrix structure at high activation levels (Petrus et al., 1984; Peng et al., 1987). Alternative methods, described by Petrus et al. (1984), produce formylmethyl cellulose derivatives. Matrix structure was preserved, even at high degrees of substitution, but large amounts of organic solvent were used.

The reduction step can be deleterious for sensitive ligands and the linkage will be substantially ionised below pH 10. For these reasons reductive amination was not considered for this study. The use of hydrazino ligands instead of amines avoids the necessity of reduction and the linkage is electrically neutral over most of the pH range. However the availability of these ligands is limited compared to amines and toxicity is often a problem. The same methodology can be used in reverse to attach ligands containing aldehyde groups, typically oxidised glycoproteins, to matrix hydrazino groups (Lamed et al., 1973).

Bifunctional etherification reagents are particularly useful for matrix activation. Epichlorohydrin (ECH), used by Axen et al. (1975), and butanediol diglycidyl ether (BDE), used by Sundberg and Porath (1974), are inexpensive. Epoxide groups are attached to the matrix by reaction with hydroxyl groups in aqueous alkaline media. Under alkaline conditions the oxirane group of epichlorohydrin reacts with the matrix producing a chlorohydroxypropyl ether, which reforms an epoxide group by intramolecular etherification. Thus it effectively reacts as a bisepoxide. These reagents have the advantage of introducing a hydrophilic spacer arm between three (ECH) and twelve (BDE) atoms long. This contrasts with the activation methods described in the preceding sections, which introduce a single atom (urethane methods), a 2 atom spacer (formylmethylation) or none at all. For these methods, extra steps are used if a spacer arm is required.

Epoxy activation and ligand attachment methods have been reviewed by Klyashchitskii and Kuznetsov (1984). Bisepoxide reagents have been used to immobilise a wide range of hydroxyl, thiol and amine containing ligands by strong ether, thioether and amine bonds (Vretblad, 1976; Simons and Vander Jagt, 1977; Gelsema et al. 1981; Murthy and Moudgal, 1986). The stability of a coupled amine ligand was excellent under strongly alkaline conditions (pH 13), even after a period of weeks at 70°C (Sundberg and Porath, 1974). Epoxy matrices have been converted to amine resins with ammonia (Matsumoto et al., 1980) and thiol resins with thiosulphate and reduction (Axen et al., 1975). The range of possible reactions of epoxidated matrices with proteins was reported by Zemanova et al. (1981). Divinylsulphone (DVS) reacts similarly to bisepoxides, albeit more readily, introducing vinyl groups to the matrix (Porath, 1974). The vinyl groups can be used for immobilisation of the ligand types described for oxirane groups. However DVS is highly toxic and the linkage is sensitive to alkali (Hermanson et al., 1992).

Cellulose etherification

Etherification of cellulose was first reported for modification of textiles to give different finishing properties. McKelvey et al. (1959) described the reaction of alkali cellulose with various epoxides, including ECH and butadiene diepoxide. Water insoluble epoxides reacted as well as soluble epoxides, water was essential for reaction and weight gains of over 200% could be obtained using cotton steeped in 3 M NaOH. An S_N2 reaction mechanism was proposed. Cellulose etherification using halide and epoxide reagents was reviewed by Savage (1971) and using activated alkenes by Bikales (1971). Preparation of ion exchange celluloses was reviewed by Peterson (1971) and Guthrie (1971). The starting material for these etherifications was dry cellulose or mercerised

cellulose of low water content. The high water content of bead cellulose was expected to complicate etherification because the greater water content will increase the level of hydrolytic side reactions.

Ion exchange derivatives of bead cellulose

The modification of bead cellulose to produce ion exchange resins has been described by Determann (1969), Peska et al. (1976), Kuga (1980) and Boeden et al. (1991). Perloza based ion exchange resins described by Peska et al. (1976) were produced from vacuum dried Perloza and are therefore expected to have lower porosity than the original cellulose (Gemeiner et al., 1989). Capacity, especially for large proteins, may therefore be lower than expected due to a reduced molecular weight cut-off. Higher charge densities were not practical because excessive swelling resulted, distorting the bead shape (Peska et al., 1976). A similar limit to charge density for fibrous cellulose was reported by Peterson (1971). Commercial Perloza ion exchange resins (Ostsorb) have excellent mechanical rigidity, high flow rates and adequate charge densities (approximately 0.1 mMoles/ml) for most applications. However resins of similar porosity to the original Perloza matrix, and therefore methods of preparation which did not involve vacuum or oven drying of cellulose beads, would be preferred. Desired characteristics are:

- (i) small ion capacity of 0.1 - 0.2 mMoles/ml,
- (ii) high protein capacity (of both the ion exchangers and their derivatives),
- (iii) similar mechanical properties, swollen volume and flow rate to original Perloza over a wide range of pH and ionic strength.

Other bead cellulose derivatives

Activation of Divicell™ with periodate, ECH and a chloroformate was described by Boeden et al. (1991). Activation of Perloza with CNBr, periodate, benzoquinone, chloroformates and epichlorohydrin was cited by Stamberg (1988) for enzyme immobilisation. Carbodiimide mediated attachment of glucose oxidase to CM cellulose was also reported. Activation with tosyl chloride was described by Gemeiner and Benes, 1983). Acid catalysed etherification with ECH to produce chlorohydroxypropyl derivatives was reported by Petrus and Gemeiner (1984). Iodohydroxypropyl derivatives were also produced. Problems were found with CNBr, bisepoxide and alkaline ECH activations of cellulose (Gemeiner and Zemek, 1981). The majority of these reactions required organic solvents. The reactivity of unmodified Perloza was insufficient for some modifications but could be improved by hydroxyethylation (Gemeiner et al., 1989).

Activation methods for this study

The CDI method is useful at a laboratory scale because high ligand densities can be obtained and amine ligands are readily attached through a neutral linkage. Previous work has demonstrated that very high levels of CDI activation (up to 10 mMoles/g) can be obtained on cellulose and agarose (Bethell et al., 1981b). However substitution of activated matrices with a variety of (aminoacyl) spacer arms and ligands was inefficient, typically in the range of 20 to 40 % (Bethell et al., 1981a/b). Methods which improve the coupling efficiency of aminoacyl spacer arms are described here. Variables tested were : reaction time, ratio of moles of spacer arm added to moles of active groups, solvent type and water content. Methods to improve and monitor solvent exchange (and hence activation) efficiency were also investigated, because solvent use would represent over half of the material cost of the activation described by Bethell et al. (1979).

The preparation of CM ion exchange resins, using "never-dried" Perloza is also described. The effect of solvating Perloza with mixtures of various organic solvents and water on the level of CM substitution is reported. The volume stability and flow properties of these CM resins is described and their protein capacities compared with commercial CM Ostsorb resins. Activation with bifunctional reagents (epihalohydrins, bisepoxides and divinylsulphone), using variations of solvation, reagent amount and temperature was also investigated. Target activation levels (for all methods) were up to 0.3 mMoles/g or 2 mMoles/g dry.

The units used in this thesis are g for suction-dried and g dry for oven-dried resin/matrix weights. Titration data are expressed accordingly in mMoles/g or mMoles/g dry.

MATERIALS AND METHODS

Reagents and Equipment

Perloza MT (various grades) and Ostsorb CM and DEAE bead celluloses were from Tessek Ltd. or ICS, both of Prague, Czechoslovakia; Sepharose CL6B from Pharmacia, Uppsala, Sweden; lysozyme (grade I), carbonyldiimidazole, divinylsulphone, butanediol diglycidyl ether (70%), 6-aminocaproic acid, 8-aminocaprylic acid and dimethyl sulphoxide (DMSO) from Sigma Chemical Co., St. Louis, MO, USA; epichlorohydrin from Dow Chemical Co., Midland, MI, USA; propylene oxide and chloroacetic acid from J.T. Baker, Phillipsburg, NJ, USA; 3-aminopropanoic acid, 4-aminobutyric acid, 5-aminovaleric acid, butadiene diepoxide and ethyleneglycol diglycidyl ether (50%) from Aldrich-Chemie, Steinheim, Germany; and haemoglobin from Life Technologies, Auckland, New Zealand. Convul NaOH and HCl were from BDH, Dorset, England. Dioxan and acetone were analytical grade. Dimethylformamide (DMF) and ethanol were technical grade. DMF was degassed and distilled under vacuum from calcium hydride before use. Lithium, sodium and potassium aminocaproate salts were prepared by dissolving 6-aminocaproic acid in an equimolar amount of 1 M LiOH, NaOH and KOH respectively, and freeze-drying the solutions. Epibromohydrin was prepared from glycerol by the methods of Braun (1943). All other reagents were analytical grade. Water was MilliQ grade.

A Radiometer ETS822 autotitrator was used for resin titrations. A Pye Unicam PU 8610 spectrophotometer was used for absorbance measurements. Reactions were mixed by rotation (Ballmill roller or Cole-Parmer Roto-Torque rotator) or shaking (Ika Vibramix). Disposable 2 ml columns were from Lab Supply Pierce, Auckland, New Zealand.

CDI activation

Matrices used were Sepharose CL6B, Perloza 100, fine and medium grades, and Perloza 200 fine. Activation of matrices and titration methods were similar to the methods of Bethell et al. (1979). Carbon dioxide was not titrated. Imidazole released by hydrolysis of active groups, was titrated from pH 5 to 8.5, after CO₂ was flushed out with nitrogen.

Solvent exchange

Initially a batch method was used. The matrix was washed in a sintered glass funnel, suction-dried, weighed and transferred back to the funnel. It was suspended in 3 volumes of water and drained. This process (suspension and draining) was repeated with

25% , 50% and 75% dioxan (5x2 volumes each) and with 100% dioxan (10x3 volumes). The rate of liquid draining was controlled (approximately 15 minutes at each solvent percentage) to allow adequate equilibration. An improved procedure used a column mode of solvent exchange. The matrix was covered with the appropriate size of Whatman filter paper, allowing solvents to be poured onto the column without disturbing the bed. Intermediate wash steps used one column volume each of 1:2 dioxan/water and 2:1 dioxan/aqueous imidazole (200mM). The eluate from the 100% dioxan wash was monitored for absorbance at 300 nm against a dioxan blank. After the absorbance returned to the range 0-0.005, the matrix was washed with a further volume of dioxan. The water content was not measured directly. If air bubbles developed in the bed during solvent exchange it was suspended in the appropriate solvent and drained. Solvent exchange back to water used the reverse of these methods but imidazole was not used.

Reaction procedure

Solvent exchanged matrix was drained of excess dioxan, transferred to a reaction vessel and slurried with dioxan (1-2 ml/g matrix). Solid CDI was added and the mixture sealed and agitated mildly using an Ika Vibra-mix shaker for 1-2 hours. The mixture was transferred to a sintered glass funnel, drained and washed with two column volumes of dioxan. A sample was kept for titration. The amount of reagent used was 35-125 mg CDI per g Perloza (original suction-dried weight) and 40-120 mg for Sepharose CL6B.

Aminoacyl spacer arm substitution

Initial experiments used a 5 molar excess (5 mMoles of spacer arm per mMole of imidazolyl groups on the activated matrix) of 6-aminocaproic acid (ACA) buffered to pH 10 with 1 M sodium carbonate, and aqueous solvated matrices (Bethell et al., 1979). Subsequently the standard amount used was a 5 molar excess, prepared as a 50% solution by dissolution with 0.95 molar equivalents of NaOH (10 M) and water. Other aminoacyl spacer arms were prepared similarly except a 4 molar excess was used. Aminopropanoic and aminobutyric acids were prepared as 45-50% solutions by dissolution of 3 mMoles (0.267g and 0.309g respectively) in 0.38 ml of 7.5 M NaOH. Aminovaleric and aminocaprylic acids were prepared as 40% solutions similarly (0.351g and 0.477g) but with addition of 0.15 and 0.38 ml water to assist dissolution.

The CDI activated matrix solvated by dioxan was used without modification or after solvent exchange to another organic solvent (1 volume of 50:50 and 4 volumes of the second organic solvent), 50% dioxan or water. The activated matrix (suction-dried) was mixed with the spacer arm solution for 24 hours followed by solvent exchange back to

water (100% organic solvated resins). The resin was washed thoroughly with water (≥ 20 volumes) to remove unbound spacer arm and imidazole. The substitution of spacer arm groups on the matrix was determined by titration (per g or per g dry). In some experiments the molar excess of the ACA solution and reaction time were varied.

Carboxymethylation of Perloza

The method of Peterson and Sober (1956) was adapted for etherification of undried Perloza (100 fine, medium and coarse and 200 fine). The first experiments used aqueous solvated Perloza, chloroacetic acid and 30% NaOH in the ratio of 10g:0.45g:1.6 ml or 10g:0.95g:3.3 ml, but these failed to generate adequate substitution levels. Subsequent experiments used Perloza solvated with 75-80% solvent/water mixtures or 100% organic solvent using acetone, ethanol or DMSO. The amount of chloroacetic acid used was varied between 0.25-0.45g. Concurrent reaction with 0.06-0.1 ml epichlorohydrin (ECH) and 0.1-0.5 ml propylene oxide (Ayers et al., 1984) was introduced in later experiments to modify the swelling properties of the CM product. The amount of NaOH required was 3.3 ml per g of chloroacetic acid and an extra 1.3 ml per ml of ECH.

Solvent exchange to 80 or 100% acetone was by the batch procedure described before for dioxan. Solvent exchange to 80% DMSO was also by a batch method. Two volumes of Perloza were mixed in a stoppered funnel with one volume of DMSO, equilibrated for 5 minutes and suction-dried. This process was repeated four times reducing the water content of the suction dried matrix from 90% to approximately 18%. Perloza solvated by 100% DMSO was prepared from this by washing, in a column, with 2 resin volumes of DMSO. Chloroacetic acid was dissolved in 7.5 M NaOH and mixed with suction-dried Perloza, ECH and propylene oxide in a stainless steel vessel (30 seconds). The vessel was then sealed and immersed in a water bath (60°C), for 4 hours.

Protein capacity, swollen volume and flow rate measurements of CM resins

The capacity of the CM Perloza matrices was compared to CM Ostsorb by frontal analysis (Turkova, 1978) using haemoglobin and/or lysozyme. Load buffers were 10 mM citrate, pH 6 for lysozyme and 20 mM acetate, pH 5.0 for haemoglobin. Proteins were prepared as 10% solutions in their respective load buffers. The haemoglobin solution was centrifuged and insoluble material discarded. Resin samples (approximately 1 ml, measured accurately by prior column calibration with water) were packed in disposable 5 ml columns (internal diameter 0.5 cm) and equilibrated with load buffer.

Protein solutions were applied at a flow rate of approximately 0.3 ml/minute. Protein loading was stopped when the absorbance (at 280 nm) of the eluate matched that of the load. Unbound protein was removed by washing the column with load buffer until absorbance returned to baseline. Elution was by a step increase of ionic strength (by inclusion of 0.5 M NaCl in the original buffer). Complete elution of haemoglobin required an extra wash with 0.1 M NaOH. Protein concentration in eluates was determined by absorbance at 280 nm, using extinction coefficients for 1% solutions of 23.5 (lysozyme) and 16.5 (haemoglobin). Extinction coefficients were determined by absorbance at 280 nm of 0.05% protein solutions (in their respective elution buffers). Protein solutions were prepared from unprocessed lysozyme and haemoglobin (dialysed and lyophilised, to a constant weight) recovered from capacity testing.

Swollen volumes were calculated by settled volume (after 24 hours) in a measuring cylinder (2-5 ml) ÷ oven-dried weight. Flow rates of CM resins and unmodified Perloza in a 20x1.2 cm column were determined with water. Column eluate (7-9 ml) was collected into a 10 ml measuring cylinder over time intervals of 0.5-5 minutes. The bed height was 6.1 +/- 0.05 cm and a "header" of 12 cm water was maintained for column packing and flow measurements. CM matrices were prepared in the Na⁺ form by washing with 0.1 M NaOH until the pH of the eluate was ≥ 10. Two CM resins were also prepared in the H⁺ form by washing with 0.1 M HCl until the eluate pH reached 2.

Epihalohydrin and bisepoxide activations

The standard method used for ECH activation was adapted from that described by Matsumoto et al. (1979). Perloza 100 fine (5g) was mixed with ECH (0.75 ml) and 0.66 M NaOH (10 ml) for 9 hours at room temperature (18°C). Epibromohydrin was used similarly to ECH except 3 ml DMSO was included after 3 hours. Variations of water content (80% DMSO solvated Perloza, 3.5 ml 2 M NaOH), temperature (37°C, 3 hour reaction) and reagent amount (2.5 ml ECH and 11 ml 2 M NaOH) were used in other ECH experiments. One Perloza sample was reacted twice with 25% ECH. The method described by Sundberg and Porath (1974) for butanediol diglycidyl ether (BDE) activation, was adapted for bisepoxide activations. Perloza 100 and 200 fine (10g) were used. Smaller proportions of base (5 ml of 1 M NaOH) and bisepoxide (2.5 ml butadiene diepoxide, 3.75 ml BDE and 4.25 ml ethyleneglycol diglycidyl ether) were used, to minimise the reaction volume. Reactions were at room temperature and sodium borohydride was not included. In other reactions 80% DMSO solvated Perloza was used : 5g cellulose was mixed with 0.5 ml butadiene diepoxide and 20g cellulose reacted with 10 ml BDE (+0.5 and 1 ml of 1 M NaOH respectively).

The concentration of matrix epoxide groups (mMoles/g dry) was determined by reaction (12-16 hours) of a 1g sample with 8 ml of a sulfite solution (prepared by dissolving 0.2g sodium sulfite in water and pH adjustment to 7 with 1 M HCl). The resulting sulphonate derivatives (Schenck and Kaizerman, 1953) were titrated to pH 8 with 0.1 M NaOH.

Divinylsulphone (DVS) activation

The standard method used was adapted from that described by Porath (1974). Perloza 500 fine was used. Perloza (40g) was washed with 5 volumes of 1 M sodium carbonate, suction-dried and mixed with 3.2 ml DVS and 20 ml of the same carbonate buffer for 2 hours at room temperature. In another experiment, 100% DMSO solvated Perloza (36g) was mixed with 0.6 ml triethylamine, 3.2 ml DVS and 20 ml DMSO for 2 hours. A second DMSO solvated reaction replaced triethylamine with 1 ml of 1 M NaOH and used 5 ml of DVS. Samples from the latter reaction were removed for titration after 1, 2 and 4 hours. The activation level was determined by addition of sodium sulphite (under the same conditions used for epoxide titration) and titration of sulphonic acid groups.

Titration methods

Sample preparation used HCl and NaOH solutions of approximate molarity. Titration was with Convol 0.1 or 1 M NaOH and HCl. Titrated samples were washed with water (50 volumes), ethanol (5 volumes), oven-dried at 110°C for 1.5 hours and weighed.

CDI activated resin samples were hydrolysed with 10 volumes of 0.1 M NaOH, acidified (to approximately pH 2) with 1 M HCl and nitrogen flushed for 5 minutes. The pH was adjusted to 5 with NaOH and the sample titrated to pH 8.5 with 0.1 or 1 M NaOH.

Resins containing carboxylate or sulphonate groups were washed extensively with water, followed by 4x10 bed volumes of 0.1 M HCl (sulphonic with 1 M HCl) to convert to the protonated form. Excess acid was removed by washing with at least 5x10 bed volumes of water, until the wash pH was neutral. The sample was suction-dried, weighed (1g) into a titration vessel and mixed with approximately 0.2g NaCl and 6 ml of water. The sample was titrated with 0.1 M NaOH to an end point of pH 8. Some spacer arm resins were not weighed, but transferred quantitatively (water washes) to the titration vessel.

Resins containing amine groups were treated similarly except they were washed with 0.1 M HCl and water to remove reagents, with 0.1 M NaOH to convert the resin to the unprotonated form, and titrated with 0.1 M HCl to pH 4.

RESULTS AND DISCUSSION

CDI activation

Initial activations of Sepharose CL6B produced highly activated resins (e.g. 6.27 mMoles/g dry, using 0.12g CDI/g Sepharose), unless solvent exchange was inadequate. In this case bubbling occurred due to reaction of CDI with residual water generating CO₂. Comparatively low activation levels of 1.25 and 1.3 mMoles/g dry were found, using 6g CDI per 60g Perloza 100 fine, presumably due to excessive water content. The wet to dry weight ratios for Perloza 100 and Sepharose CL6B are approximately 10:1 and 16:1 respectively, and 1g CDI is approximately 6 mMoles. Therefore the efficiency of CDI use was 20% for Perloza and 50% for Sepharose. Sepharose CL6B was reduced to approximately 60% of original volume, when exchanged to dioxan, which might increase the effectiveness of solvent exchange. The volume of Perloza was not altered. Perloza matrices drained faster than Sepharose, which reduced equilibration time during solvent exchange. Therefore the equilibration time was increased by using finer (grade 2) sintered glass funnels. Thus an activation level of 3.3 mMoles/g dry was obtained using 6.5g CDI/60g Perloza 100 medium (50% efficiency). Although similar reactivity of Perloza to Sepharose was demonstrated following improved solvent exchange, solvent exchange was still qualitative, prone to variation and required large amounts of dioxan. The results of several activations using batch solvent exchange are recorded in Table 2.1.

Efficient and reproducible solvent exchange has been obtained by use of column rather than batch methods of solvent exchange and spectrophotometric monitoring of the completeness of exchange. Effective solvent exchange by the batch mode required 5x2 resin volumes of each intermediate wash and 10x3 resin volumes of the final dioxan wash. This method would therefore use 45 litres of dioxan per litre of matrix. Because the matrix was not disturbed during column exchange the change in solvent percentage was sudden and occurred over a small volume. Thus the matrix was equilibrated to the solvent change within 1-2 matrix volumes. One resin volume was used for each of two intermediate wash steps and two to three volumes for the final wash (3-4 litres of dioxan per litre of matrix). Despite the great reduction in solvent use, greater consistency of activation levels (Table 2.2) was obtained and significant CO₂ evolution was not detected. The consistency was presumably because completeness of solvent exchange was determined quantitatively using the imidazole tracer. The absorbance of a solution of 3 parts dioxan and 1 part 200 mM imidazole was 0.110 at 260 nm, 0.227 at 280 nm, 0.272 at 300 nm and 0.176 at 320 nm. Therefore 300 nm was used for monitoring. The intermediate (2:1) wash containing imidazole had an absorbance of 0.384 at 300 nm.

Table 2.1 : CDI activation levels for batch solvent exchanged matrices

The dry weight of matrix used for activation was estimated. Suction-dried weight was multiplied by 0.1 and 0.06 to obtain the dry weight estimate for Perloza and Sepharose respectively. The activation level data used measured dry weights. Except for the first two Perloza activations, the relationship between CDI used and activation level was reasonably consistent. For Perloza approximately 3 mMoles of imidazolyl groups were attached per g CDI used. The Sepharose efficiency was slightly higher (3-3.5).

Amount of CDI used (g/g dry matrix)	Activation level (mMoles/g dry)
Perloza	
1.0	1.25
1.0	1.3
1.1	3.3
1.2	3.5
0.8	2.57
Sepharose CL6B	
2.0	6.27
1.5	4.5
0.7	2.47
0.6	2.28

Table 2.2 : CDI activation levels using imidazole tracer, column exchange

Perloza 100 fine was used unless otherwise noted. The amount of CDI used is expressed per g (drained) dioxan solvated Perloza. Assuming 10g of dioxan solvated Perloza was equivalent to 1g dry, the activation level range was 2.5-3 mMoles/g CDI used.

Amount of CDI g/10g Perloza	Activation level (mMoles/g dry)
0.4	1.22
0.67	1.9
0.87 (200 fine)	2.47
1.0	2.69
1.25	3.07

A compound with a larger absorbance than imidazole would be more sensitive and a compound absorbing in the visible range would facilitate monitoring. Any compound used should have good water solubility (to minimise the risk of partitioning into the 100% dioxan wash), not interact significantly with the matrix and not be likely to interfere with the activation if residual amounts are present. The first two possibilities should be detected as a prolonged low absorbance level in the eluate while the third possibility could be tested by including the compound in an activation mixture.

Imidazole was chosen because it is cheap and soluble in both dioxan and water. Residual imidazole in the exchanged matrix should not have a significant effect on the reaction, since imidazole is produced in large amounts as a by-product of activation. Using imidazole the absorbance returned to zero rapidly, indicating efficient removal. The absorbance peak of the effluent was equivalent to that of the applied solvent mixture. Imidazole or a similar "tracer" could be used for other solvent exchange requirements (e.g. tosyl chloride activation) to monitor water removal.

Attachment of aminoacyl spacer arms to CDI activated matrices

Aminocaproic acid

Using carbonate buffered solutions of aminocaproic acid (ACA) and aqueous solvated matrices the efficiency of substitution of activated groups varied between 20 and 40%. Because hydrolysis of activated groups would reduce efficiency, conditions were altered to reduce the water content of the ACA solution. Using a 5 molar excess of ligand at pH 11.3, extra buffering was not required because the ligand was predominantly (90-95%) in the unprotonated (reactive) form and in considerable excess. This allowed preparation of concentrated solutions (40-50%) of ACA, the use of which increased substitution efficiency to between 50 and 60%.

The remaining option to reduce water content was to use an organic rather than aqueous solvated matrix. The sodium, potassium and lithium salts of aminocaproic acid were found to be insoluble, using a variety of solvents, in the absence of water. Therefore 50% ACA solutions were prepared as before but water content could be minimised by using organic solvated matrices. A steady increase in substitution efficiency was found going from water solvated matrices through 50% organic solvated to 100% organic solvated matrices (Table 2.3). Dioxan was replaced by DMSO and DMF with similar results. Substitution efficiencies ranged from 50-65% for water solvation to 90-95% for DMSO, DMF and dioxan solvation (Table 2.4). These efficiencies were determined using corrected substitution values, which are verified in Table 2.4.

Table 2.3 : Coupling yields of CDI activated matrices with 6-aminocaproic acid

Activation and aminocaproic acid (ACA) substitution levels units are mMoles/g dry. Corrected substitution values were calculated using dry weights corrected for the weight of the spacer arm (mMoles titrated x 0.179 g/mMole). The accuracy of these corrections was confirmed by the correlation between corrected substitution and titration/g values, expressed as a percentage of CDI activation level (Table 2.4). Reaction efficiency (%) was calculated using corrected substitution values, compared to CDI titration values.

Matrix	CDI titration	Media water %	ACA Substitution		Efficiency %
			(a) Titrated	(b) Corrected	
Bead cellulose	3.50	100	1.60	2.20	63
		50	1.88	2.85	82
		0	2.05	3.25	93
Sepharose CL6B	2.47	100	1.01	1.23	50
		50	1.40	1.87	76
		0	1.67	2.38	96

Table 2.4 : Solvent variations and verification of corrected ACA substitutions

Weighed samples of CDI activated matrices in dioxan were solvent exchanged using mixtures of DMSO, DMF or water with dioxan, to produce samples in 100% DMSO, 100% DMF, 100% water, 50% dioxan and the original 100% dioxan form. These were reacted with ACA (50% solution) and the product quantitatively titrated. Another weighed sample (CDI*), solvent exchanged to 100% water, was used to determine the CDI activation level. Substitution# is the "corrected" titration value. The proportion of caproic groups is expressed as a percentage of the possible (CDI titration) level.

Sample	Titration (mMoles/g)	%	Titration (mMoles/g dry)	Substitution#	%
CDI*	0.295		2.54		
Dioxan	0.248	84	1.61	2.26	89
50 %	0.191	65	1.30	1.69	66
Water	0.144	49	1.03	1.27	50
DMSO	0.276	94	1.70	2.44	96
DMF	0.261	89	1.62	2.29	90

Other solvents were less effective. The maximum efficiency using acetone was 79%. The efficiency using ethanol was approximately the same as for water, although this was not investigated further or quantified precisely. Although reactions were usually mixed for 24 hours, only small increases of substitution (1.14 to 1.19 mMoles/g dry, water solvated; 1.40 to 1.49 mMoles/g dry, acetone solvated) were found after 0.5 hours. Although a 5 molar excess was used routinely, a 3 molar excess was found to give the same result (1.49 mMoles/g dry, acetone solvated). Contrary to an earlier report (Burton et al., 1991), some precipitation did occur when concentrated ACA solutions were mixed with organic solvent, but this did not appear to adversely affect the reaction.

Other aminoacyl spacer arms

The substitution efficiencies of other aminoacyl spacer arms in dioxan/DMSO mixtures were compared. Results for aminopropanoic and aminobutyric acids were even higher than for ACA. The solubilities of aminovaleric (AVA) and aminocaproic acids were poorer and this was reflected by lower substitution efficiency. Considerable precipitation occurred when the latter spacer was mixed with organic solvated Perloza. Nevertheless, substitution efficiency was still greater than 75%, and superior to the maximum efficiency (67%) described in an earlier report (Burton et al., 1991). A small amount of precipitation also occurred with AVA and ACA, but not with the other two spacer arms. The data for these experiments are summarised in Table 2.5.

Table 2.5 : Substitution efficiencies of various aminoacyl spacer arms

Approximately 3g (accurately weighed) of activated matrix was reacted with each spacer arm and the product transferred quantitatively for titration. CDI* is the reference CDI titration and substitution# the corrected substitution calculated from the titration value (mMoles/g dry) as described before. The spacer arm weights used for corrected value calculations were 0.137, 0.151, 0.165 and 0.207 g/mMole for aminopropanoic, aminobutyric, aminovaleric and aminocaproic acids respectively.

Sample	Titration (mMoles/g)	%	Titration (mMoles/g dry)	Substitution#	%
CDI*	0.230		2.40		
Aminopropanoic acid	0.218	95	1.76	2.35	98
Aminobutyric acid	0.215	93	1.69	2.27	95
Aminovaleric acid	0.181	79	1.45	1.91	80
Aminocaproic acid	0.205	89	1.56	2.17	90
Aminocaprylic acid	0.177	77	1.35	1.88	78

The much improved reaction efficiency, for both CDI activation and ligand attachment, allows greater **predictability** of ligand density and the option of **very high substitution** levels. Aminoacyl spacer arm resins are usually used to attach amine ligands for affinity chromatography. The substitution levels reported here are much higher than is usually recommended for this purpose. However these highly substituted resins have been found useful for forms of chromatography other than affinity, described in Chapters 5 and 7.

Carboxymethylation

The method initially used was based on that used for dry cellulose by Peterson and Sober (1956). The reaction of water swollen Perloza with similar quantities of reagents (45 mg/g Perloza, equivalent to 0.45 g/g dry) resulted in a low substitution level (0.4 mMoles/g dry). This presumably occurred because the volume of water in the mixture was much higher resulting in a greater proportion of the side reaction (hydrolysis of chloroacetic acid to hydroxyacetic acid) rather than cellulose carboxymethylation. The substitution level (0.37 mMoles/g dry) was not increased using a much greater amount of chloroacetic acid (95 mg/g Perloza). However, using 75% acetone solvated Perloza and unchanged amounts of NaOH and chloroacetic acid, a substitution of 1.6 mMoles/g dry was obtained.

Other features of this reaction were considerable shrinkage of beads in the alkaline acetone medium and excessive swelling of the product when solvent exchanged back to water. The latter problem was highlighted by a CM product of 100% acetone solvated Perloza 500 fine which had a substitution of 2.4 mMoles/g dry but swelled so much in base that it became an amorphous gel. Excessive swelling was somewhat alleviated by using less chloroacetic acid and including epichlorohydrin in the reaction mixture, to crosslink the matrix (Perloza is stabilised by hydrogen bonding alone). Propylene oxide was included in the activation mixture to counteract any reduction in swollen volume due to crosslinking (Ayers et al., 1984).

It was believed that the extreme shrinkage and swelling involved with acetone use could have a detrimental effect on the matrix properties. When Perloza was solvated in DMSO, an organic solvent of much greater polarity, there was no apparent change in matrix volume after addition of alkali. The dielectric constant of DMSO is 46.7 and of the common organic solvents this is the closest to that of water (78.4). Oxidative side reactions between DMSO and halide reagents (Johnson and Pelter, 1964) were considered unlikely under the conditions used. The effects of reagent proportions on substitution level, swollen volumes and flow rate are summarised in Table 2.6.

Table 2.6 : Substitution levels, volumes and flow rates of CM Perloza resins

P100F, P100M and P100C are unmodified Perloza 100 fine, medium and coarse matrices. All CM resins were prepared from 80% DMSO solvated Perloza, suction-dried. The CM resins are identified by the amount of chloroacetic acid (g)/propylene oxide (ml)/epichlorohydrin (ml) used per 100g Perloza. Bead grades are identified by F, M or C. Flow rate figures in brackets are for the H⁺ resin form.

Resin	Substitution mMoles/g dry	Volume (H ⁺) (ml/g dry)	Volume (Na ⁺) (ml/g dry)	Flow rate (ml/hr)
P100F				186
P100M				414
P100C				1080
2.4/3.8/0.85 F	1.03	8.3	8.9	134 (150)
2.5/3/0.6 F	1.32	9.2	11.1	89 (140)
2.5/3/0.8 F	1.33	9.1	10.0	100
2.5/4/0.8 F	1.30	9.1	10.2	n.d.
2.5/4/0.6 F	1.37	9.1	10.7	97
2.8/4/1 M	1.40	7.6	8.2	204
2.8/4/1 C	1.22	8.3	9.2	852
3.6/5.3/1.3 C	1.65	8.4	9.6	n.d.

The substitution level of the first CM 2.4/3.8/0.85 F was surprisingly low compared to the other CM (fine) resins. This was presumably due to an experimental error, but it did demonstrate the beneficial effect of lower substitution on resin flow rate. The flow rate of all CM resins were reduced compared to unmodified Perloza. The least affected, in this respect, were the above CM resin and 2.8/4/1 C (also lower than average substitution). There also appeared to be a correlation between swollen volume and flow rate (i.e. an increase in swollen volume resulted in a decrease in flow rate). This was exemplified by the large increase in flow rate for 2.5/3/0.6 F after titration of CM groups to the H⁺ form. The swollen volumes of this resin in the base and acid forms differed considerably. A much smaller effect on flow rate was found for the less swollen 2.4/3.8/0.85 F resin. Swollen volume was apparently stabilised (least difference between H⁺ and Na⁺ forms) by increasing both propylene oxide and epichlorohydrin content. In the case of propylene oxide this was unexpected and was attributed to formation of a "layer" of hydroxypropyl groups between the cellulose backbone and the CM groups. This layer could reduce the destabilising effect of a high charge density on the backbone.

The capacities, for lysozyme and haemoglobin, of some of these CM Perloza resins were compared with CM Ostsorb (Table 2.7). High protein capacities were obtained with CM Perloza resins, even for haemoglobin (molecular weight of 66 kDa), and these data compared well with CM Ostsorb results. Haemoglobin capacities approached the value of 100 mg/ml reported for CM Sepharose CL6B (Pharmacia catalogue).

Table 2.7 : Lysozyme and haemoglobin capacities

The identification of CM Perloza resins is described before (Table 2.7). Substitution values are per ml of the Na⁺ resin form.

Resin	Substitution mMoles/ml	Lysozyme mg/ml resin	Haemoglobin mg/ml resin
2.4/3.8/0.85 F	0.116	n.d.	89
2.5/4/0.8 F	0.133	140	n.d.
2.8/4/1 M	0.171	118	72
2.8/4/1 C	0.133	116	67
Ostsorb 1	0.161	65	22
Ostsorb 2	0.102	37	n.d.

Although protein capacities were high, it was apparent that careful control of activation level and the modifying reagents (ECH and propylene oxide) was required to produce CM resins with the desired volume and physical strength characteristics. The preparation of these CM resins also involved considerable solvent use. Ligand attachment to produce other resins would require expensive carbodiimide catalysis. Therefore these methods were **not ideal** for the preparation of cheap industrial resins.

Etherification with bifunctional reagents

ECH activation levels of up to 0.8 mMoles/g dry were reported by Matsumoto et al. (1979) using Sepharose. However application of these methods to Perloza resulted in low activation levels (0.11-0.15 mMoles/g dry). Activation with epibromohydrin (18°C) and ECH (37°C) gave slightly lower results than ECH (18°C). Because of the success of etherification in DMSO mixtures for preparation of ion exchange resins this approach was applied to ECH activation. The activation level was increased but still low (0.36

mMoles/g dry) using 80% DMSO solvated Perloza and more concentrated (2M) NaOH solutions. Higher activation levels (0.39 mMoles/g dry) were also obtained by using larger amounts of ECH (0.5 ml/g Perloza) and NaOH.

The maximum activation level obtained on Perloza (by repeat activation) was 0.63 mMoles/g dry but consumed 0.5 ml ECH per g cellulose. The large reagent use was not ideal and could give rise to excessive crosslinking, yet it still failed to meet target activation levels. The sample prepared using 0.5 ml ECH/g Perloza had a significant dry weight increase (20% higher than the 0.15 ml/g samples) which could not be explained by the relatively low activation level. This was attributed to side reactions (hydrolysis and crosslinking). Relatively low activation levels were also reported by Boeden et al. (1991) for a bead cellulose using the method of Matsumoto et al. (1979).

Aqueous bisepoxide activation of Perloza resulted in low activation levels despite using large amounts of reagent (10-17 ml/40g Perloza). Titrated values were 0.5, 0.39 and 0.27 mMoles/g dry for butadiene diepoxide (BD), ethyleneglycol diglycidyl ether and butanediol diglycidyl ether (BDE) respectively. Results for 4-8 hour BDE activations in 80% DMSO gave activation levels < 0.1 mMoles/g dry (several reactions). A similar reaction using BD was also unsuccessful (0.13 mMoles/g dry). The most effective bisepoxide (BD) is volatile, dangerous and expensive. Molar prices of these and other commonly used reagents are included in Appendix 1.

Divinylsulphone reaction with Perloza 500 fine in 1 M sodium carbonate resulted in an activation level of 0.73 mMoles/g dry. Very little reaction occurred using triethylamine instead of sodium carbonate and DMSO solvation (0.05 mMoles/g dry). A higher activation level (1.13 mMoles/g dry) was obtained in DMSO using a catalytic amount of 1 M NaOH and 55% more DVS. Although these levels were higher than could be obtained with the epoxide reagents, this activation is less desirable for general use because of reagent toxicity and base instability of the vinyl sulphone linkage.

None of these bifunctional reagents produced the desired result : efficient activation of Perloza to high levels. It was assumed that side reactions were limiting activation levels. New etherification reagents or methods, which limit these side reactions, were required to produce high activation levels on Perloza. High activation levels were necessary for chromatographic methods described in later chapters.

SUMMARY

Perloza matrices have been activated with CDI, in anhydrous media, and very high activation levels obtained. The reactivity with CDI was only slightly lower than that of Sepharose CL6B. Reliable levels of activation have been obtained by improvements to the solvent exchange procedure. These improvements greatly reduce solvent use and hence expense. These CDI activated matrices were used to attach ligands with amine groups. Substitution efficiency was significantly improved by minimising the water content of reaction mixtures. This was exemplified by 90-95% efficiency for sodium aminocaproate (50% solution) using DMSO, DMF or dioxan solvated matrices.

Perloza was etherified with chloroacetic acid to produce CM resins. Organic solvents were required to avoid excessive reagent hydrolysis, but the solvent requirement was lower (75-80%) than for CDI activation. Preparation of anhydrous Perloza, even with improved techniques, consumed four times as much solvent as the preparation of Perloza in 80% organic solvent/water mixtures. Highly substituted ion exchange resins were produced using 75% organic solvation but were physically unstable in the charged form. Co-reaction with epichlorohydrin (to form crosslinks) and propylene oxide allowed preparation of stable, moderately substituted resins. The capacity of these "never-dried" ion exchange resins compared favourably with commercial products. However a less complex activation method, without solvents, would be preferred. Also, further reaction (ligand attachment) of CM resins is comparatively expensive.

Perloza activation with bifunctional etherification reagents was much less successful. The activation levels obtained were much lower than both the original target and those obtained on Sepharose. A brief study of DVS activation demonstrated activation levels up to 1.1 mMoles/g dry could be obtained. However the favoured ECH and bisepoxide reactions gave low activation levels despite using large amounts of reagent and solvents. This implied that epoxide reactivity with Perloza was low compared to hydrolytic and crosslinking side reactions. Epoxide activation was preferred for general use, because of cost and the strength of the chemical attachment for ligands. However the activation levels obtained were marginal for many applications and unsatisfactory for high ligand density applications.

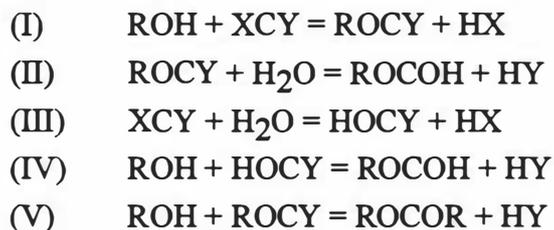
A new method(s) was required for Perloza with the preferred features of bifunctional etherification but which could produce high activation levels. Successful Perloza activation and ligand coupling using alternative reagents is described in the Chapter 3.

CHAPTER 3 MATRIX ACTIVATION WITH ALLYL REAGENTS AND METHODS OF LIGAND ATTACHMENT

INTRODUCTION

Moderate activation levels have been reported for Sepharose using bifunctional etherification reagents (Axen et al., 1975; Sundberg and Porath, 1974) but much lower activation levels were obtained with Perloza bead cellulose. The anticipated reactions for a mixture of a polysaccharide matrix, aqueous alkali and a bifunctional reagent (e.g. epichlorohydrin) are summarised in Figure 3.1. A number of side reactions would occur beside the desired matrix activation (I) using these methods. Firstly, water competes for the reagent, causing hydrolysis of the reagent in solution and of the immobilised functional groups (II-IV). Secondly, the bifunctional reagent may react with the matrix at each functional group resulting in matrix crosslinking (V) rather than activation.

Figure 3.1 : Possible reactions of cellulose with a bifunctional reagent



ROH = matrix (hydroxyl), XCY = a bifunctional reagent of which X and Y are reactive groups and C the linkage. ROCY is the desired product, ROCOH is an immobilised but inactive (hydrolysed) group and ROCOR is a crosslink.

These side reactions limit the efficiency of reagent use and hence the activation level which can be obtained. They may also have deleterious effects on chromatographic properties e.g. steric restrictions and reduced porosity. Reaction temperature, time and hydroxide concentration must be carefully controlled for consistent activation, due to the lability of the immobilised functional groups.

A preferred strategy for activation with bifunctional reagents would be to "block" one functional group for the activation and subsequently remove the "blocking group". This would be analogous to the techniques for coupling amino acids in solid phase peptide

synthesis (SPPS), first reported in detail by Merrifield (1963). This approach would allow only reactions I and III to occur. The only reaction involving the matrix is the desired activation. The "blocked" reagents used for SPPS are very expensive and the chemical linkage is an amide, which is less stable than the preferred ether. However a similar effect can be obtained by using bifunctional reagents whose functional groups (and their reactivities) are substantially different. Ideally one functional group reacts readily with the matrix while the other is relatively inert towards the matrix or any solvent or reagent used in activation. The second group should nevertheless be reactive with a wide range of ligands with or without prior conversion to a more reactive form. An example of this is carboxymethylation with chloroacetic acid followed by a carbodiimide catalysed condensation with an amine ligand. However this method suffers practical disadvantages of carbodiimide expense, amide bond sensitivity to strong alkali and a very short spacer arm. A preferred bifunctional reagent would activate the matrix by etherification, introducing a spacer arm of at least three atoms. The less reactive group would then be used to immobilise ligands by O-, S- or N-alkylation. Simple, efficient methods and aqueous or largely aqueous reaction mixtures would be preferred for all stages of resin preparation : activation, intermediate reaction (if required) and ligand attachment.

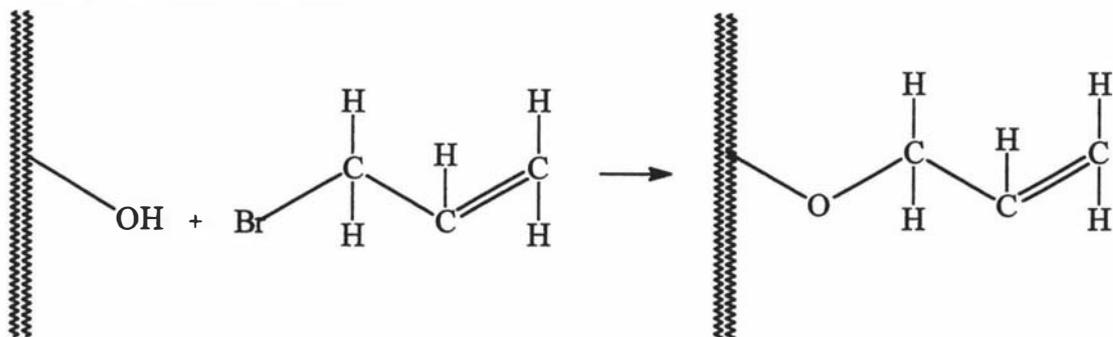
An activation method of this type was described by Ellingboe et al. (1970). The bifunctional reagent was epichlorohydrin but an acid catalyst was used. The epoxide group reacted with the matrix (Sephadex) forming a chlorohydroxypropyl ether but under acidic conditions an epoxide was not reformed. Therefore crosslinking did not occur. The chlorohydroxypropyl group could subsequently react as a halide, with suitable ligands, or as an epoxide under alkaline conditions. Petrus and Gemeiner (1984) described the application of this method to bead cellulose. However Ellingboe's reaction was carried out in dry dichloromethane and Petrus and Gemeiner's cellulose activation used a 25:1 mixture of dioxan and water. The large requirement for organic solvents would contribute significantly to the cost of these methods.

An alternative approach would be etherification of matrices with an allyl halide or allylglycidyl ether. At alkaline pH the halide or glycidyl group should react with polymeric hydroxyl groups (Figure 3.2). Under these conditions the allyl group is expected to have limited reactivity with the matrix or water. Therefore high activation levels should be possible without crosslinking and hydrolysed groups (reactions II, IV and V of Figure 3.1) should not be attached to the matrix. After activation the allyl groups could be "activated" by aqueous bromination to form bromohydroxypropyl groups (Figure 3.3a), which could be used like epoxides to attach nucleophilic ligands

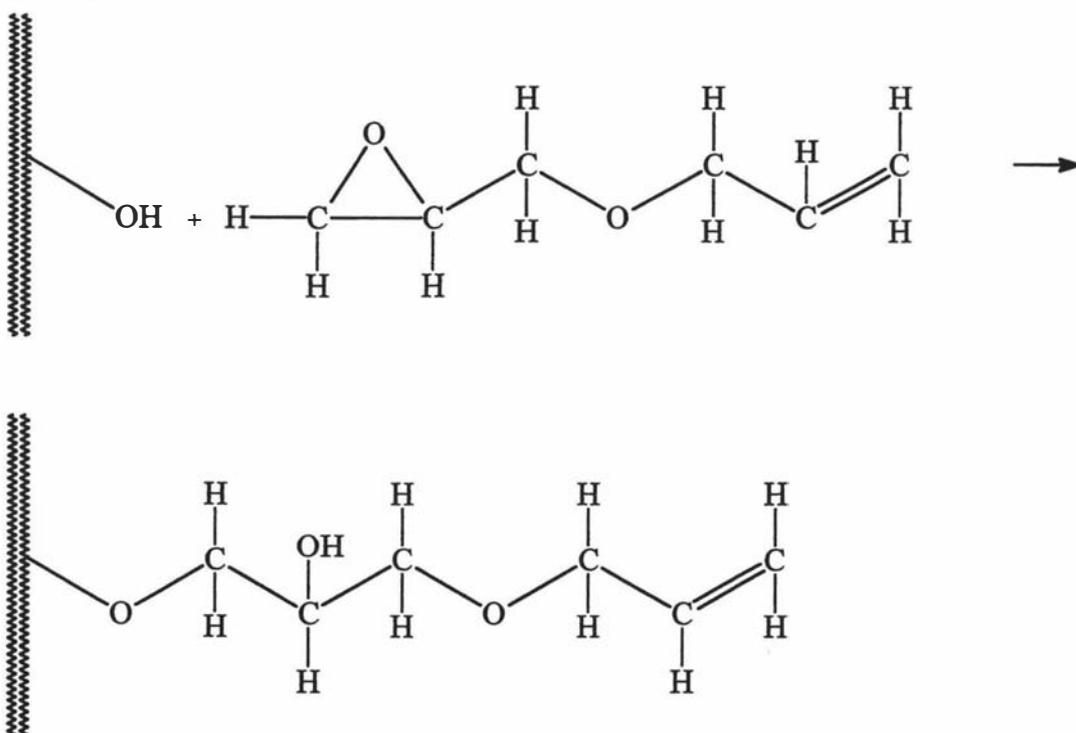
(Figure 3.3b). Hydrolysis and crosslinking side reactions should be limited if the pH of ligand substitution is ≤ 12 . Alternatively allyl reagents could be used for activation of ligands followed by a one step reaction with matrix hydroxyl groups. Allyl hydroxypropyl thioglycosides have been produced in this fashion using allyl glycidyl ether and attached to a chitosan matrix after ozonolysis (Holme et al., 1988).

Figure 3.2 : Attachment of allyl groups to matrices

A : Allyl bromide activation



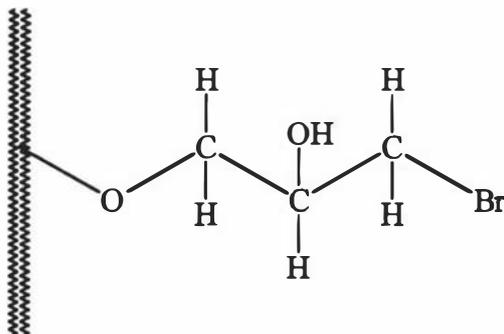
B : Allylglycidyl ether activation



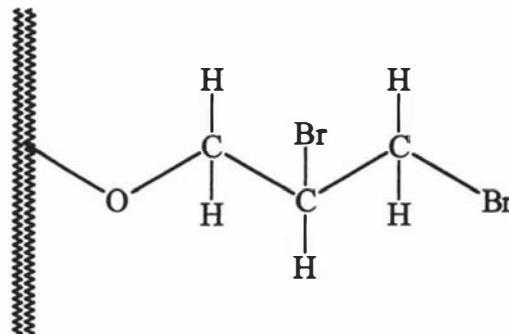
[hydroxypropyl - ether - propenyl]

Figure 3.3 : (A) Bromination of allyl matrices and (B) ligand substitution

A1 : Bromohydroxypropyl

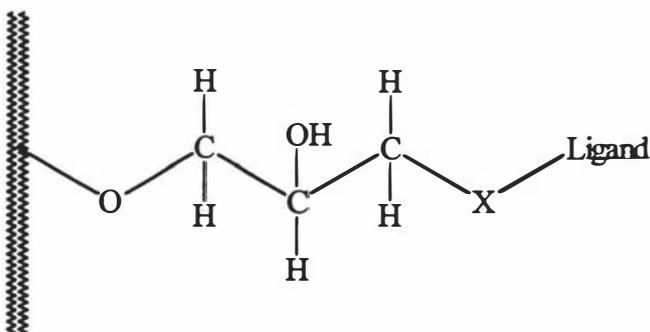


A2: Dibromopropyl



Product A1 is expected to predominate greatly if dilute bromine water is used

B : Ligand-XH attachment (X = NH, S or O)



The halide group of allyl halides is very reactive (De Wolfe and Young, 1964). Allyl bromide has been used to modify Sepharose for use as a hydrophobic adsorbent (Caldwell et al., 1975) but the possibility of further reaction of the allyl group was not exploited. Longer chain haloalkenes (e.g. 4-bromobut-1-ene) could also be used but the halide groups of these reagents will be much less reactive and hydrophobic effects of the linkage may become significant. Recently, activation of cellulose and agarose with allyl halides and allyl glycidyl ether and subsequent ligand substitution was described (Sawato and Yagi, 1992; Lindgren, 1994). The activated matrices were brominated with bromine water and substituted with thiol and amine ligands. However experimental details were limited. Bromine water has been used before to oxidise polysaccharide hydroxyl groups to aldehydes (Larm and Scholander, 1977). This reaction is unlikely to compete significantly because the hydroxyl oxidation is slow whereas reaction with alkene groups is rapid.

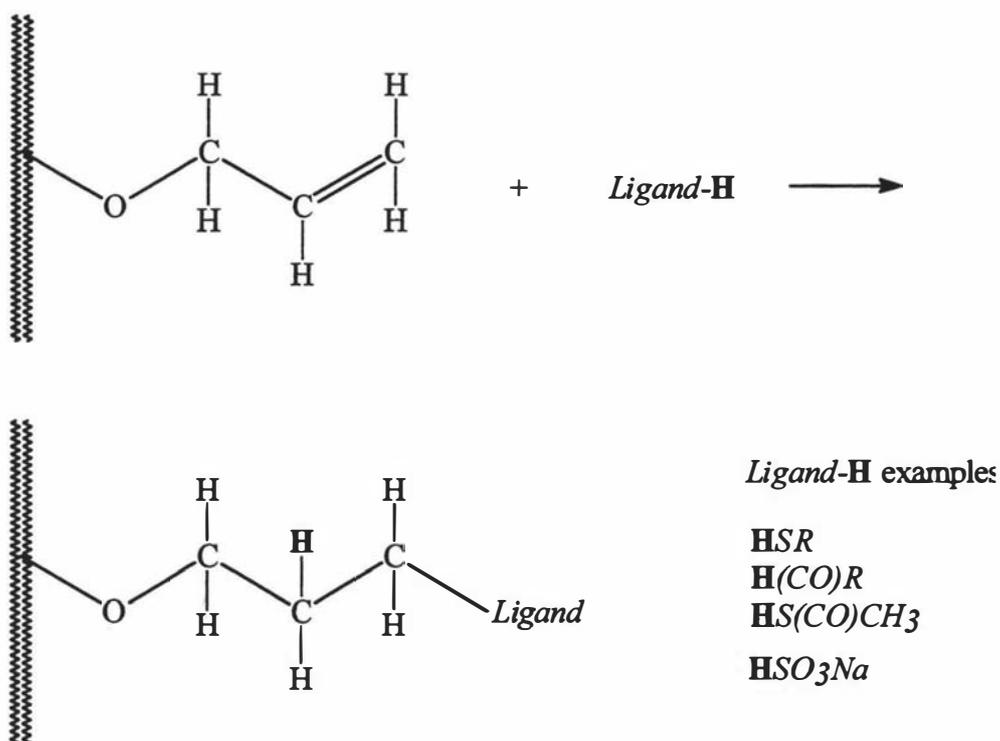
An alternative reagent for bromohydrin formation is N-bromosuccinimide (NBS). This has been used in water (Guss and Rosenthal, 1955) and DMSO/water mixtures (Dalton et al., 1968) to convert alkenes to bromohydrins. Halohydrin derivatives could also be produced with N-bromoacetamide (Schmidt et al., 1926), iodine (Cornforth and Green, 1970) or N-iodosuccinimide (Adinolfi et al., 1976). N-chlorosuccinimide could be used although the mechanism is apparently different from that of NBS (Belucci et al., 1978).

Useful reactions of the allyl group are not limited to halogenation. Alkene groups have been epoxidated with peroxy acids (Imuta and Ziffer, 1979) or alkaline hydrogen peroxide (Payne, 1959). Epoxidation of allyl cellulose with peracetic acid has been described by Lin and Huang (1992). However this procedure required anhydrous solvents and side reactions occurred. If allyl groups could be epoxidated in aqueous media without significant side reactions this could be an industrially useful reaction.

Another possible reaction is direct addition of ligands to the double bond. Activated (conjugated) alkenes will react with thiols and amines by base catalysed addition but unconjugated alkenes like the allyl group react poorly by this mechanism. However unconjugated alkenes can add suitable ligands by a free radical mechanism. Useful reactions (Figure 3.4) should include addition of bisulphite to form alkylsulphonate groups (Kharasch et al., 1938b), thiolacetic acid to form a thiolacetate intermediate (Cunneen, 1947b), thiol ligands by a thioether linkage (Kharasch et al., 1938a) and aldehydes by a ketone linkage (Kharasch et al., 1949). The thioether linkage could be easily modified by bromine water (oxidation) to a sulphoxide (Smith and Hernestam, 1954), which would be more polar and still a strong linkage. Resins for covalent chromatography could be produced by hydrolysis of thiolacetate groups to thiols. Thiol groups are also useful for further chemical modification. Other free radical reactions, e.g. alkyl phosphite addition (Stiles et al., 1957) may also be useful.

Free radical addition to allyl groups is potentially very cheap and specific (e.g. a ligand containing an amine and a thiol group should only react via the thiol). Free radical addition of alkene ligands to thiol agarose has been reported by Brandt et al. (1977) and Caron et al. (1979), catalysed by γ -radiation and potassium persulphate respectively. In general, alkenylated matrices are preferred for ligand immobilisation because they are simpler to prepare than thiol matrices. Thiol Sepharose is prepared by ECH activation, thiosulphate substitution and reduction (Axen et al., 1975). The recommended reducing reagent, dithiothreitol, is expensive and thiol groups are readily oxidised, necessitating careful storage or reduction immediately prior to use (Axen et al., 1975). By contrast allyl groups are expected to be stable under typical aqueous storage conditions.

Figure 3.4 : Possible addition reactions of allyl matrices



Many thiol ligands are commercially available, while others can be prepared using simple organic chemistry reactions (Reid, 1958). Radical addition to alkenes has been reviewed by Walling (1957), Stacey and Harris (1963) and Sosnovsky (1964). Radical addition of thiols has been reviewed by Reid (1960), Pryor (1962) and Griesbaum (1970). Some additions occur rapidly due to traces of peroxides or oxygen in the reaction (Walling, 1957). Other reactions require heat, chemical (peroxide) radical initiators and/or irradiation. (Cunneen, 1947a/b; Brown et al., 1951; Walling, 1957).

Allylglycidyl ether activation would introduce a 7 atom spacer arm. The spacer arm would include a hydroxypropyl group, ether linked to a propenyl group (Figure 3.2). The propenyl group would be converted to an hydroxypropyl or a propyl group (bromohydrin/ligand or radical addition derivatives). This would compare favourably to other methods for introducing a relatively hydrophilic spacer arm (e.g. bisepoxides).

In this chapter, Perloza etherification by allyl bromide and allylglycidyl ether is reported. The aim was to develop one step, aqueous solvated and inexpensive methods. The target range of activation levels was again 0.6 to 2 mMoles/g dry weight. The activated products were used to attach amine and thiol ligands, and sulphite/bisulphite. These ligands were attached by halogenation and substitution or addition methods.

MATERIALS AND METHODS

Reagents :

Allyl glycidyl ether, thiolacetic acid, mercaptoacetic acid, thiosalicylic acid, cysteamine HCl, mercaptopropionic acid and 6-bromohexanoic acid were from Janssen Chimica, Geel, Belgium; 4-mercaptopyridine, mercaptosuccinic acid, diethylaminopropylamine, thiophenol and sodium bisulphide from Aldrich-Chemie, Steinheim, Germany or Aldrich Chemical Co., Milwaukee, WI, USA; glutathione reduced free acid, iminodiacetic acid and butyrolactone from Sigma Chemical Co., St. Louis, MO, USA; calcium hydroxide, allyl bromide, N-bromosuccinimide, n-butylamine, ethanolamine, cysteine, sodium borohydride, benzoyl peroxide, ammonium persulphate, tetraethylammonium hydroxide, tetrabutylammonium iodide, 4-methoxyphenol and diethylamine from BDH, Dorset, England; bromine from Hopkin and Williams, Essex, England; 2-mercaptoethanol, barium hydroxide octahydrate and cysteine HCl from Riedel-de Hahn, Selze, Germany; sodium sulphite from May and Baker, Manchester, England; sodium metabisulphite from Ajax Chemicals Ltd., Auburn, NSW, Australia; and thiourea and 45% trimethylamine from Merck, Munich, Germany. Allyl bromide was distilled prior to use unless otherwise noted; 4-mercaptobutyric acid prepared from butyrolactone and thiourea by the method of Kharasch and Langford (1963); and 6-mercaptohexanoic acid prepared by the method of Ivanovics and Vargha (1947) from 6-bromohexanoic acid and sodium bisulphide.

Synthesis of 4-mercaptoethylpyridine HCl

4-Mercaptoethylpyridine HCl was prepared from 4-vinyl pyridine and thiolacetic acid by an adaptation of the method described by Vinton (1952) for preparation of 2-mercaptoethylpyridine. Under stirring, 4-vinyl pyridine (95%, 250 ml) was prechilled to -30°C (methanol/ice bath) and thiolacetic acid (170 ml) added dropwise over a period of about 1 hour. The solution froze after 5-10 minutes and the addition rate was increased to raise the temperature to 15-25°C and allow efficient stirring. Further addition was controlled to maintain reaction temperature between 15-25°C and not exceeding 30°C. The reaction was stirred for a further 1 hour to room temperature and then overnight.

The product was mixed with ether and extracted with saturated sodium bicarbonate solution - 3 times (note on the third extraction no gas was evolved). The ether layer was washed once with saturated salt solution, treated with activated charcoal to reduce colour and dried over anhydrous magnesium sulphate. It was then filtered and evaporated under vacuum with a bath temp about 30°C. The resulting oil was stirred for 4 hours with 800 ml of 6 M HCl and then the acid layer reduced under vacuum. The

dried solid was slurried with isopropyl alcohol, filtered and dried to give 328g (theoretical 387g) of creamy white 4-mercaptoethylpyridine hydrochloride (yield 85%), m.p. 190-191°C (lit. 189°C, Bauer and Gardella, 1961). A repeat preparation using 206 ml vinyl pyridine, 140 ml thiolacetic acid and more efficient HCl removal gave 308g (theoretical 318g, yield 97%) of a creamy solid, m.p. 189-190°C.

Mercapatan syntheses and allyl bromide distillation were carried out by Dr. D.R.K. Harding, Massey University. All other chemicals and matrices were described in Chapter 2 or were analytical grade.

General reaction methods :

Reactions at room temperature were mixed mechanically (Chapter 2) unless otherwise indicated. Reactions at elevated temperature were incubated in a water bath without external mixing. Glass vials (25 ml) were used routinely for matrix samples up to 15g. Glass jars, up to 1250 ml capacity, were used for larger reaction mixtures. Perloza 100 fine was used for activation reactions unless otherwise indicated. Perloza, solvated with water or DMSO/water mixtures, was prepared and suction-dried by the methods described in Chapter 2. Activation mixtures were identified by the volume of allyl reagent (ml) per 100g suction-dried matrix, expressed as a percentage.

Crosslinking of Perloza with epichlorohydrin

Perloza 100 fine, washed with water and suction-dried (200g) was mixed with 120 ml of 2 M NaOH and 2 ml epichlorohydrin (ECH) for 72 hours at room temperature. This matrix is described as 1% crosslinked, on the basis of the ECH (ml) : cellulose (g) ratio. Perloza 100 medium was 1% crosslinked similarly, using 380g Perloza with 120 ml of 2 M NaOH and 3.8 ml ECH.

Allyl bromide (AB) activation :

Initial conditions

The initial activation was 75% DMSO solvated Perloza (10g), 0.3 ml AB and 3.75 M NaOH (1 ml) mixed for 4 hours at 60°C. A second activation used greater amounts of AB (0.6 ml) and sodium hydroxide (2 ml) for 3 hours at 60°C (a "6%" activation mixture). The same 6% mixture was used for activation at room temperature for 1 hour and a 14% mixture (0.7 ml AB, 2.3 ml of 3.75 M NaOH per 5 g 75% DMSO solvated Perloza) was used for a 24 hour room temperature activation. All subsequent activations were at room temperature. Reaction was for 24 hours unless otherwise indicated.

Base reagent choice

In some (6%) activations sodium hydroxide solutions were replaced by barium hydroxide (2g), calcium hydroxide (0.5g) or potassium phosphate (3g) per ml AB used. Barium hydroxide was also used for a 10% activation of 75% DMSO solvated Perloza and a 25% activation of water solvated Perloza, both at room temperature for 24 hours.

Reagent proportion

Water solvated Perloza (5g), 0.5 ml water and 1 ml DMSO were mixed with 0.25 ml, 0.5 ml or 0.75 ml AB (+0.5g, 1g or 1.5g Ba(OH)₂ respectively). Further mixtures used 10g Perloza, 2 ml DMSO and 0.8, 1 or 1.2 ml AB (1.6, 2 or 2.4 g Ba(OH)₂).

Solvent proportion

Water solvated Perloza (5g), 0.5 ml AB and 1g Ba(OH)₂ were mixed with 3 ml water or 2 ml of 12.5, 50 or 100% DMSO.

Solvent and base type effects

The standard reaction (24 hour) mixture was 10g water solvated Perloza, 1.5 ml solvent, 0.7 ml AB and 2.4 ml of 3.75 M NaOH. Solvents used were : DMSO, dioxan, acetone, ethanol and water. A second DMSO sample was reacted for 7 hours. A further DMSO reaction used 1.4g Ba(OH)₂ and 2 ml water instead of NaOH solution.

Sepharose activations

Sepharose 6B (10g) was reacted with 0.5 ml AB, 0.1 ml ECH (crosslinker) and 6 ml of 1.7 M NaOH for 48 hours at room temperature. Another 10g sample of Sepharose 6B was reacted likewise, except that 1 ml AB and 6 ml of 2.5 M NaOH were used. Sepharose CL6B (10g) was reacted with 0.6 ml AB and 4.5 ml of 2 M NaOH for 18 hours at room temperature.

Optimised method

Water solvated Perloza (10g), with or without 1.5 ml DMSO, was mixed with 0.7 ml AB and 3 ml of 3M NaOH, 4.5 ml of 2 M NaOH or 5 ml of 2 M NaOH. The optimal mixture for a 7% activation used 4.5 ml of 2 M NaOH (no DMSO). A "stock 7%" activated Perloza was prepared by reaction of 100g Perloza with 7 ml AB and 45 ml of 2 M NaOH. For other AB percentages, the volume of NaOH used was maintained between 0.4-0.45 ml of solution/g Perloza. However the NaOH molarity was varied such that 12.5-13 mMoles of hydroxide were used per ml (approximately 12 mMoles) of AB.

A 2% AB activation of 10g Perloza used (4.5 ml) 0.6 M NaOH. A 6% activation of 50g Perloza used 20 ml of 2 M NaOH. For a 7.5% activation of 400g Perloza, 30 ml AB and 180 ml of 2.1 M NaOH were used. A stock 8% activation of 80g Perloza used 6.4 ml AB and 35 ml of 2.3 M NaOH. For "stock 10%" activation of 1% crosslinked and uncrosslinked Perloza (50g) 5 ml AB and 21.5 ml of 3 M NaOH were used. A second stock 10% activation of uncrosslinked Perloza (75g) used 7.5 ml AB and 32 ml of 3 M NaOH. A 7.5% activation of 700g Perloza 100 medium used 52.5 ml AB and 300 ml of 2.2 M NaOH.

Allyl glycidyl ether activation :

Initial methods

The initial standard was a "30%" activation of base pretreated Perloza. Perloza was washed with 0.3 M NaOH, suction-dried and 10g mixed with 3 ml AGE. Reaction mixtures were shaken manually and formed a "gel-like" suspension. These were incubated without mechanical mixing for up to 48 hours at room temperature, 40°C or 60°C. Samples (approximately 1g) were removed at various times for assay. The standard method adopted was 48 hours at room temperature. Activation mixtures containing 10, 20 and 40% AGE were prepared and reacted in the same manner. The reaction mixtures used were not amenable to mixing although the "fluidity" of the 30% and 40% samples improved after 24 hours of the reaction. These samples were mixed for the second half of the reaction by mechanical shaking.

Use of organic solvents

Reaction mixtures containing an organic solvent were prepared similarly, except that the Perloza (10g) was pretreated with a mixture of 0.3 M NaOH and solvent. The solvent mixtures used were 1:2 (acetone/NaOH) and 1:1 (DMSO/NaOH). These reaction mixtures had superior "fluid" properties to aqueous mixtures and were mixed by mechanical shaking.

Aqueous slurry activation

Another aqueous activation was prepared without pretreatment by mixing water solvated Perloza (8g) with 2 ml of 1.5 M NaOH and 1.2 ml AGE. This produced a more "fluid" slurry. A stock AGE Perloza was prepared in analogous fashion by repeat aqueous slurry activation of 50g cellulose using 15% AGE followed by 12% AGE (and 10 ml of 2 M NaOH on each occasion). Reagent and products of the first reaction were washed out (in a sintered glass funnel) with water before the second addition of reagent and NaOH.

Titration methods :

Acid/base titration

The equipment and methods used were described in Chapter 2. Titrated substitutions were again expressed as mMoles/g (suction-dried) and/or mMoles/g dry (oven-dried). Diethylamine, trimethylamine and cysteamine resins were titrated with 0.1 or 1 M HCl to pH 4. Cysteine resins were titrated with 0.1 M NaOH to pH 11 and corrected by subtraction of an unmodified Perloza control titration to the same pH. Other resins were titrated with 0.1 M NaOH to pH 8 unless otherwise indicated.

For titration curves, 1g of a mercaptopropionic acid (MPA) and 0.6g of a mercaptoacetic acid (MAA) Perloza resin were mixed with 5 ml of 1 M NaCl. Titrant (0.1 M NaOH) was added in suitable increments (10-200 μ l, using the smallest volumes as the end point was approached). An iminodiacetic acid (IDA) Perloza resin (1g) was prepared and titrated similarly.

Bromine water titration of matrix allyl groups

Bromine water titration was by incremental addition (using 100 μ l, 200 μ l or 1 ml Gilson pipettes) of calibrated (approximately 2%) bromine water to a 0.5 - 2 gram sample of activated matrix. The smallest increments (50 μ l) were used once the rate of bromine decolourisation had slowed noticeably, usually after about 75% of the total addition. The endpoint was determined visually. Titration was usually completed in 2-5 minutes. Bromine water was calibrated by two methods :

(I) Bromine water of 1.5 - 2.5 % was prepared as accurately as possible by weighing bromine (1.5-2.5 g) into a (100 ml) volumetric flask and (rapidly) making the volume up to 100 ml with water. The molarity of the bromine water was calculated assuming that the initial Br₂ concentration was maintained (no vapour loss) at the time of titration.

(II) Bromine water of similar concentration range to the previous method was prepared as a stock solution and assayed immediately before use. Bromine water (0.5 ml) was diluted with water to 25 ml and its absorbance measured at 410 nm. Bromine content was calculated by comparison with the values of standards, freshly prepared by method I.

Mercaptoacid titration of allyl groups

Activated matrix (1g) was mixed with 100 μ l of mercaptoacetic acid (MAA) or mercaptopropionic acid (MPA), and 1-5 ml water. The mixture was incubated for 4-16 hours at 60°C or for 24-48 hours at room temperature (MAA only). Samples were

transferred to a sintered glass funnel and excess reagent washed out with 20 ml water, 20 ml 0.1 M NaOH and 10x20 ml water. Preparation and titration of carboxylic acid groups was otherwise the same as described previously.

Elemental analysis

Analyses of samples, oven-dried for 1.5 hours at 110°C, were carried out by the Chemistry Department, University of Otago, Dunedin, New Zealand.

Halogenation methods:

Bromine Water

Bromination of (unbuffered) allyl matrices was similar to the titration method described above except that a surplus (approximately 10%) was used to ensure completeness. Bicarbonate buffered matrices (0.5 M, pH 6.5) were used for bromine water addition in the experiment comparing this method with N-bromosuccinimide addition.

Iodine addition

Iodine (1g) was dissolved in (a) 75 ml of absolute ethanol followed by dilution with water to 100 ml or (b) 100 ml of 1% NaI solution. The iodine solutions were mixed with 1g resin samples for up to 30 minutes. Residual iodine was washed out with ethanol.

N-bromosuccinimide (NBS)

A 1.1 to 1.5 molar excess over allyl groups (estimated or determined by bromine water or MAA titration) was used. The first reaction used solvation with 1:1 DMSO/water (8 ml/g wet matrix). Subsequent experiments used 1-10 ml of water or 0.1 M phosphoric acid/g wet matrix. The solubility of NBS is limited in water and dissolution was aided by crushing larger particles with a glass rod. Reaction was for 0.5-1 hours and all NBS dissolved if the molar excess used was at the lower end of the range (1.1-1.2). Excess NBS was consumed at the end of reaction by KBr (for mixtures containing H₃PO₄) or 0.1 M HBr to produce bromine and succinimide. In another experiment excess NBS was washed out with water and the completeness of allyl group reaction determined by bromine water titration.

Substitution methods for brominated resins:

Amine ligands

These were mixed with brominated resins for 24 hours at 60°C or 24-96 hours at room temperature. Diethylamine, trimethylamine, butylamine and diethylaminopropylamine (DEAPA) were used without modification. Iminodiacetic acid (IDA) was prepared as a 1.5 M solution by dissolution of 4g in approximately 7.5 ml of 7.5 M NaOH and water to a final pH of 11 and volume of 20 ml. The molar excess of amine used was between 5 and 50, usually 5-10.

Thiol ligands

For initial reactions, mercaptoacids (MAA, MPA) and mercaptoethanol were adjusted to pH 7 or 10.5 with 2 M NaOH. Cysteamine and mercaptoethylpyridine (MEP) hydrochlorides were dissolved in water (3 ml/g ligand) and the pH adjusted to 7.5, 9 or 10 with 2 M NaOH. A 5 molar excess (over original allyl groups) of thiol ligand was used. Reaction mixtures also contained 3-5 ml of 1 M phosphate buffer (pH 7, 7.5 or 9) or 1 M carbonate (pH 10) and 1-10 mg NaBH₄ per g brominated matrix. Reactions were for 24 hours at 60°C, except for those samples at pH 10, which were reacted at room temperature. A 3 molar excess of MEP was used once its substitution was optimised at pH 10. The reaction volume of the above examples varied between 8 and 12 ml/g matrix.

For later reactions, using the NBS derivative of stock 8% AB Perloza (1g), MAA and cysteamine were adjusted to pH 10 as before, but using 10 M NaOH. Less buffer (1 M carbonate, pH 11, 0.5-1 ml/g matrix) was used. Total reaction volumes were approximately 2 and 3 ml/g (MAA and cysteamine respectively). A similar procedure was used for reaction of the same activated matrix (2g) with 0.3 ml of mercaptohexanoic acid (MHA) and 0.4g of mercaptobutyric acid (MBA), but extra water was used for dissolution of MBA and its reaction volume was approximately 4 ml/g matrix.

Sodium sulphite

The standard mixture was 200 mg sodium sulphite, 1g brominated matrix and 5 ml water. Reaction was either at 60°C for 8 hours, or room temperature for 24 hours. Variations were the use of less water (matrix + sulphite predissolved in 1 ml of water), pH adjustment (1 ml of 1 M sodium carbonate), solvent inclusion (1 ml of dioxan or DMF), or addition of tetraethylammonium hydroxide (0.1 ml). Another variation was the inclusion of tetrabutylammonium iodide (0.1g) : solid or dissolved in 2 ml of dioxan.

Oxidation of allyl Perloza :

Alkaline

Stock 27% AGE Perloza (1g) was mixed with 100 μ l of H₂O₂, 5 ml water and 1 ml DEAPA for 48 hours at (i) 60°C and (ii) room temperature. The washed products were titrated for amine groups.

Acidic

The same AGE Perloza (1g) was mixed with 100 μ l of H₂O₂, 5 ml water and 1 ml of glacial acetic acid at room temperature for 48 hours. The washed product was reacted for 24 hours at room temperature with 1 ml DEAPA and titrated as above.

Addition methods:

Mercaptoacids

Initial addition of mercaptopropionic acid (MPA) to allyl Perloza used 75 μ l/g wet matrix and included 2.5 mg ammonium persulphate (APS). Subsequent additions used 100 μ l/g. Reaction was for 3-4 hours at 60°C. Variations were room temperature reaction for 16 hours, inclusion of methoxyphenol (20 mg) and exclusion of APS. Reaction at 60°C for at least 4 hours, without APS, was the standard method adopted for MPA and mercaptoacetic acid (MAA) addition. Water (1-5 ml) was included to ensure adequate mixing. The same conditions were used for control reactions with unmodified Perloza. Variations tested were reagent proportion (30 and 60 μ l/g), room temperature (24 or 48 hours) and pH adjustment to 6 (with 7.5 M NaOH).

Mercaptosuccinic acid (MSA) was reacted with allyl Perloza and water (1:1) for 16 hours at 60°C or 64 hours at room temperature. MSA (0.15g/g matrix) was dissolved in the reaction mixture. Glutathione (GSH, free acid form) was reacted under the same conditions, but 0.2g/g matrix was used. GSH was also reacted (at 60°C) with Perloza, using 0.2 M H₃PO₄ instead of water.

Mercaptohexanoic acid (0.2 ml), mercaptobutyric acid (0.5g) and thiosalicylic acid (0.2 ml) were reacted with allyl Perloza (1g) for 48 hours at 60°C.

Mercaptoethanol

Addition of mercaptoethanol (100 μ l) was at 60°C (16 hours) or room temperature (24 or 48 hours). All samples were "back-titrated" by the MAA method. The addition level was determined by difference (original MAA titration - back titration).

Bisulphite

The initial reaction mixture was 0.1g each of sodium sulphite and sodium metabisulphite and 5-10 ml water per g allyl Perloza. Reactions were at room temperature or 60°C and for 4 to 48 hours, as indicated. In other experiments 0.2g of sodium metabisulphite was used, and the pH of the reaction mixture adjusted to 5 or 5.5 with 7.5 M NaOH. For the latter reaction, 1 ml of 4 M acetate buffer, pH 5.5, was included. One sample, prepared at pH 5.5 was reacted for 48 hours without modification. A second sample, prepared at pH 5.5, was readjusted to pH 6 with 7.5 M NaOH after 24 hours and the reaction continued for a further 24 hours. In another experiment a 5 molar excess (each) of sodium metabisulphite and sodium sulphite, adjusted to pH 7 with 7.5 M NaOH, was used and the pH maintained with excess 4 M acetate buffer (2 ml/g Perloza). In a final pH variation, a 10 molar excess of sodium sulphite, adjusted to pH 8 with 3 M HCl, was used without extra buffering.

The addition reaction was optimised by using a smaller reaction volume. Solutions containing equal parts (by weight) of sodium metabisulphite and sodium sulphite were used. Initially, the total sulphite and bisulphite percentage was 32% and 1 ml of "bisulphite" solution was used per g matrix. Later reactions used a 40% sulphite/bisulphite solution (7-1.0 ml/g matrix). Up to half of this solution (0.2-0.5 ml/g) was used to prewash the matrix, which was then suction-dried. The remainder (0.5 ml/g) was mixed with the suction-dried matrix. All larger scale reactions (20-250g allyl Perloza) were carried out by this method. It was also used for a control reaction with unmodified Perloza.

Cysteine, cysteamine and MEP

The first addition reactions used unbuffered cysteine hydrochloride. Reaction was for 16 hours at 60°C using AGE activated Perloza (1g), 0.08g cysteine HCl and 5 ml water. Subsequently, cysteine (5 molar excess, +/- 50 µl formic acid) was used rather than its hydrochloride. Trichloroacetic acid, oxalic acid or acetic acid were also used instead of formic acid (1 mole/mole of cysteine). Acetic and formic acids were also used in larger amounts (5 or 10 molar equivalents). Cysteamine and MEP hydrochlorides (5 molar excess) were neutralised with a 1 molar equivalent of (7.5 M) NaOH and reacidified with a 2-5 molar excess of an organic acid. For initial addition reactions, acetic acid was used and the solutions were reacted with AGE Perloza for 16 hours at 60°C. These are referred to as cysteamine/acetate and MEP/acetate addition mixtures. Formic acid was used for subsequent additions (cysteamine/formate and MEP/formate mixtures).

The initial addition of cysteamine/formate (5 molar excess) to stock 7% AB activated Perloza was for 48 hours at 60°C +/- ammonium persulphate (2.5 mg) or benzoyl peroxide (3.6 mg). A further (uncatalysed) sample was reacted for 96 hours at 60°C. Similar additions (to stock 10% AB Perloza) were carried out for 24, 48, 96 and 144 hours at 70-75°C, using a 10 molar excess of cysteamine/formate. Allyl Perloza (6% AB stock) was reacted likewise with cysteamine/formate. A control sample of 10% stock AB Perloza and cysteamine HCl was also reacted for 144 hours at 70°C. The same temperature was used for addition (96 hours) of a 10 molar excess of cysteine/formic acid to stock 10% AB Perloza (1.7g). Control reactions (96 hours, 60°C) with allyl Perloza used ethanolamine (+/- 1 molar equivalent of formic acid). A control reaction was also carried out using unmodified Perloza and cysteamine/formate.

Other additions

AGE activated Perloza was reacted with thiolacetic acid (100 µl) and 5 ml water for 24 hours at room temperature or 16 hours at 60°C. Another room temperature reaction used ethanol solvation rather than water. The AGE activated Perloza was washed with 5 volumes each of 50% and 100% ethanol, suction-dried, and mixed with thiolacetic acid and 5 ml of ethanol. Water solvated AB Perloza (7% stock) was used for addition of thiolacetic acid (0.2 ml), together with 2 ml each of water and ethanol, for 90 hours at room temperature. Thiophenol (0.2 ml) was mixed with 7% stock AB Perloza (1g), 2 ml water and 3 ml acetone or ethanol, for 90 hours at room temperature.

Thiolacetate resins were washed with 20 volumes of water. Thiophenol resins were washed with 10 volumes each of ethanol, 0.1 M NaOH and water. Unreacted allyl groups were determined by MAA "back" titration.

RESULTS AND DISCUSSION

Allyl bromide activation of Perloza and titration methods :

Initial experiments

An initial investigation of allyl bromide reaction with Perloza demonstrated significantly higher activation levels (between 0.13 and 0.15 mMoles/g, by bromine water titration) than could be obtained using ECH activation. These results were obtained using 0.3 ml allyl bromide per 10g of 75% DMSO solvated Perloza. Activation with 0.6 ml AB ("6% AB mixture") for 3 hours, bromination and substitution with sodium sulphite or ethanolamine resulted in ligand densities of 0.20 mMoles/g (1.22-1.28 mMoles/g dry). By contrast, the highest substitution obtained by ECH activation of Perloza was 0.66 mMoles/g dry, using a far greater amount of reagent.

These activation conditions were chosen to minimise water content and provide a slight (approximately 1.05) molar excess of hydroxide over AB. A significant reduction in matrix swollen volume (10-15 % less weight of suction dried matrix after activation) was found for highly activated samples. Samples did not regain their original swollen volume upon ligand substitution. It was considered that reduced swollen volume might be caused by extensive crosslinking or by hydrophobic effects of the allyl groups. AB should not cause crosslinking itself (Lindgren, 1994) but impurities might do so. The AB used was slightly brown coloured whereas it should be colourless. A colourless reagent was obtained, by distillation, and used for subsequent experiments.

Room temperature activation

Room temperature was apparently adequate for activation because a high level (0.15 mMoles/g wet weight) was obtained after a 1 hour reaction, using the 6% AB mixture described for the 60°C reaction. All subsequent allyl bromide activations were at room temperature but the reaction time was increased to 24 hours.

Bromine water titration

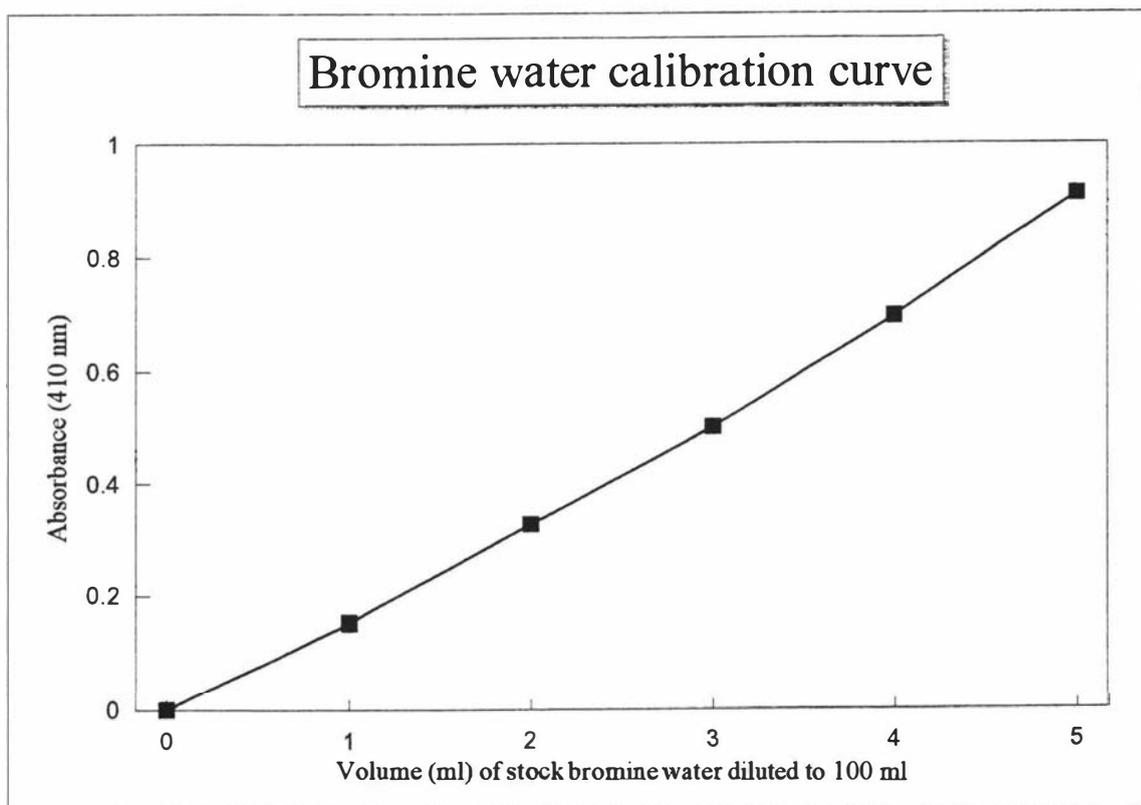
Initial assays of activation level were hampered by variability, especially if bromine water was not freshly prepared. Quantification was improved by using a spectrophotometric assay of the bromine water immediately before titration. A series of dilutions of a stock bromine water solution (1:99, 2:98, 3:97, 4:96 and 5:95) had absorbances of 0.154, 0.327, 0.500, 0.698 and 0.910 respectively (Figure 3.5). A reasonably linear increase in absorbance was found, up to 0.5. Therefore measurements were confined to this range using the appropriate dilution. The absorbance of freshly prepared bromine water at 410

nm was 0.117 for a 0.014% solution and 0.305 for a 0.036% solution. These values equated to : absorbance (x dilution) x 0.12 = bromine water %. This conversion was used for subsequent "quantitative bromination titration". This bromine water titration was still less than ideal for a routine laboratory assay because of accuracy limitations and noxious fumes associated with preparation and use of bromine water.

Alternatives to sodium hydroxide

The initial pH of reaction mixtures described above would be very high (estimated to be > 14). Lower reaction pH might reduce the rate of allyl bromide hydrolysis relative to the desired etherification. Therefore the 2 ml of 3.75 M NaOH solution used for 6% AB activation was replaced by barium/calcium hydroxide or potassium phosphate (solids) and 2 ml water. High levels of activation were obtained using Ba(OH)₂ (0.46 mMoles/g wet weight) but lower levels were obtained using Ca(OH)₂ and K₃PO₄ (each 0.1 mMoles/g wet weight). Ca(OH)₂ has a lower solubility than Ba(OH)₂ and the saturated hydroxide concentrations with respect to water were expected to be approximately 0.01

Figure 3.5 : Absorbance versus concentration of bromine water



M and 0.2 M respectively (CRC, 1971). The pH of a 1 M K_3PO_4 solution was 13 but the starting pH would not be maintained during the reaction because of buffering effects. Therefore it seemed that a base of sufficient strength to maintain the reaction pH at approximately 13-13.5 was required for optimal activation but that higher pH reduced activation efficiency. However the swollen volumes of the lower activated matrices produced using the weaker bases was not significantly altered, indicating that high levels of activation were responsible for matrix shrinkage. A very highly activated matrix (0.71 mMoles/g) was produced by increasing the proportion of allyl bromide to 1 ml per 10g suction-dried (75% DMSO) Perloza.

Another highly substituted matrix (0.43 mMoles/g) was produced using (10g) water solvated Perloza and 25% AB. Although reagent consumption was much higher, this reaction demonstrated that high activation levels could be obtained in aqueous media. Effective reaction occurred despite the limited solubility of AB in water.

The relationship of activation level to reagent proportion

The relationship between reagent : cellulose and activation level for 1:6 DMSO:water solvated cellulose was determined using 5, 10 and 15 % AB and $Ba(OH)_2$. The titration values increased in a roughly linear fashion (Table 3.1). There was no indication of reduced reactivity of cellulose at increasing levels of activation. This was consistent with the extremely high activation levels obtained previously.

Table 3.1 : Comparison of titration methods for allyl bromide activation levels

Sulphonate derivatives were prepared by sulphite substitution of the brominated matrix

AB %	Titration (mMoles/g)		
	Bromine	MPA	Sulphonate
5	0.12	0.116	0.111
10	0.18	0.174	0.166
15	0.30	0.273	0.172
10 (no DMSO)	0.10	0.093	0.096

Allyl group quantification by base titration of mercaptoacid derivatives

The 5-15% AB activation levels include values determined by mercaptopropionic acid (MPA) addition and titration. Correlation with bromine water titration values (Table 3.1) was reasonable and the variations due to visual assessment of titration endpoint and falling bromine content over time were avoided. By contrast sulphonate titration values gave a low estimate of activation level, especially at high AB percentages. The MPA titration method was used henceforth for routine assay of activation levels until MPA was replaced by mercaptoacetic acid (MAA). Completeness of reaction has been assumed because titration values have always matched or exceeded those with other ligands and were repeatable. However completeness could not be assessed by bromine water titration of unreacted allyl groups because bromine would also be decolourised by reaction (oxidation) with the thioether linkage between the mercaptoacid and the matrix (Smith and Hernestam, 1954).

Organic solvent (%) effects

AB (10%) activation of water solvated Perloza resulted in an activation level of 0.79 mMoles/g, suggesting that a small percentage of DMSO in the reaction mixture greatly enhanced efficiency. This difference was attributed to improved reagent mixing. The effect of increasing the proportion of DMSO to cellulose, using a constant amount of water and AB (10%), was to increase activation level initially but no improvement was found above 20% DMSO (Table 3.2). Sulphonate titration values were again low.

Table 3.2 : Titration values for variations of reagent and cosolvent proportion

The base used was Ba(OH)₂. DMSO % = DMSO used (ml)/g Perloza

AB %	DMSO %	Titration (mMoles/g)	
		MPA	Sulphonate
10	5	0.153	n.d.
10	20	0.203	n.d.
10	40	0.197	n.d.
8	20	0.188	0.116
10	20	0.232	0.126
12	20	0.269	0.119

Variation of solvent (type), using NaOH solutions

Because the water content of reaction mixtures had been increased, NaOH could be used without the initial pH exceeding 14. A 7% AB reaction (24 hour) with Ba(OH)₂, water and 15% DMSO resulted in an activation level of 0.183 mMoles/g. A greater activation level (0.202 mMoles/g) was obtained using 2.4 ml of 3.75 M NaOH instead of Ba(OH)₂/water. The effect of solvent type, using NaOH is summarised in Table 3.3. Equivalent activation was obtained using acetone instead of DMSO. Slightly lower levels resulted with dioxan and water and much lower with ethanol. A 7 hour reaction sample (DMSO) had an activation level of approximately 70 % of the 24 hour reaction. Because extra water was used in the 100% aqueous sample, the difference between the aqueous and DMSO/acetone activation level may be simply attributed to the increased water : cellulose rather than the reaction being enhanced by organic solvents.

Table 3.3 : Activation levels with solvent and base variations

Uncalibrated bromine water, approximately 0.1 mMoles/ml was used. Good correlation was again found between bromine water and mercaptopropionic acid, whereas the sulphonate results were consistently lower. There appeared to be an upper limit to the sulphonate level of approximately 0.16 mMoles/g.

Cosolvent	Bromine titration ml/g	Titration (mMoles/g)	
		MPA	Sulphonate
Ethanol	1.0	0.121	0.104
Water	1.5	0.175	0.156
Dioxan	1.6	0.190	0.156
Acetone	1.7	0.204	0.157
DMSO	1.7	0.202	0.158
DMSO, 7 hour	1.2	0.145	0.124
DMSO, Ba(OH) ₂	1.6	0.183	0.139

Sepharose activation

Reaction of Sepharose 6B with 5 and 10% AB resulted in (MPA) titrations of 0.088 and 0.139 mMoles/g (1.60 and 1.97 mMoles/g dry). ECH (included to crosslink Sepharose) was not expected to affect the titration level significantly because its proportion was low and it should react to completion with the base excess used. Reaction of Sepharose CL6B with 6% AB also produced a highly activated matrix (1.42 mMoles/g dry).

Optimisation of aqueous activation of Perloza

For subsequent allyl bromide activations aqueous solvation was standard. To provide optimal mixing by "external" methods (e.g. rotation rather than a paddle stirrer), 4 to 5 ml of NaOH solution was required per 10g cellulose. This produced a free flowing slurry. Therefore the molarity rather than volume of NaOH solution was varied for different AB proportions. Similar activation (0.163-0.169 mMoles/g) of Perloza (10g) with 0.7 ml AB was obtained using 3 ml of 3 M NaOH or 4.5 ml of 2 M NaOH (+/- 1.5 ml DMSO). The activation using 3 M NaOH was expected to give a higher result but the mixing was unsatisfactory. When 1.5 ml of DMSO was included to improve mixing, a higher activation level was obtained (0.199 mMoles/g). A much lower level (0.11 mMoles/g) was obtained when a slightly greater proportion (5 ml) of 2 M NaOH was used. At the 10g (cellulose) reaction level, a typical activation increased 0.023 - 0.025 mMoles/g for every percentage increase of AB. Slightly higher levels were found at the 100-700g reaction level. These results are summarised in Table 3.4.

Table 3.4 : Titration values for various AB% and 10-700g cellulose scales

Unless otherwise noted, 10g Perloza fine was used, . Medium Perloza was used for the 700g reaction.

Reaction mixture NaOH, solvent	AB %	MAA titration (mMoles/g)	(mMoles/g dry)
4.5 ml, 0.6M	2	0.051	n.d.
20 ml, 2M (50g)	6	0.139	n.d.
4.5 ml, 2M	7	0.169	1.22
4.5 ml, 2M, 1 ml DMSO	7	0.164	1.25
3 ml, 3M	7	0.168	1.23
5 ml, 2M	7	0.111	0.84
3 ml, 3 M, 1.5 ml DMSO	7	0.199	1.35
45 ml, 2 M (100g)	7	0.181	1.36
35 ml, 2.3 M (80g)	8	0.202	1.51
180 ml, 2.1 M (400g)	7.5	0.188	1.46
20.5 ml, 3 M (50g)	10	0.252	1.67
300 ml, 2.2 M (700g)	7.5	0.260	1.46

Comparison of AB with other activation methods

The efficiency of AB use in aqueous media was 20-25% (mMoles of allyl groups attached to the matrix per 100 mMoles AB used) using optimised reaction conditions. High activation levels were obtained using small amounts of this cheap reagent (laboratory price ~ US\$ 4 per mole). Only tosyl chloride and ECH of the commonly used reagents are of similarly low cost (Appendix 1). The former reagent requires anhydrous solvents while the latter proved ineffective for activation of Perloza.

Activation was analysed simply by bromine water titration (semi-quantitative) or by mercaptoacid addition and subsequent titration of carboxyl groups. Although swollen volume reduction was found initially for highly activated resins, this effect was not significant once activation methods were optimised. Consistent results were obtained when the preferred reaction volume and base excess were adhered to.

Allyl glycidyl ether (AGE) activation :

Initial reaction conditions

AGE was reacted with Perloza solvated with 0.3 M NaOH, by analogy to the optimal hydroxide concentration reported by Sundberg and Porath (1974) for bisepoxide activation. Reaction mixtures were prepared as thick suspensions by mixing AGE with suction dried (NaOH solvated) cellulose. The standard amount of AGE used was 0.3 ml/g (30%). Reaction at 60°C was complete by 5 hours. The (unassayed) bromine water titration of 1.7 ml/g suction-dried Perloza was unchanged after a further 19 hours reaction. Slightly higher activation levels were obtained at 40°C (1.8 ml/g after 24 hours) and room temperature (1.5 and 2 ml/g at 24 and 48 hours), using the same bromine water solution. The 48 hour, room temperature activation level was 0.183 mMoles/g (using assayed bromine water). This activated cellulose had a similar wet weight to the original cellulose. It was therefore assumed that matrix swollen volume was not significantly altered by activation. A sulphonate resin (0.178 mMoles/g) was prepared by reaction of sodium sulphite with the brominated matrix. This suggested reasonable accuracy of the bromine water titration value and that substitution reactions of the brominated AGE matrix was more efficient (95-100%) than for AB Perloza.

Reagent proportion and activation level

A comparison of activation level for 10%, 20% and 40% AGE mixtures demonstrated a lower efficiency of reagent use as the percentage was increased (Table 3.5). However a very high activation level (0.315 mMoles/g by bromine water assay) was obtained by repeat activation using 30% AGE.

Table 3.5 : AGE% versus activation level

AGE %	MPA titration (mMoles/g)
10	0.078
20	0.133
30	0.172
40	0.168

Solvation effects

AGE (15%) activation (72 hours at room temperature) efficiency was compared for Perloza (10g), solvated by 0.3 M NaOH or a 50:50 DMSO/0.3 M NaOH mixture. The activation levels (MPA titration) were 0.109 and 0.194 mMoles/g respectively. The higher activation level was attributed to a lower rate of hydrolysis. Activation was compared for a 33% acetone solvated, 15% AGE slurry and an aqueous solvated, 12% suspension. The respective titrations after 96 hours, were 1.05 and 0.703 mMoles/g dry. No significant change was found after 48 hours for the aqueous method but the 33% acetone samples required 72 hours for complete reaction (Table 3.6).

Table 3.6 : MPA titrations for varying AGE reaction time and solvation

Reaction time	Titration (mMoles/g dry)	
	Water	33% Acetone
48	0.72	0.90
72	0.74	1.04
96	0.70	1.05

Aqueous slurry activation

The use of acetone or DMSO improves the "fluidity" of the reaction mixture allowing efficient mixing by rotation or shaking. Extra 0.3 M NaOH (2 ml per 8g of Perloza) was required for similar mixing of 100% aqueous samples. A 48 hour activation by this method with 15% AGE resulted in an activation level of 0.113 mMoles/g. Stock AGE (27%) was prepared by this aqueous slurry method, using 15% AGE followed by a second activation step with 12% AGE. The activation level was 0.153 mMoles/g.

Comparison of AGE with other methods

Although Perloza activation with AGE was less efficient than with AB, especially at high levels, it was still a great improvement over previous methods. High activation levels were obtained by use of large amounts of AGE, partial organic solvation or repeat activation. Activation was therefore more expensive than for AB. These disadvantages are offset by the lower toxicity and flammability of AGE. Furthermore, substitution efficiency of brominated AGE Perloza was higher than for the corresponding AB matrices and the 7 atom AGE spacer arm should be beneficial for chromatography.

Reactions of allyl matrices :

Stock AB and AGE Perloza activation levels

The MAA titration values of stock resins used to study ligand substitution and addition reactions are summarised in Table 3.7.

Table 3.7 : Stock allyl resin activation data (MAA titration)

Matrix	Titration	
	(mMoles/g)	(mMoles/g dry)
AGE 27%	0.153	1.17
AB 6%	0.139	n.d.
AB 7%	0.180	1.36
AB 8%	0.202	1.51
AB 10%	0.252	1.67

Bromination:

Initially reaction with bromine water was used both to titrate activated matrices and to prepare reactive bromohydrin intermediates for substitution of amine, thiol and sulphite nucleophiles. Although the predominant reaction with bromine water is expected to be bromohydrin formation (HOBr addition), some dibromide formation will occur (Figure 3.3 a,b), due to competition between bromide ions and water for the bromonium intermediate (McMurray, 1988). Bromohydrin groups are preferred because they react readily with nucleophiles at alkaline pH, by formation of an epoxide intermediate (Ellingboe et al., 1970). Dibromide groups do not form this intermediate. Although water solvation was used, an S_N2 mechanism was expected using strong amine and thiol

nucleophiles (McMurray, 1988). Therefore the secondary bromide group will be less reactive than the primary, which in turn may have reduced reactivity due to the inductive effect of the second halide group (Kemp and Vellaccio, 1980). Steric restriction effects due to the greater bulk of two bromine groups may also effect reaction yield. Therefore dibromide groups may react less efficiently than bromohydrins and the level of residual (especially secondary) bromide groups may be greater. Blocking of unreacted groups may thus be more arduous. Bromine water cannot be prepared and stored in bulk because it decolourises slowly, even in the dark. Noxious fumes due to bromine vapour are a hazard during preparation and use.

Other halogen additions

Halohydrins could also be formed by chlorine or iodine addition. The reactivity of alkyl halides decreases in the order : iodides > bromides > chlorides. Therefore chlorine was not favoured but successful iodine addition would be useful. However iodine solutions react poorly with alkenes (Cornforth and Green, 1970) and no evidence of addition (decolourisation) to allyl matrices was found. Bromine water titration values of iodine treated and untreated samples were equivalent, indicating that no reaction had occurred with iodine. Iodine addition might be improved by use of iodate (Adinolfi et al., 1976) but this was not tried because of the comparative ease of bromination.

Bromohydrin formation using N-bromosuccinimide (NBS)

Reaction of allyl Perloza with a 1.1 -1.5 molar excess of NBS, for 0.5-1 hours, resulted in complete reaction of allyl groups (negative alkene test with bromine water), whether using water or a mixture of DMSO and water for solvation. Unless Br^+ is reduced, there is no Br^- to compete with water for the bromonium intermediate. Dibromide formation should therefore be minimised or eliminated (Dalton and Dutta, 1971). Direct proof was not obtained but small differences in ligand substitution levels were found after the respective brominations (Table 3.8). Amine substitutions of NBS modified matrices were higher but MAA substitution levels were lower, compared to bromine water treated matrices. These results indicated real differences between the NBS and bromine water reaction, as expected. The different trend between the amine and the mercaptan may be due to higher reactivity of the mercaptide ion compared to the amine allowing a greater level of reaction with dibromide groups. Nevertheless the results obtained by the two methods were relatively similar and other factors influenced the selection between the two methods.

Table 3.8 : Ligand substitution levels of NBS and bromine water intermediates

The AGE Perloza sample used for ethanolamine was from a 30% activation (MAA titration of 0.177 mMoles/g). Elemental analysis of the ethanolamine samples gave lower results (0.6 and 0.55 mMoles/g dry) but the difference was maintained. DEAPA is diethylaminopropylamine.

Ligand / matrix	Titration (mMoles/g dry)	
	(NBS)	(Br ₂)
ethanolamine / AGE	0.83	0.79
DEAPA / AGE stock	0.67	0.62
DEAPA / 7% AB stock	0.69	0.66
DEAPA / 10% AB stock	0.88	0.79
MAA / AGE stock	0.94	0.99
MAA / 7% AB stock	0.94	1.00
MAA / 10% AB stock	0.87	1.06

Aqueous bromination with NBS was adopted as the method of choice. In addition to the proposed reaction advantages, solution preparation was not required and noxious fumes were avoided. Buffering was not required, whereas bromine water addition is an acid producing reaction and the importance of pH control was reported by Lindgren (1994). Acids which do not interfere with bromination may promote the action of NBS (Heasley et al., 1983). The products of NBS reaction with an AGE Perloza matrix at acidic (pH 2) and neutral pH were indistinguishable by MAA titration (both 0.125 mMoles/g) of the sulphite derivative, although no attempt was made to compare reaction rates. No indication of bromine formation occurred in either reaction but a bright orange colour appeared upon addition of bromide ions, at acidic pH, indicating both that NBS had not been exhausted and that bromide ion formation during the reaction, if any, was insignificant. Other halosuccinimides might have been used, but there was no perceived advantage for use of N-chlorosuccinimide and N-iodosuccinimide was rejected because of expense. The Aldrich catalogue prices of bromine and NBS are similar and inexpensive (US\$8/Mole). The Janssen catalogue price for bromine is lower (approximately US\$4/Mole but the difference compared to NBS is likely to be a small consideration in terms of overall resin cost. Even at high activation levels, the requirement of bromine or NBS per litre of resin is unlikely to exceed 0.3 Moles.

Substitution reactions of brominated allyl Perloza :

Reactivity differences between AB and AGE Perloza derivatives

For AGE activated matrices the reaction efficiency of matrices treated with bromine water was apparently very good (sulphite substitution up to 95% of the bromine titration value). However some residual bromine was found by elemental analysis, especially for highly activated samples. The sulphite derivative of a repeat 30% activated matrix (0.315 mMoles/g bromine water titration) was titrated at 0.272 mMoles/g (1.61 mMoles/g dry, elemental analysis 1.32 mMoles/g dry). The apparent sulphite substitution efficiency was 86%. The small amount of unreacted bromine (0.19 mMoles/g dry) detected in the elemental analysis was consistent with the titrated substitution efficiency. The small proportion of unreacted bromine might be due to steric restriction of brominated groups or the presence of dibromide groups.

The first AB matrices were highly activated (bromine water titration) but the ligand substitution level following bromination was low. For example an AB matrix (0.46 mMoles/g) produced a sulphonate resin of 0.22 mMoles/g (48% efficiency). This suggested that either the bromine water titration was misleading or that the reactivity of brominated AB Perloza was less than that of brominated AGE Perloza. The bromine water titration could have overestimated activation if removal of non-covalently bound allyl groups (allyl bromide or allyl alcohol produced by hydrolysis) was inadequate. However MPA titration later confirmed the bromine water titration values and the residual bromine (12.8%) found by elemental analysis was substantial.

This poor reactivity could be due to steric factors (exacerbated by the shorter spacer arm) or different proportions of bromohydrin and dibromide formation. Steric factors could include ligand exclusion effects caused by the formation of a "ligand layer" as the reaction proceeds. Substitution of sodium sulphite will result in formation of a negatively charged surface which will repel sulphite ions, limiting access to remaining activated groups. By contrast amine ligands would be reacted in the uncharged (unprotonated) form and if the reaction pH was maintained above 11 the amine linkages formed would also be neutral. Charge shielding effects should be most noticeable on AB activated matrices because distances between groups and flexibility will be lower. Charge shielding effects might be reduced by increasing the ionic strength of the reaction medium, especially by using concentrated ligand solutions. Surfactants and organic solvents might also facilitate access of ligands to the matrix surface.

Amine substitution

Low substitution efficiencies were found for both NBS and bromine water modified allyl Perloza (1g), reacted with DEAPA (1 ml) and 5-6 ml of water (Table 3.8). Substitution of AGE Perloza derivatives was slightly more efficient than for AB derivatives. The substitution data for amine ligands other than DEAPA is summarised in Table 3.9. Superior substitution efficiencies were obtained using diethylamine (DEA), trimethylamine (TMA) and n-butylamine at the same ratio of amine to cellulose (1:1) but without added water. Whether due to use of more concentrated reaction mixtures or greater reactivity of the amines, substitution levels were consistently high (80-90% based on MAA titration results, Table 3.7). There was no indication of a limit of substitution for AB activated matrices. Subsequent reactions using less reagent (15 ml DEA + 35 ml water or 25 ml of 45% TMA + 25 ml water per 100g activated matrix) were equally effective, suggesting that superior reactivity was responsible.

Table 3.9: Substitution levels of various amine ligands on brominated allyl Perloza

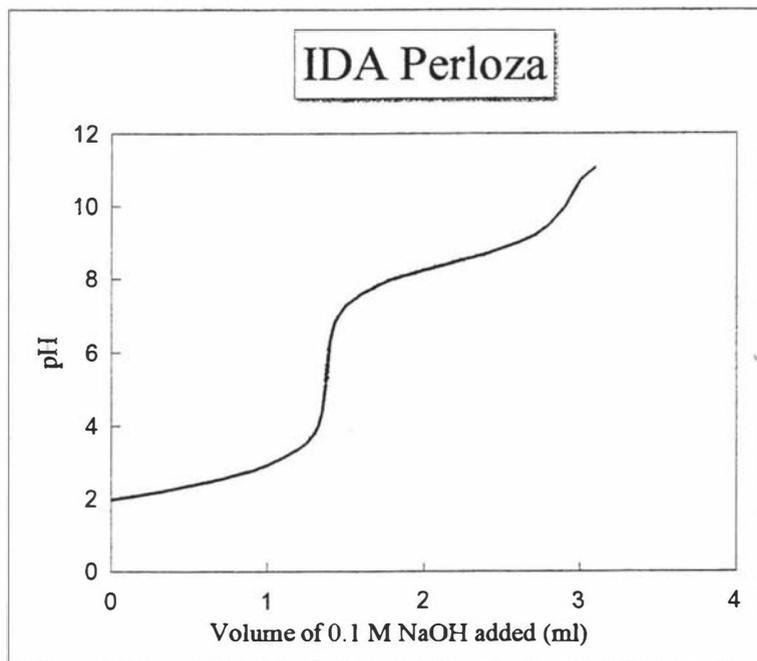
Stock resins were Perloza activated with 7% AB, 10% AB or 27% AGE. All samples were brominated by the NBS/water method.

Chemistry (%)	Ligand	Titration (mMoles/g)	Dry weight (g dry)	Substitution (mMoles/g dry)
AB (10)	butylamine	0.192	0.134	1.43
AB (7)	butylamine	0.146	0.125	1.17
AGE	butylamine	0.136	0.128	1.07
AB (10)	trimethylamine	0.212	0.150	1.42
AB (7)	trimethylamine	0.147	0.126	1.17
AB (7)	diethylamine	0.159	0.138	1.15
AB (10)	iminodiacetate	0.187	0.163	1.15
AB (7)	iminodiacetate	0.139	0.134	1.04
AGE	iminodiacetate	0.145	0.143	1.01

High substitution levels were also found with disodium iminodiacetate (IDA) solutions (5 molar excess), although the efficiencies were somewhat lower, especially for a 10% AB matrix. This occurred despite the amine group being secondary and the likelihood of greater steric restriction effects. Charge repulsion effects could occur but the use of 1.5

M IDA solutions might have reduced this effect. The level of IDA substitution was determined by titration with NaOH to pH's 7 and 11. The titration curve of an IDA resin (Figure 3.6) confirmed that only one carboxyl group was titrated (to pH 7). It also suggested an end point of pH 10 would be adequate for titration of the imine group.

Figure 3.6 : Titration curve of (AGE, NBS) IDA Perloza



IDA resins can be used for immobilised metal ion affinity chromatography (IMAC). The products of TMA and DEA substitution are useful for ion exchange chromatography and their properties are described further in Chapter 4.

Thiol substitution

Initial substitutions of brominated allyl Perloza with the sodium salt of MAA (Table 3.8) were inefficient compared to the corresponding MAA addition data (Table 3.7). The reaction mixture used was relatively dilute to facilitate mixing and charge shielding may have caused the low yield. These reactions were at room temperature and pH 10.5.

Other thiol substitution data are summarised in Table 3.10. Similar substitution levels were found at pH 8.5, but lower substitution levels were found at pH 7 (both 60°C). Substitution of cysteamine at room temperature, pH 10, was also higher than at 60°C, pH 7. Charge shielding by amine hydrochloride groups may affect the result at pH 7.

However the amine group could compete with the thiol for bromohydrin groups at pH 10-11. The significance of this competition is not expected to be great due to the far greater reactivity of thiols (Friedman et al., 1965). Subsequent results for sulphite substitution showed that efficiency could be increased by use of minimal reaction volumes. The efficiency of MAA and cysteamine substitution were also increased by this approach, approaching the values obtained for MAA addition titrations. Similar efficiency was found using mercaptohexanoic acid (MHA). A larger reaction volume was used for mercaptobutyric acid (MBA) and consequently the substitution efficiency was lower. These products could be used for ion exchange chromatography, mixed mode chromatography (Chapter 5) or for attachment of amine/carboxyl groups by condensation reactions. Aminopropyl cellulose has been used as a support for solid phase peptide synthesis (Englebretsen and Harding, 1992). The preparation of aminopropyl Perloza included a 3 hour (reflux) anhydrous reduction with diborane. Perloza (allyl/NBS) cysteamine derivatives could be used instead of the aminopropyl resin and are simpler, safer and cheaper to prepare. They could also be used for the protein immobilisation and immunoaffinity chromatography (Stubbings et al., 1993).

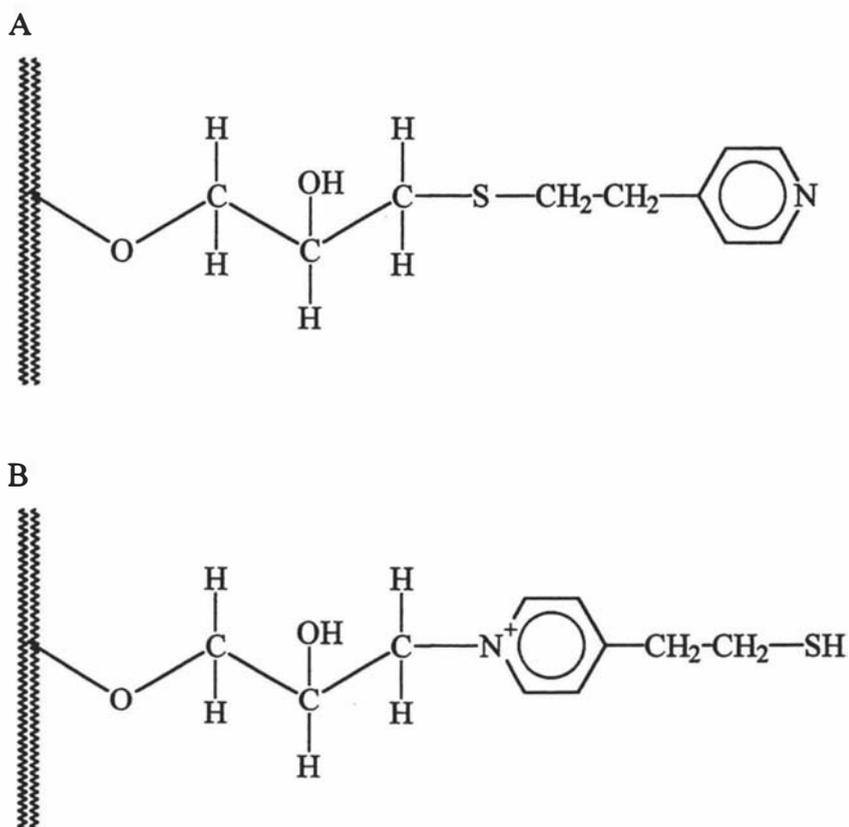
Table 3.10 : Substitution data for thiol ligands

The reaction pH was 10.5-11 unless otherwise stated. Minimal reaction volumes were used for matrices marked *. Stock AGE (27%) and AB matrices were used.

Matrix	Ligand	Titration (mMoles/g)	Weight (g dry)	Substitution (mMoles/g dry)
AGE, pH 7.0	MAA	0.119	n.d.	n.d.
AGE, pH 8.5	MAA	0.137	n.d.	n.d.
AB 8%*	MAA	0.183	0.125	1.47
AB 8%*	MHA	0.178	0.156	1.14
AB 8%	MBA	0.144	0.135	1.08
AGE, pH 7	cysteamine	0.141	n.d.	n.d.
AGE, pH 10	cysteamine	0.120	n.d.	n.d.
AB 8%	cysteamine	0.153	n.d.	n.d.
AB 8%*	cysteamine	0.207	n.d.	n.d.
AB 10%	MEP	0.209	0.130	1.49
AB 7%	MEP	0.162	0.134	1.20
AGE	MEP	0.166	0.150	1.11

Substitution efficiency of NBS modified allyl matrices with 4-mercaptopyridine (4MP) and mercaptoethylpyridine (MEP) at pH 7.5 (60°C) was also inefficient, especially using AB activation chemistry. The titrations (0.08 and 0.1 mMoles/g respectively) for an AB Perloza were much lower than that obtained by MPA addition (0.175 mMoles/g). The starting pH values were 10.25 and 10.86 respectively. A substantial proportion of the titrations (6% for 4MP and 20% for MEP) occurred above pH 8, and therefore was above the range expected for the pyridyl group. This indicated that a substantial side reaction between activated groups and pyridine had occurred, producing (quaternary) pyridinium groups (Figure 3.7b) rather than thioether linked pyridine (Figure 3.7a).

Figure 3.7 : (A) Thioether linked MEP ; (B) Pyridinium linked MEP



A similar result (0.098 mMoles/g) was obtained using MEP and 30% AGE Perloza (MPA titration 0.198 mMoles/g), although the start pH of titration was much closer to neutral (8.45). Improved efficiency was obtained by reaction at pH 9, using the same AGE matrix (titration 0.168 mMoles/g, starting pH 7.72). Reaction at pH 10, room temperature gave a similar result (titration 0.164 mMoles/g, starting pH 7.04,). No evidence of a significant side reaction was found for these substitutions, presumably

because the thiolate ion reacted much faster than thiol or pyridyl groups. Room temperature substitution at pH 10 was adopted as the standard method. The use of a lower (3) molar excess of MEP did not greatly effect the outcome (start pH of 7.66, titration of 0.163 mMoles/g).

Substitution levels for stock AB and AGE matrices ranged between 1.11 and 1.49 mMoles/g dry (Table 3.10). If the dry weight was corrected for the added weight of MEP groups (compared to MAA), the substitution level was similar to the MAA addition level (Table 3.7). Corrected substitutions were 1.21, 1.32 and 1.68 mMoles/g dry for AGE, AB 7% and AB 10% matrices respectively. The corrected values were obtained by multiplying the moles of NaOH used to titrate the sample by the molar weight difference of MEP hydrochloride (175.5 +16 g/Mole for the oxygen of the hydroxypropyl linkage) and sodium mercaptoacetate (114 g/Mole). The correction may be excessive because some HCl may be washed from the resin before weighing. The superior substitution efficiency compared to the other thiols tested was attributed to the absence of charge shielding effects. Hydrophobic interactions between pyridyl groups and the activated matrix might also assist this reaction. The titration of 4MP and MEP resins and their chromatographic properties are discussed in Chapter 6.

Substitution of AB activated matrices by sodium sulphite

Initial reactions of sodium sulphite at 60°C (16 hours) with AB Perloza matrices of various substitution level were inefficient compared to MPA addition (Tables 3.1, 3.2 and 3.3). Reinvestigation of sulphite substitution, using stock 10% AB Perloza, showed that substitution levels could be increased by altering reaction conditions. Higher substitution levels were obtained by reaction at 60°C than at room temperature. Substitution efficiency was further improved by use of a smaller reaction volume (the sulphite concentration in the reaction mixture was approximately 1 molar) at 60°C. Other reagents were used to try to overcome the effect of charge shielding by analogy to the methods of Paul and Ranby (1976). The room temperature reaction was slightly enhanced by inclusion of tetrabutylammonium iodide (TBI) in the reaction mixture. However this difference may have been due to variation of reaction volume. Inclusion of dioxan had no effect on the room temperature reaction but DMF resulted in a lower level. The use of tetraethylammonium hydroxide (TEOH) also depressed the substitution level. Addition of dioxan solvated TBI (0.5 ml of a saturated solution) or NaCl to the 60 °C reaction resulted in slightly lower substitution levels. Therefore the best method for substitution of AB activated matrices was reaction with concentrated sulphite solutions at 60°C. These results are recorded in Table 3.11.

Table 3.11 : Substitution levels for sodium sulphite on stock 10% AB Perloza

Method	Titration (mMoles/g)	Titration (mMoles/g dry)
room temperature	0.163	1.18
+ dioxan	0.164	n.d.
+ DMF	0.149	1.08
+ TBI	0.177	1.29
+ TEOH	0.149	n.d.
concentrated mixture	0.175	n.d.
60°C	0.180	1.27
+ carbonate buffer	0.184	1.39
concentrated mixture	0.202	1.49
+ TBI, dioxan	0.178	1.34
+ NaCl	0.185	1.25

Oxidation of matrix allyl groups :

Attempts to convert the allyl group to an epoxypropyl group (and substitution of this with DEAPA) using either alkaline H₂O₂ or aqueous acetic acid/H₂O₂ were apparently unsuccessful, despite using an excess (about 5 molar) of H₂O₂. The titrations were 0.003 - 0.004 mMoles/g (compared to the MAA titration of 0.177 mMoles/g). These were consistent with a low level of ionised groups on the original matrix (a control titration of Perloza was 0.005 mMoles/g) rather than any DEAPA groups covalently attached. The titration values certainly demonstrated that no significant reaction had occurred. Although efficient epoxidation of allyl cellulose (in anhydrous solvents) has been reported by Lin and Huang (1992) the failure to demonstrate any reaction in aqueous conditions suggested this would be a much less economic method than bromohydrin formation for "activation" of the double bond. Therefore the epoxidation reaction was not pursued further.

Free radical addition reactions :

Mercaptoacids

Reaction of mercaptopropionic acid (MPA) with 30% AGE Perloza at 60°C for 4 hours, with catalytic ammonium persulphate, resulted in an addition level of 0.198 mMoles/g. The same AGE activated Perloza, reacted with bromine water followed by substitution with sodium sulphite, gave a titration of 0.177 mMoles/g. Addition at 60°C was similarly effective without ammonium persulphate (0.186 mMoles/g) but was largely inhibited by methoxyphenol (0.044 mMoles/g). A 16 hour addition at room temperature resulted in an intermediate titration value (0.136 mMoles/g). No attempt was made to exclude oxygen from the reaction mixture and it was assumed that light, dissolved oxygen and/or trace amounts of peroxides effectively initiated the reaction, without requirement of any extra catalyst. Therefore peroxides were not used for subsequent mercaptoacid additions. The inhibitory effect of methoxyphenol was consistent with the anticipated free radical mechanism. These results are summarised in Table 3.12. MPA addition (60°C) to unmodified Perloza gave a titration of 0.004 mMoles/g, demonstrating the requirement of allyl groups for reaction. Titration of unmodified Perloza gave a similar value (0.005 mMoles/g), which suggested that the small level of charged groups was not due to reaction of unmodified Perloza with MPA.

Table 3.12 : Catalyst and inhibitor effects on mercaptoacid addition

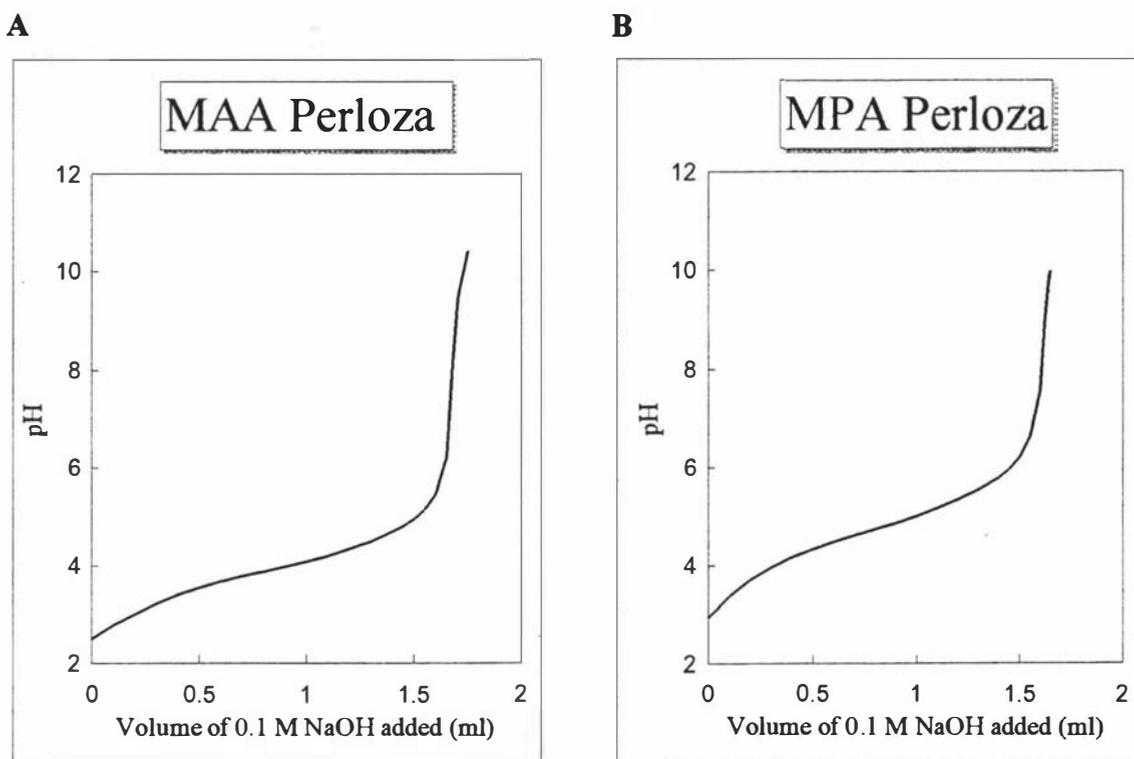
AGE (30%) Perloza was used except for the control sample (Perloza + MPA). MPA was the ligand used, unless otherwise stated. The free radical inhibitor used was methoxyphenol. Efficiency was calculated by correction for the control titration value and comparison to the "possible" addition level found with MAA.

Reaction sample	Titration (mMoles/g)	Efficiency (%)
control	0.004	n.a.
60°C + catalyst (APS)	0.198	97
60°C	0.186	91
60°C + inhibitor	0.044	20
room temperature + APS	0.136	66
MAA, 60°C	0.204	100

Addition (at 60°C) of mercaptoacetic acid (MAA) was also demonstrated, using 30% AGE Perloza. The titration of 0.204 mMoles/g was even higher than the result with MPA (Table 3.12), suggesting superior reactivity and/or more complete titration by pH 8. Titration curves (Figure 3.8) showed that in 1 M NaCl the pKa of MAA Perloza was significantly lower than that of MPA Perloza (3.9 compared to 4.9). However neither resin titrated significantly above pH 7. Superior reactivity was therefore assumed and MAA replaced MPA (addition) for allyl group titration. Insignificant addition was found for a control reaction of unmodified Perloza with MAA and addition of MAA at pH 6 to stock 7% AB Perloza (both 0.004 mMoles/g). These titration values were again attributed to carboxyl groups existing on the original Perloza.

Figure 3.8 : Titration curves of (A) MAA 10% AB and (B) MPA 7% AB Perloza

Samples of MAA (0.7g) and MPA (1g) were suspended in 5 ml of 1 M NaCl.



Optimisation of MAA addition

The addition reactions of MAA described before were carried out at 60°C. An equivalent level of addition to an AGE Perloza sample (at 60°C) was found after a 48 hour reaction at room temperature (both 0.135 mMoles/g). The addition level to another

AGE matrix was 0.191 mMoles/g after 24 hours at room temperature (compared to 0.194 mMoles/g at 60°C). The standard amount of MAA used was 100µl/g allyl Perloza. This represented a 5 molar excess or greater over allyl groups for activated matrices of 0.28 mMoles/g or less. Addition of lower amounts to the more highly activated AGE matrix resulted in titrations of 0.119 mMoles/g (2 molar excess of MAA) and 0.184 mMoles/g (4 molar excess). These levels equated to 61 and 95% of the standard titration respectively. Therefore a 5 molar excess or greater was considered preferable for quantitative MAA addition, and room temperature was sufficient for complete reaction.

Mercaptoethanol addition

Addition of 2-mercaptoethanol (BME) to AGE activated Perloza (MAA titration of 0.144 mMoles/g) at 60°C (16 hours) was apparently quantitative. BME addition to another AGE Perloza (MAA titration of 0.191 mMoles/g; 1.35 mMoles/g dry) at room temperature (24 hours) was equally effective, indicating complete reaction of allyl groups (with mercaptoethanol). The "back" titration of both resins, with MAA, was 0.001 mMoles/g. The elemental (sulphur) analysis of the latter sample was 4.45% (1.39 mMoles/g dry), consistent with the figure obtained by titration for the MAA derivative. BME addition at room temperature would thus represent a simple and effective method for blocking unreacted allyl groups, following addition of another ligand (providing the other ligand groups do not cause steric restriction).

Glutathione and mercaptosuccinic acid addition

Significant addition of glutathione (GSH) to the above AGE Perloza (MAA, 0.191 mMoles/g) was also effected by reaction for 16 hours at 60°C. The titration of 0.115 mMoles/g (up) to pH 7 indicated that although addition occurred it was less efficient than for MPA or MAA. However, a lower molar excess of GSH was used.

Addition of GSH to an AGE matrix of lower activation (MAA titration 0.134 mMoles/g) resulted in a titration of 0.098 mMoles/g to pH 8 and 0.203 mMoles/g to pH 11. This resin was prepared by addition of the reduced free acid form of GSH. Addition of GSH in dilute phosphoric acid was much less effective (0.046 mMoles/g to pH 11). For GSH resins it was assumed that titration to pH 7 would represent one carboxyl group per GSH immobilised. Titration to pH 11 would also include the amino group of GSH. This presumed that the second carboxyl group of glutathione would be associated with the amino group in the carboxylate ion form at the start of titration, analogous to IDA derivatives (Figure 3.6). Allowing for dilution effects (a blank titration to pH 11 was 0.017 mMoles/g), an addition level of 0.093 mMoles/g (70% of MAA) was calculated.

Addition of mercaptosuccinic acid (MSA) to the same AGE matrix resulted in a titration of 0.189 meq/g to pH 10. Because MSA has two carboxyl groups this was equivalent to 0.095 mMoles/g. The MSA resin was titrated to a higher pH than the MAA resin because it appeared that titration was still occurring at pH 8. Dilution effects were insignificant for the pH 10 endpoint used. The apparent addition level of MSA was approximately 75% of the possible (MAA level).

The efficiency of both MSA and GSH addition was improved by reaction at room temperature for 60 hours. Stock 7% AB activated Perloza (MAA titration of 0.174/g) was used. The titration value for MSA was 0.313 meq/g (0.157 mMoles/g) to pH 8. Although the thiol group of MSA is secondary (steric restriction more likely), a very high addition level was thus obtained. In contrast to earlier results, the titration value was not found to increase significantly above pH 8 (0.317 meq/g to pH 9). The MSA derivative could be used for ion exchange chromatography. Specific ionic interactions may occur with the dicarboxylic acid moiety. The titrations for the GSH resin were 0.136 and 0.300 meq/g to pH 7 and 11 respectively. The addition level was calculated to be 0.142 mMoles/g after subtraction of the blank titration value. The high efficiency of GSH addition at room temperature suggested that this method could be used for immobilisation of other thiol containing peptides and possibly proteins, without requiring elevated temperature. GSH is an acidic peptide and it is not yet known how well neutral and basic peptides or proteins might react. Free radical addition of GSH represents a very simple method for production of GSH affinity resins. Unreacted allyl groups are readily blocked using mercaptoethanol. The results for MSA and GSH addition are summarised in Table 3.13.

Table 3.13 : Efficiency of MSA and glutathione addition compared to MAA

Stock 7% AB Perloza was used. Efficiency was determined by comparison with the "possible" value obtained by MAA addition.

Thiol ligand	Titration (mMoles/g)	Efficiency (%)
MAA	0.174	100
MSA	0.157	90
GSH	0.142	82

The slightly lower level of addition of GSH and MSA compared to MAA might be due to inhibition by the amine group (GSH), greater ionisation of the diacidic ligands (compared to MAA), steric effects or simply poorer reactivity. The addition level of GSH might also be increased if a higher molar excess (than 3) were used.

Addition of mercaptobutyric, mercaptohexanoic and thiosalicylic acids

No evidence of addition of thiosalicylic acid was obtained after a 48 hour reaction at 60°C (titration of 0.002 mMoles/g). Addition of the other mercaptoacids did occur but was limited by comparison with MPA and MAA, despite a 96 hour reaction at 60°C. The respective titration values were 0.131 and 0.026 mMoles/g for mercaptobutyric acid and mercaptohexanoic acid (MAA titration of 0.180 mMoles/g). No attempt was made to optimise these additions.

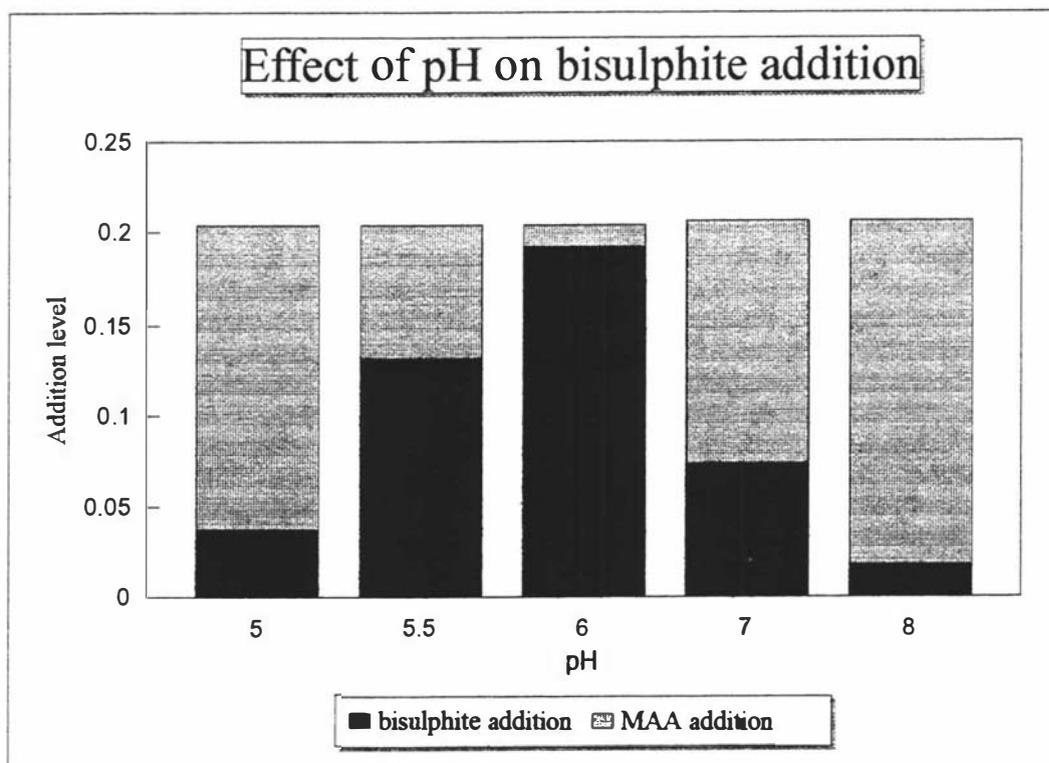
Bisulphite addition

Initial experiments used a 1:1:10 ratio by weight of sodium sulphite, sodium metabisulphite and AGE Perloza (24 hours at room temperature). The titrated addition level was 0.163 mMoles/g (bromine water value 0.18 mMoles/g). Although addition of a peroxide catalyst was not required it was presumed that the free radical mechanism of addition was responsible and that dissolved oxygen or trace amounts of peroxide acted as initiators (Kharasch et al., 1938b). The reactive species was reported to be bisulphite and the preferred range for bisulphite addition to be pH 5-6 by Kharasch et al. (1938b). However, using unbuffered bisulphite at pH 5, the pH dropped during the reaction and low addition yields were obtained. The pH drop was presumably due to oxidation of bisulphite to the more acidic bisulphate ion. Reaction at elevated (60°C) temperatures gave a low addition (0.031 mMoles/g for a 30% AGE matrix, MAA titration 0.204 mMoles/g) and addition results at room temperature were little better (0.037 mMoles/g).

A 48 hour, room temperature addition of bisulphite (adjusted to pH 5.5) to AGE Perloza resulted in a much higher yield (0.139 mMoles/g). An equivalent addition, adjusted up to pH 6 after 24 hours, had a titration of 0.192 mMoles/g (1.30/g dry). The comparable values for MAA were 0.204 mMoles/g (1.31/g dry). This indicated nearly quantitative reaction of allyl groups. Therefore sodium sulphite or acetate was used for subsequent additions to buffer the reaction above pH 5. Little reaction occurred when sodium sulphite, buffered at pH 8, was mixed with another AGE Perloza sample. The titration was 0.018 mMoles/g (compared to 0.206 mMoles/g by MAA titration). Addition occurred more readily at pH 7 but was low (titration of 0.074/g). The effect of initial pH on bisulphite addition to allyl Perloza is depicted in Figure 3.9.

Figure 3.9 : Bisulphite addition levels at various pH values

The pH 6 example was initially reacted at pH 5.5 for 24 hours, followed by adjustment to pH 6 and a further 24 hour reaction. All other samples were reacted for 48 hours without pH adjustment. AGE Perloza (MAA titration of 0.204 mMoles/g) was used for additions at pH 5-6. AGE Perloza (MAA titration of 0.206 mMoles/g) was used for addition at pH 7 and 8.



The preceding experiments concentrated on pH effects and reaction volumes were variable. The same approach with AB activated Perloza gave significantly lower results than those obtained by MPA addition or bromination and sulphite substitution. The titration values after bisulphite addition to the 7 and 24 hour DMSO samples of Table 3.3 were 0.067 and 0.142 mMoles/g. The corresponding MPA titration values were significantly higher (0.145 and 0.202 mMoles/g). Optimal **substitution** of brominated matrices by sodium sulphite, obtained using small reaction volumes, led to a reassessment of reaction volume and bisulphite concentration for the addition reaction. High addition levels were thus obtained by reaction of allyl Perloza (1g) with 1 ml of bisulphite reagent (containing 0.16g each of sodium metabisulphite and bisulphite). Titration values of 0.160, 0.161 and 0.213 mMoles/g were obtained for stock AGE, 7% AB and 10% AB Perloza, compared to 0.153, 0.180, 0.252 for MAA addition. The addition level on AB activated Perloza was still lower than for MAA, but was a

significant improvement over earlier results. Using a bisulphite solution containing 2g each of sodium sulphite and sodium metabisulphite per 10 ml, addition to AB Perloza was improved. However, scale-up to a 20g (Perloza) reaction gave a poorer addition to 10% AB Perloza (0.196 mMoles/g). Therefore the effect of time, heat, and catalysts on addition to this 10% AB Perloza were compared. Elevated temperature gave a much lower addition level (0.070 mMoles/g). This might be due to a greater rate of sulphite oxidation compared to addition at elevated temperature. Inclusion of catalysts (air or hydrogen peroxide) provided no advantage. There was a small increase between 24 and 48 hours for room temperature addition. A resin with a lower titration value (air catalysed, 0.200 mMoles/g) was reacted again with bisulphite and the titration value increased to 0.218 mMoles/g. Likewise repeat addition of bisulphite to the 20g (10% AB) Perloza described before resulted in a titration of 0.216 mMoles/g. This repeat addition involved preparation of 1 ml bisulphite solution per g resin, half of which was used to presolvate the resin (displacing water), which was then suction-dried. The remaining half of the bisulphite solution was mixed with the suction-dried resin for 48 hours. This results in a lower reaction volume and higher reagent concentration and was used for subsequent bisulphite additions. These results are summarised in Table 3.14.

Table 3.14 : Optimisation of sodium bisulphite addition

* = result of repeat addition. # = medium Perloza resin.

Chemistry	Ligand	Titration (mMoles)	Dry weight (g dry)	Substitution (mMoles/g dry)
AB 10%	32% bisulphite	0.213	0.141	1.52
AB 7%		0.161	0.128	1.26
AGE		0.160	0.133	1.21
AB 10%, 60°C	40% bisulphite	0.070	n.d.	n.d.
AB 10%, air		0.200	n.d.	n.d.
AB 10%, H ₂ O ₂		0.197	n.d.	n.d.
AB 10%, 24 hr		0.195	0.138	1.42
AB 10%, 48 hr		0.212	0.142	1.50
AB 10%, 20g *		0.216	0.136	1.59
AB 7%, 20g		0.179	0.140	1.28
AB 7.5%, 200g #		0.204	0.141	1.44

At high AB activation levels bisulphite cannot match MAA addition. Nevertheless 85-90% of the possible level has been obtained and the reaction is no longer prone to significant variation. A probable cause of the drop in performance at high activation levels is charge shielding. Whereas the addition species and resin product are uncharged for MAA, both bisulphite and the sulphonate resin product are negatively charged. Therefore electrostatic repulsion may restrict access to a small proportion of allyl groups. This proportion of restricted groups would be expected to rise if total allyl group density increases. Reaction of a stock 7% AB Perloza with bisulphite produced a titration value of 1.25 mMoles/g dry compared to 1.36 mMoles/g dry for MAA addition. However the difference could not be made up by addition of MAA to the sulphonate resin, either in the protonated or Na^+ form (both unchanged at 1.25 mMoles/g dry).

This suggested that the excess groups had reacted with a neutral contaminant of the bisulphite solution or that sulphonate groups restrict access to some allyl groups, irrespective of the nature of the addition reagent. Because addition to AGE Perloza was apparently complete, it seemed unlikely that a contaminant was interfering with bisulphite addition. Allyl groups were not detected by bromine water titration, but the allyl group level may be too low to be reliably detected by this method. Access of bromine to unreacted groups might also be restricted. A thiol (e.g. BME) might be used to block unreacted groups although MAA did not appear to react with a sulphonated allyl resin. Residual groups, which do not react with bisulphite or small thiols, are not expected to interact with macromolecules (and thus effect protein chromatography).

The product of sulphite addition, Perloza-propyl-sulphonate (SP Perloza), can be used for ion exchange chromatography. The physical, chemical and chromatographic properties of SP Perloza resins are described in Chapter 4.

Nitrogen containing thiols :

Addition of cysteine HCl to an AGE Perloza matrix was limited (0.013 mMoles/g, MAA titration of 0.134 mMoles/g), using conditions (16 hours, 60°C) which were effective for the thiols described before. An even lower addition level was found for cysteamine HCl (0.006 mMoles/g), on another AGE Perloza sample (MAA titration of 0.204 mMoles/g). Because addition of GSH was limited in the presence of phosphoric acid, cysteine HCl was replaced by cysteine, used with or without a one molar excess of formic acid. The addition in the presence of formic acid was more successful (0.063 compared to 0.012 mMoles/g). Cysteine/formic acid addition resulted in a titration of 0.035 mMoles/g on another AGE matrix (0.082 mMoles/g by MAA titration). Formic acid was replaced by acetic acid without a significant change (0.033 mMoles/g) but trichloroacetic and oxalic

acids were less effective (0.025 and 0.007 mMoles/g). These results might indicate an effect of acid strength because the latter two are stronger acids (than formic and acetic). Oxalic acid decomposition in water and its second carboxyl group might have negative effects on the reaction. Little difference resulted from use of a 1 or 10 molar excess of formic or acetic acid (range of 0.033 to 0.035 mMoles/g). A repeat addition at 60°C increased the titration level to 0.055 mMoles/g, whereas addition at room temperature gave no increase in titration. Similar results were obtained using cysteamine/acetate (titration of 0.032 mMoles/g) and 4-mercaptoethylpyridine/acetate (0.043 mMoles/g).

Reaction of cysteamine/formate with AB Perloza (0.180 mMoles/g MAA titration) for 48 hours at 60°C gave higher results but these were not increased by inclusion of ammonium persulphate (APS) or benzoyl peroxide (Table 3.15). The titration of an uncatalysed sample reacted for 96 hours at the same temperature was 0.103 mMoles/g. A limit to addition (0.128 mMoles/g) was found on a highly activated AB Perloza (MAA titration, 0.249 mMoles/g) after a prolonged (96 hour) reaction with cysteamine/formate at 70°C. Other titrations were 0.054, 0.098 and 0.129 mMoles/g after 24, 48 and 144 hours respectively. Little increase was found when the reaction was repeated with fresh cysteamine/formate (0.135 mMoles/g). However this might be improved by use of smaller reaction volumes. Use of cysteamine HCl (no formic acid) gave a much lower result. Addition of cysteine (10 molar excess)/ formic acid at 70°C gave a similar result (0.114 mMoles/g) to that with cysteamine. Addition of cysteamine/formic acid to a lower substituted AB resin (0.139 mMoles/g MAA titration) gave a titration of 0.110 mMoles/g (79% efficiency). This demonstrated that efficiency was much greater at lower activation levels such as those likely to be used for affinity chromatography. These results are summarised in Table 3.15.

Although addition of cysteine and cysteamine occurred, their reactivity was much poorer than another amine containing ligand, glutathione. Glutathione has a surplus of acidic over amine groups (solutions used had a pH of 2) and the nitrogen most adjacent to the thiol group is part of a neutral amide bond. The significance of these differences could be tested by reaction of allyl Perloza with cysteine containing peptides.

Although reaction of formic acid with double bonds has been documented (Knight et al., 1953) there is no doubt from the observed titration that amine groups have been attached to Perloza. No evidence of reaction was found between allyl Perloza and amine groups, using ethanolamine +/- formic acid (60°C, 96 hours), or between cysteamine/formate and unmodified Perloza. The titration values of these samples were all 0.001 mMoles/g and the starting pH between 4.88 and 4.96. The titration for cysteamine/formate addition to

the same allyl Perloza, was 0.080 mMoles/g and the starting pH 10.20. Both ligand thiol groups and matrix allyl groups were necessary for addition. Free radical addition of an amine to allyl groups was not expected (Cadogan and Perkins, 1964) and the results with ethanolamine supported this. The effect of formic acid/acetic acid may be catalytic or due to "masking" of amine groups.

Table 3.15 : Addition levels for cysteamine and cysteine on allyl Perloza

Cysteamine refers to cysteamine/formate mixtures except for one sample using cysteamine HCl. APS = ammonium persulphate, BP = benzoyl peroxide. Stock 7% AB Perloza was used for cysteamine additions at 60°C, stock 10% AB Perloza for additions at 70°C unless otherwise indicated.

Ligand	temperature (°C)	time (h)	titration (mMoles/g)
Cysteamine	60	48	0.063
Cysteamine/APS	60	48	0.063
Cysteamine/BP	60	48	0.050
Cysteamine	60	96	0.103
Cysteamine	70	24	0.054
Cysteamine	70	48	0.098
Cysteamine	70	96	0.128
Cysteamine	70	144	0.129
Cysteamine HCl	70	144	0.027
Cysteine/formate	70	144	0.114
Cysteamine (6% AB)	70	144	0.112

Simple methods of catalysis were used for these experiments, with no attempt to exclude oxygen. Under these conditions no advantage has been observed for use of chemical catalysts. Reaction temperatures above 70°C may improve reaction yields. Superior reactions may be obtained by use of radiation catalysis (ultraviolet or gamma) but these methods have not been tried. Although these reactions were less efficient than those of mercaptoethanol and the mercaptoacids they may be economically viable with superior catalytic techniques. The specificity of reaction through the thiol group is an advantage over conventional methods of thiol attachment.

Thiolacetic acid and thiophenol addition

Results with thiolacetic acid indicated that some reaction occurred but was well short of completion. Analysis was by "back" titration with MAA, which may hydrolyse the thiol ester, displace a thiolacetate radical (Walling 1957) and/or oxidise thiol groups. The back titrations of aqueous reaction samples were 0.105 (60°C) and 0.112 (room temperature) mMoles/g, compared to the original MAA titration of 0.135 mMoles/g. This was equivalent to 0.029 and 0.023 mMoles/g of thiolacetate groups. A similar result was obtained for addition with an ethanol/water mixture (0.023 mMoles/g of thiolacetate). These low addition levels might be due to the high acidity of thiolacetic acid (the pH of a 1% solution was 1.95). The indirect method of analysis might result in an underestimate of thiolacetate groups, although it was satisfactory for analysis of mercaptoethanol addition. A third possibility is that stronger catalytic measures are required. The thiolacetic acid radical is comparatively stable and addition can be reversed (Walling, 1957). Thiolacetic acid is reported to be especially reactive with many alkenes in solution (Cunneen, 1947b; Sosnovsky, 1964). Indeed Cunneen reported that its reactivity was greater than that of several thiols, including MAA. Therefore it would be surprising if the problems of this reaction could not be overcome. Because the thiol ester is readily hydrolysed this approach, if successful, would represent a new and simple method for production of thiol resins (Axen et al., 1975).

Some addition of thiophenol to 7% AB Perloza apparently occurred, although it was only 20-25% of the possible (MAA) level. The back titrations were 0.137 and 0.128 mMoles/g for acetone and ethanol solvated samples respectively. The original MAA titration was 0.174 mMoles/g, therefore the thiophenol addition levels were 0.037 and 0.046 mMoles/g. Thiophenol was reported to be less reactive than MAA with various alkenes (Cunneen, 1947a). Cunneen obtained high addition yields by using ultraviolet irradiation and similar methods may be necessary for efficient addition to allyl Perloza.

Accuracy of ligand density values

Elemental analyses of selected AGE sulphonate samples (0.91 and 0.98 mMoles sulphur/g) were lower than titrated substitution values (1.08 and 1.16 mMoles/g dry respectively). Retitration of these resins with fresh Convul NaOH gave unchanged results. The contribution of charged groups on unmodified Perloza was not high enough to explain this level of discrepancy. Other elemental analyses, especially of amine and sulphite substitution derivatives, were also (about 10%) lower than expected and variations of up to 0.1 mMoles/g dry were found between duplicate analyses. Lower than expected elemental analysis values were also found by Englebretsen (1992) for

aminopropyl Perloza resins. Nevertheless, the elemental analyses did confirm that very high ligand densities were obtained using allyl chemistry.

Some variation could occur with suction-dried resin weights due to differences of vacuum applied, suction time and sintered glass funnel used. Also some titration results reported here refer to the weight of suction-dried allyl Perloza before reaction with a ligand whereas others refer to the weight of the matrix-ligand derivative. In the former case the resin product was transferred quantitatively and comparisons could be made between different ligands using weights of the same starting material. When comparisons were made, samples weights were obtained consistently (i.e. all referred to the weight of original matrix or all referred to the weight of resin derivative).

SUMMARY

Allyl matrices have been produced by alkaline etherification with allyl bromide or allyl glycidyl ether. Side reactions (crosslinking or hydrolysis) on the matrix were not evident. Activations were reproducible once methods were optimised. High activation levels were obtainable, especially using allyl bromide (up to 0.7 mMoles/g). The activation methods described were cheap and did not require organic solvents. Activation levels could be assayed with bromine water but more reliable methods were developed. These methods were apparently well suited to large scale use.

Allyl matrices were modified with bromine water to produce bromohydroxypropyl (BHP) matrices. However, facile reaction of allyl groups with N-bromosuccinimide was preferred to prepare the BHP derivatives because the reagent was less hazardous and side reactions were not anticipated. BHP derivatives were used to attach amine or thiol containing ligands, or sodium sulphite, by **nucleophilic substitution**. Optimisation of these reactions resulted in high substitution efficiencies, even at high activation levels. Other resins were produced by **free radical addition** reactions between allyl matrices and suitable ligands. High addition levels were obtained with mercaptoacids, glutathione, mercaptoethanol and sodium bisulphite. Addition of other thiol ligands, including cysteine and cysteamine was also effected, although with greater difficulty. The range of useful addition reactions might be extended by use of radiation catalysis.

Iminodiacetic acid was attached to BHP matrices to prepare IMAC resins. Attachment of cysteamine or mercaptoacids (by addition or substitution chemistry) produced amine or carboxyl resins, which could be used for further ligand attachment chemistry. Glutathione derivatives could be used for affinity chromatography of GSH enzymes. A wide range of ligands, including proteins could be attached by these methods to produce resins for mixed mode, dye ligand and affinity chromatography. Resins for hydrophobic interaction chromatography could be produced by reaction of phenol with bromohydrin resins or reaction of thiol ligands (e.g. butyl mercaptan) with allyl or bromohydrin resins. β -mercaptoethanol could be used to block unreacted allyl or brominated groups (unless the ligand is sensitive to such reagents). The use of mixed mode and novel hydrophobic resins, produced using allyl chemistry, is described in Chapters 6 and 7.

Amine, thiol and (bi)sulphite ligand attachment to allyl or modified resin forms produced strong and weak (anion and cation) ion exchange resins. Highly substituted resins were obtained despite the use of aqueous solvation. The physical and chromatographic properties of these resins is described in Chapter 4.

CHAPTER 4 PROPERTIES OF ALLYL PERLOZA ION EXCHANGE RESINS

INTRODUCTION

Activation of Perloza, with allyl bromide (AB) and allylglycidyl ether (AGE), and subsequent ligand attachment, was described in the previous chapter. High ligand densities were obtained using substitution or addition methods. The results suggested that these methods would be extremely useful for the preparation of inexpensive resins for laboratory or industrial scale use, if their physical and chromatographic properties met with expectations. These properties could be assessed by preparation of allyl Perloza derivatives analogous to conventional resins. Therefore ion exchange derivatives of allyl Perloza have been produced for comparison with commercial resins.

Anion exchange resins were prepared by bromination of AB Perloza (with NBS), followed by substitution with diethylamine (DEA) or trimethylamine (TMA). Although the preparation was a three step process, the methods were simple and the first two steps could be used for the production of a wide range of other derivatives (Chapter 3). High amine substitution levels were obtained without difficulty.

Addition of bisulphite to allyl matrices was found to be a simple method for preparation of medium to highly substituted sulphopropyl (SP) cation exchange resins. This compared favourably to the cellulose sulphopropylation method of Goethals and Natus (1966), which used propane sultone (a highly toxic reagent) and required large amounts of organic solvent. Sulphohydroxypropyl derivatives were also obtained by sulphite substitution of bromohydroxypropyl derivatives of allyl activated matrices. Epoxide activated Perloza could also be substituted with sulphite but the substitution level would be limited by the low activation levels that have been obtained (Chapter 2). Activation with allyl bromide allowed efficient sulphonation to high levels on Perloza. The addition reaction was preferred over substitution because the intermediate bromination step was not required, reducing reagent and production costs (assuming similar costs of the respective sulphonation steps). The SP Perloza resins described in the following sections were all obtained by the addition method.

Addition chemistry was also used to prepare weak cation exchange resins. Resins produced by addition of mercaptoacetic acid (MAA) were expected to have similar chromatographic properties to conventional CM resins, although the carboxymethyl group was attached by a propyl thioether spacer arm. Resins produced by

mercaptosuccinic acid (MSA) addition could also be used for ion exchange chromatography, although the juxtaposition of carboxyl groups might produce stronger interactions.

The economics of the above manufacturing methods are assessed here. The physical and chromatographic properties of these ion exchange resins are described. Ligand densities, swollen volume (stability) and flow properties data are reported. Titration curves are shown for each type of ion exchange resin. The elution kinetics and protein capacities are compared with Otsorb and Sepharose Fast Flow ion exchange resins and unmodified Perloza.

Only AB derivatives have been analysed here, although the longer spacer arm of AGE is expected to be beneficial for protein chromatography, due to less steric hindrance of protein access. The AGE spacer arm should be sufficiently hydrophilic for ion exchange chromatography and might provide some "tentacular" effect (Muller, 1990). The convention used for naming resins was the abbreviation of the ligand type, followed by the percentage of allyl bromide used for activation and F for the fine grade and M for the medium grade of Perloza 100. Hence SP7.5F was an SP cation exchange resin produced by bisulphite addition to Perloza 100 fine activated with 7.5% AB.

MATERIALS AND METHODS

Reagents and equipment

Ribonuclease, ovalbumin and immunoglobulin G were from Sigma Chemical Co., St. Louis, MO, USA; β -lactoglobulin (batch MP589) from NZDRI, Palmerston North, New Zealand; and bovine plasma and sulphated and SP Indion resins from Life Technologies, Auckland, New Zealand. SHP, DEAE and Q Ostsorb resins were from Tessek or ICS, Prague, Czechoslovakia; and SP and Q Sepharose Fast Flow from Pharmacia, Uppsala, Sweden. Perloza (7, 7.5 and 10% AB) fine (+/- 1% ECH crosslinking) and medium grade resins were prepared by the methods of Chapter 3. Ion exchange derivatives of AB Perloza were obtained by bromination (NBS) and amine (DEA, TMA) substitution, or addition (MAA or bisulphite), using the methods of Chapter 3. Other proteins and resins used were described before. Buffer reagents were analytical grade.

Haemoglobin was further purified by CM Perloza ion exchange chromatography. Bovine serum albumin (BSA) was prepared from plasma by chromatographic methods (Ayers et al., 1984a). Sulphated cellulose was used to remove lipoproteins (Ayers et al., 1984b). The flowthrough fraction was dialysed (3 changes) against 10 mM Tris + 20 mM NaCl, pH 7.5 (Buffer I). The dialysed material was loaded onto a DEAE Ostsorb column (equilibrated with buffer I) and the column washed, after loading, with the same buffer to remove immunoglobulins. The column was washed with 5 volumes of 20 mM acetate, pH 7.5, and eluted with 20 mM acetate, pH 4.5 (Buffer II). The eluate was loaded onto an SP Indion column, equilibrated with Buffer II. BSA was eluted with 20 mM acetate + 400 mM NaCl, dialysed (3 changes) against water, and lyophilised. The BSA preparation was carried out by Mr. G. Taylor, Massey University.

A Pharmacia P500 FPLC pump was used for flow rate experiments. Capacity tests and BSA elution were monitored with a Biorad Econosystem. Other equipment used was described previously.

Physical and chromatographic properties of ion exchange resins :

Titration methods

Ligand density was determined by endpoint titration of 1g samples and the volume and oven-dried weight obtained, as described before. Titration curves were obtained using resin (1g) mixed with 5 ml of 1.2 M NaCl or KCl (cation and anion exchange resins respectively), or 5 ml of 0.01 M NaCl (one MAA resin).

Resin volume at high and low ionic strength

The tip of a 2 ml pipette was sealed with parafilm and perforated with a 26 gauge needle. This permitted flow of aqueous solutions but not resin particles. A 5 ml glass funnel was attached to the top using silicone tubing and the pipette filled with water (SP and MAA resins) or 1M NaCl (DEA and TMA resins). The titrated resin sample was transferred quantitatively to the pipette and washed with water or NaCl solution until the bed volume stabilised. Salt was included with anion exchange resin samples to counteract the tendency of these resins to adhere to glass surfaces. Resin beads adhering to the wall of the pipette above the bed were physically dislodged with wire. The volume was determined in both water and 1 M NaCl. MAA resins were washed with 20 mM NaCl instead of water. All volume analyses were of the charged resin form (Na^+ for cation exchange and Cl^- for anion exchange resins). After stable volumes had been obtained, the resin was transferred quantitatively to a sintered glass funnel. It was then washed, oven-dried and weighed, as described previously.

Flow rate

Resins were gravity packed into a 30x0.8 cm glass column to a bed depth of 28-29.5 cm. Removable, fritted, plastic end-pieces were used. The column was connected to the P500 pump, with a pressure gauge (0-4 Bar) placed in line between the pump and the column. Bed volume, flow rate (elution) and back pressure were monitored at increasing pump rates up to a maximum of 499 ml/hr. The pump rate was not increased once the back pressure exceeded 1.7 Bar. The pump rate was returned to zero, incrementally, and changes of back pressure, flow rate and bed (volume) recorded and graphed. The column height : volume ratio was determined, with water, to be 1.85:1. This conversion factor (1.85) was used to convert flow rate from ml/h to cm/h.

Chromatography

SP7F Perloza and Sepharose fast flow resins were packed in 2 ml columns (internal diameter 0.7 cm) and equilibrated with 10 mM citrate, pH 3.5 (Buffer A). The bed volumes were 0.95 and 1 ml for respectively. Bovine serum albumin (BSA, 3 mg), was dissolved in Buffer A and adsorbed to the resin. Any unbound material was washed out with Buffer A and BSA was eluted with 0.02 M phosphate + 0.5 M NaCl, pH 8 (Buffer B). The same method was used with BSA on TMA Perloza and Q Sepharose fast flow (1 ml bed volume), except that 10 mM Tris, pH 8 (Buffer C) was used for resin equilibration, BSA dissolution and adsorption. BSA was eluted with 10 mM Tris + 0.5 M NaCl, pH 8 (Buffer D). The flow rate used, for all resins, was 20 ml/hour.

Batch capacity testing

The capacity of anion exchange resins was determined by batch adsorption and column desorption. Proteins tested were β -lactoglobulin, ovalbumin and BSA. Buffer C was used to equilibrate resins and prepare protein solutions. Resin (0.5g) was mixed with 6 ml of 1.5% BSA, 2% ovalbumin or 2% β -lactoglobulin in a 25 ml glass vial (2 hours at room temperature). Other resin samples (1.5g) were mixed with 18 ml of protein solution and aliquots (approximately 5 ml) were removed after 0.25, 0.5, 1 and 2 hours. Samples were transferred to 5 ml columns, excess protein rapidly washed out with load buffer and the resin volume calculated from the bed height (conversion was height \times 0.4 = volume, calibrated in Chapter 2). Washing with load buffer was continued until the absorbance (280 nm) returned to baseline. Protein was eluted with Buffer D and collected in 10 or 25 ml volumetric flasks. Protein concentration and capacity was determined by the methods described previously. The extinction coefficients used were those described previously, plus 9.6 for β -lactoglobulin (Eigel et al., 1984) and 7 for BSA. The latter extinction coefficient was determined by absorbance measurements, at 280 nm, of dialysed and lyophilised BSA (0.1% solution in Buffer D) and its half and quarter dilutions.

Batch BSA capacity testing of SP resins was similar, except Buffer A was used for sample preparation, resin equilibration and adsorption, and Buffer B for elution.

Column protein capacities of SP7F Perloza

SP7F Perloza (0.95 ml) was packed in a 2 ml column, as described above. The load and equilibration buffers used were Buffer A (BSA) and 10 mM acetate, pH 5 (all other proteins). Proteins were prepared as 4% (BSA), 5% (immunoglobulin G, IgG) or 10% solutions. Elution was with Buffer B. The columns were regenerated with 0.1 M NaOH. The flow rate was maintained at approximately 0.3 ml/minute. Elution samples were collected and analysed, as described before. The extinction coefficients used were 5.7 (Ribonuclease), 12.8 (IgG) and those described before. The former values were determined, as described before for BSA, using 0.1% solutions in Buffer B. Haemoglobin capacity was determined by dialysis and freeze-drying of the eluate to constant weight, rather than by absorbance.

RESULTS AND DISCUSSION

Resin preparation cost

Cost estimates (materials only) are based on a 7% AB activation mixture, using Janssen and Aldrich catalogue prices (US\$, Appendix 1). The amounts of reagents relate to 1 kg of suction-dried Perloza, which produces 1-1.2 l of ion exchange resin. For all resins, 70 ml of AB (\$2.50) is used. For anion exchange resins, 35g of NBS (\$1.60) and 100-200 ml of DEA or TMA (\$1-2) is used. For SP resins, 140-200g each of sodium sulphite and sodium metabisulphite [or 300-400g of the latter, pH adjusted to 6.3] (\$2-3) is required. The requirements for other resins are: 80 ml MAA (\$2.50) and 150g MSA (\$27). It is apparent that reagent costs are very low (\leq \$6/kg resin), for all except the MSA derivative. Although conditions might be optimised to reduce reagent use, the impact of this is likely to be minor compared to initial matrix cost, freight, equipment and labour costs. Although 2 or 3 steps are involved, the low cost of materials and simplicity of methods (no heating or organic solvent requirement) suggest that production would be economically competitive.

Physical properties

Swollen volumes

Substitution levels of 129-190 μ Moles/ml were obtained without significant alteration of swollen volume for SP, DEA and TMA Perloza (Table 4.1). This contrasted with reported instability of DEAE, sulphoethyl and sulphomethyl cellulose derivatives at high ligand densities (Peterson and Sober, 1956; Guthrie, 1971). The weight of the suction-dried product was similar to that of the original Perloza. The volume/g dry was reduced due to the extra weight of ion exchange groups. The values indicated little change in swollen volume, after correction for the extra weight of the ion exchange groups.

For example 1g dry of SP10F (1.59 mMoles/g dry) includes weight due to SP groups, calculated at 0.144g (weight of 1 millimole of $C_3O_3H_5SNa$) \times 1.59 = 0.229g. Therefore the weight of the cellulose backbone per g dry is 1-0.229 = 0.771g. The "corrected" swollen volume/g dry obtained by measured volume, 8.34/g dry, \div 0.771g = 10.8 ml/g dry. Corrected values of 10.7, 10.9 and 11.0 were found likewise, for SP7.5F, SP7F and DEA7F (using a millimolar weight of 0.165g for the latter resin). These swollen volumes were very similar to that of the original Perloza 100 fine (11 ml/g dry). The swollen volumes were significantly lower than those of CM Perloza resins described in Chapter 2.

Table 4.1 : Charge density and swollen volume of AB Perloza ion exchange resins

The resin "XL" was produced from Perloza 100 fine, crosslinked with 1% epichlorohydrin. The Na⁺ form of SP and MAA resins and the Cl⁻ form of TMA and DEA resins were used. Swollen volumes of MAA Perloza were measured in 1 M and 20 mM NaCl (lower and upper volume limits). All other resin volumes were measured in 1 M NaCl and water, without variation.

Resin	Titration (mMoles/g dry)	Swollen volume (ml/g dry)	Charge density (mMoles/ml)
SP7F	1.28	8.92	0.143
SP7.5F	1.44	8.49	0.170
SP10F	1.59	8.34	0.190
SP7.5M	1.38	7.55	0.183
TMA7F	1.17	8.74	0.134
TMA7.5F	1.33	8.73	0.152
TMA10F	1.42	7.90	0.187
DEA7F	1.15	8.90	0.129
DEA7.5M	1.12	7.30	0.153
MAA7F	1.32	10.4-10.9	0.121-0.126
MAA10F	1.67	12.4-14.6	0.115-0.135
XL MAA10F	1.69	8.9-9.2	0.184-0.190

The volume of these Perloza derivatives did not vary with ionic strength (between water and 1 M NaCl). This volume stability was found despite the fact that these resins were not deliberately crosslinked. However some crosslinking could occur during the substitution step for TMA and DEA resins, due to the high pH of the amine reagents. The starting pH for the DEA reaction is approximately 12.5, but this will fall due to displacement of HBr by the substitution reaction. TMA has a lower pKa value and its start pH is approximately 12, but this will not drop significantly during reaction, because a quaternary ammonium bromide is produced rather than HBr. Crosslinking (due to reaction of matrix bromohydrin and hydroxyl groups) should require very high pH conditions. It is uncertain if the conditions of amine substitution would be alkaline enough for significant crosslinking to occur.

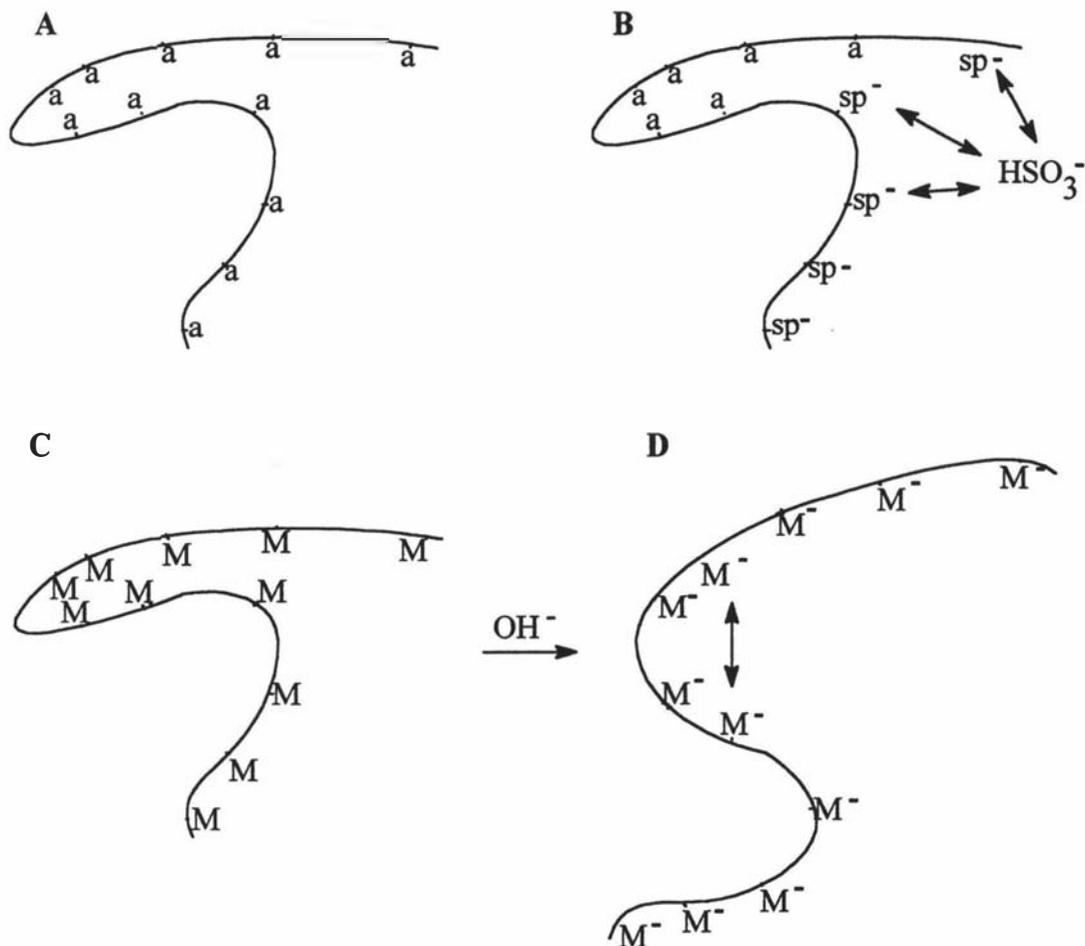
The swollen volume of the base form of MAA Perloza was significantly greater than the acid form, especially for highly substituted, uncrosslinked resins (Table 4.1). However the gelation found with medium to highly substituted, uncrosslinked, CM Perloza (Chapter 2) and physical instability reported for highly substituted CM celluloses (Peterson and Sober, 1956) did not occur with MAA Perloza. Flow properties were compromised but not lost (Figure 4.6). The stability compared to CM Perloza was attributed to the longer spacer arm which distances the carboxyl group from the matrix backbone. The bed volumes of MAA Perloza resins (Table 4.1) increased when ionic strength was decreased (from 1 M to 20 mM NaCl), especially for uncrosslinked MAA10F Perloza. The crosslinked equivalent and MAA7F Perloza were much more stable, but all MAA resins were more swollen (base form) than any SP Perloza resin.

The volume stability of SP, DEA and TMA Perloza resins was thus surprisingly greater than for MAA Perloza. This could be due to attachment of MAA groups to the cellulose backbone at critical sites where charge repulsion could have a destabilising effect. Steric effects were expected to limit sulphonation because of charge repulsion between resin sulphonate groups and bisulphite ions. Under the conditions of MAA addition both the resin and the reagent were uncharged, which might allow greater (reagent) access to allyl groups. This is reflected in the higher addition levels obtained (for MAA) on the same allyl Perloza. When charged groups are attached at "crowded" sites electrostatic repulsion might disrupt the hydrogen bonding, which gives Perloza its strength. These possibilities are depicted in Figure 4.1. Charge repulsion may also affect TMA substitution. Enhanced stability might also be due to unreacted allyl, bromohydrin or propanediol (hydrolysed bromohydrin) groups which could form new hydrogen bonds or π - π interactions. Because good nucleophiles and addition reagents did not react with the (presumed sterically hindered) groups, it was expected that large, charged molecules (proteins) would not get close enough to form covalent or non-covalent bonds. Therefore unreacted groups are unlikely to influence protein chromatography.

Titration of ion exchange resins

Titration curves of MAA10F Perloza (XL) were determined at high and low ionic strengths to assess the preferred pH range of MAA resins for use cation exchange chromatography (Figure 4.2a). The high ionic strength titration curve was similar to that described for CM Sepharose (Scopes, 1987). At low ionic strength the resin was predominantly (90-95%) ionised at pH 6. Due to the high ligand density the concentration of ionised carboxyl groups was still high at pH 5 (0.16 mMoles/g, estimated to be 0.12 mMoles/ml) and pH 4.5 (0.11 mMoles/g). The titration curve of SP10F Perloza (Figure 4.2b) was equivalent to that of SP Sephadex (Scopes, 1987).

Figure 4.1 : Possible effect of ligand attachment at a sterically restricted site for (A) allyl Perloza, (B) SP Perloza, (C) MAA Perloza, (D) base washed MAA Perloza

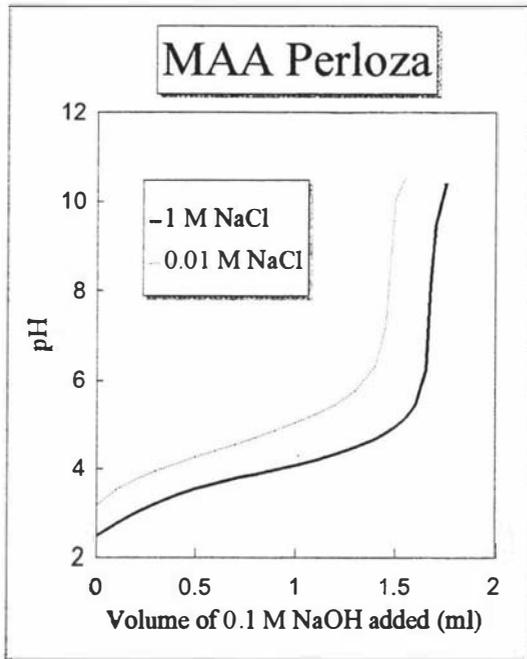
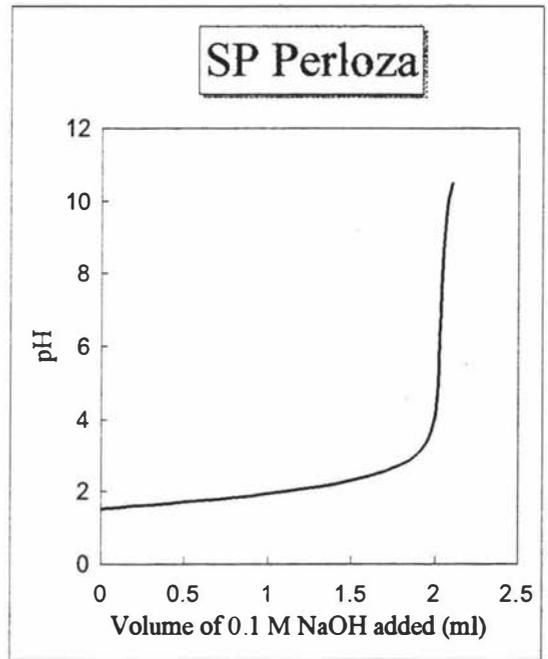
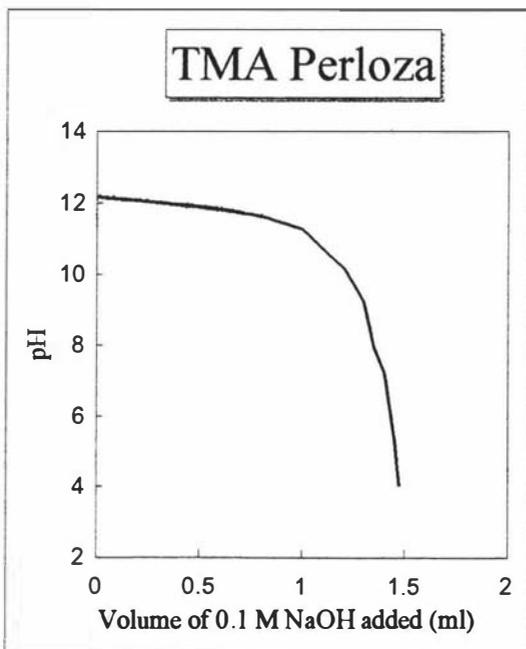
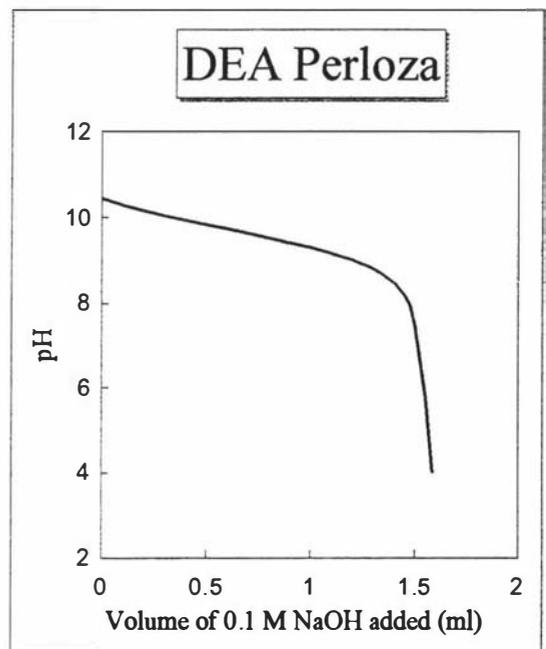


a = allyl, sp⁻ = propyl sulphonate, M = MAA (COOH), M⁻ = MAA (COO⁻Na⁺)

These diagrams represent possible outcomes of ligand addition to a sterically restricted site ("pocket"). Addition of bisulphite (B) results in the formation of a charged surface which repels the negatively charged HSO_3^- ion, preventing addition to sterically restricted sites. Addition of MAA is not restricted thus, because both the addition species and the resin surface formed are neutral (C). The groups inside the pocket are electrically neutral (A, B and C). The addition of base to the MAA resin will destabilise the structure, because of the proximity of like charges. The backbone adopts a new, more favourable conformation, opening up the "pocket" (D).

Figure 4.2 : Titration curves of (A) MAA, (B) SP, (C) TMA and (D) DEA Perloza ion exchange resins

MAA10F XL (0.7g, high and 0.61g, low ionic strength), SP10F, TMA7F and DEA7F Perloza (1g each) resins were titrated.

A**B****C****D**

The titration curve of TMA7F Perloza (Figure 4.2c) was comparable to commercial strong anion (Q) exchange resins (Scopes, 1987). The buffering region between pH 9 and 6 (also found with Q Sephadex) was attributed to the presence of carbonate ions bound during the NaOH wash before titration. The titration curve of DEA7F Perloza (Figure 4.2d) indicated homogeneity of ionic groups. This contrasted with the heterogeneity of traditional DEAE anion exchange resins (Peterson and Sober, 1956). This might cause dissimilar chromatographic properties on some occasions.

The similarity of titration curves to commercial equivalents indicated that these resins should be useful for ion exchange chromatography.

Flow rates of Perloza ion exchange resins

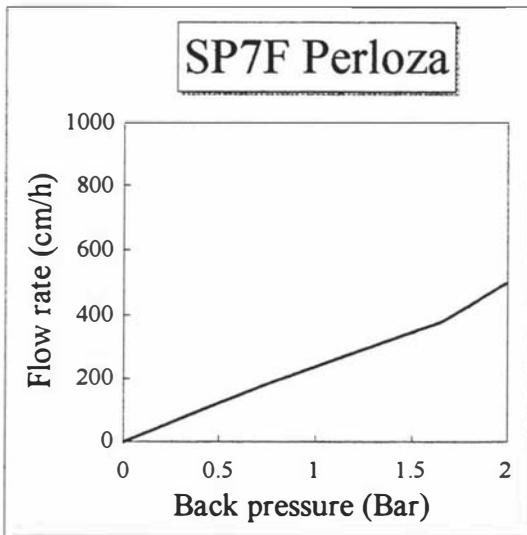
The flow rate versus back pressure curves depicted in Figures 4.3 to 4.6, were based on the values obtained (at decreasing pump speed) after maximum flow rate, pressure and bed compression had been reached. Up to 20% bed compression was found at maximum back pressure. High flow rates at low back pressures were found with unmodified Perloza (Figure 4.3C) in agreement with the description of Stamberg (1988). Although greater back pressures were found for Perloza derivatives, the performance of most resins was still very good. The performance of SP7F Perloza was superior to that of SP Sepharose fast flow (Figure 4.3, A and B), despite the fact that Perloza resins were uncrosslinked, whereas the Sepharose resin was extensively crosslinked. The results for an uncharged Perloza AB derivative were intermediate between those of unmodified and SP7F Perloza (Figure 4.3D). The flow rates of other SP Perloza fine resins, prepared from more highly activated AB Perloza, were better than SP7F (Figure 4.4 a-c). A TMA Perloza resin (Figure 4.4d) had similar performance to SP7F. SP and DEA derivatives of Perloza medium developed minimal back pressure at high flow rates. Their performance was better than that of Ostsorb (especially SHP) equivalents (Figure 4.5).

Much poorer performance was found with the MAA Perloza resins (Figure 4.6). The flow rates obtained for the crosslinked resin were about an order of magnitude higher than for the uncrosslinked resin. However, even this crosslinked MAA resin was still about three times slower than for the worst performed SP Perloza (SP7F, uncrosslinked) at equivalent back pressures. These results seem to reflect the greater swollen volume and hence greater compressibility of the MAA resins. The flow performance of MAA resins should be improved if crosslinking and ligand density (lower) can be optimised to produce less swollen resins. Other studies have indicated this might be achieved by addition at pH 3-4 (MAA partially charged) or by MAA substitution of BHP Perloza.

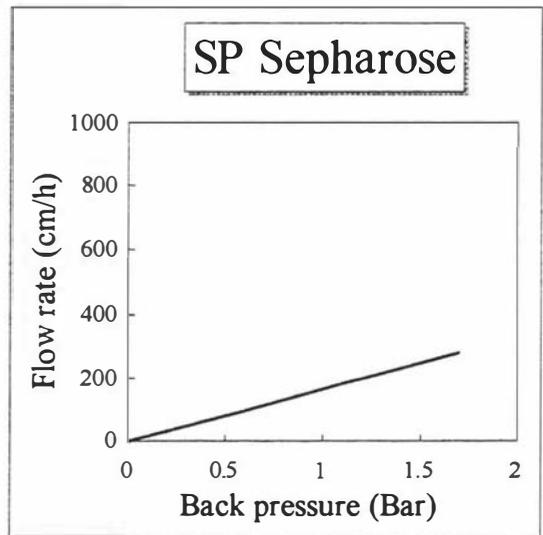
Figure 4.3 : Flow rates of SP7F Perloza, SP Sepharose and neutral Perloza resins

Steeper curves represent better flow performance. Some loss of performance was found when Perloza (C) was modified by attachment of a neutral ligand (D). A further reduction in performance was found if a charged (SP) ligand was attached (A). Nevertheless, the performance of this SP Perloza was superior to that of SP Sepharose Fast Flow (B). Initial bed height (29.5 cm) was reduced to 23.8 (A), 25.5 (B), 25.4 (C) and 25.5 (D) at the maximum flow rate. These compressed bed heights were maintained as flow rate was decreased.

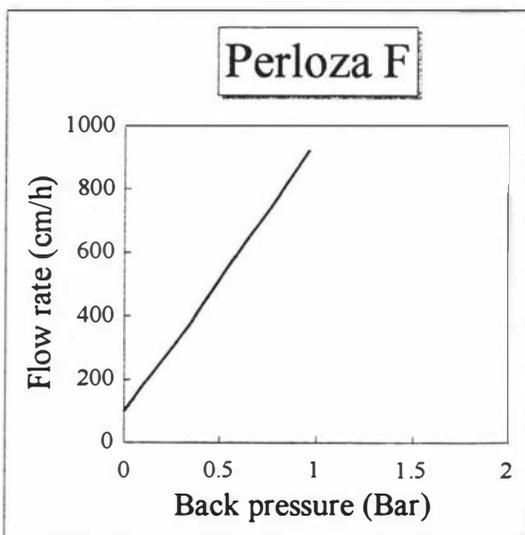
A



B



C



D

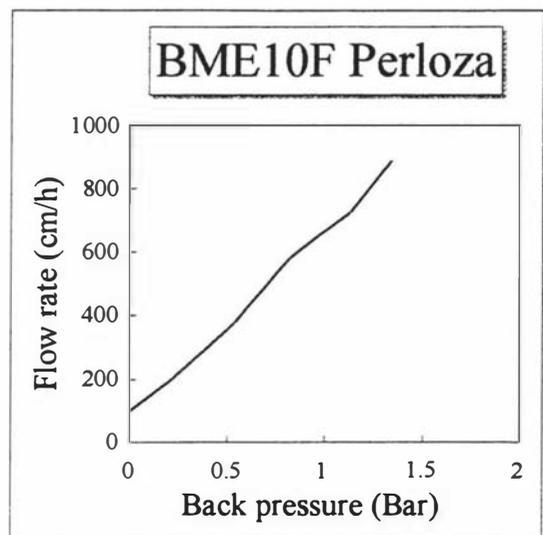
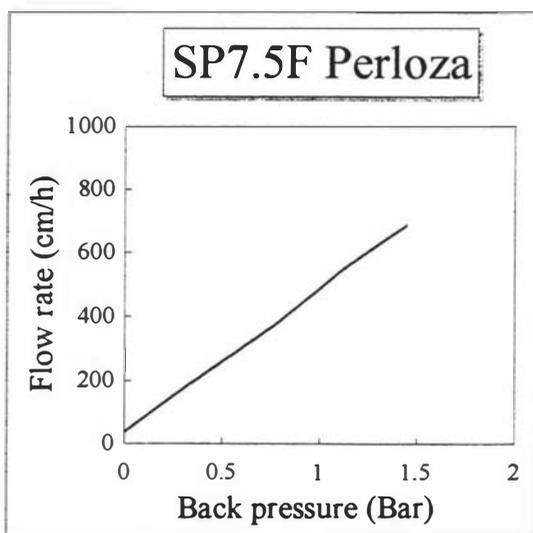


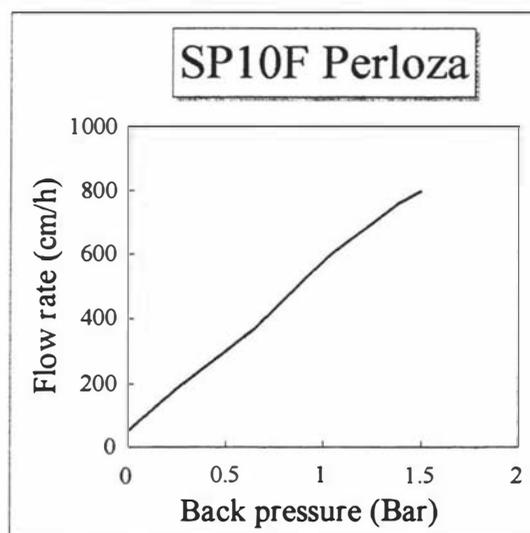
Figure 4.4 : Flow properties of various SP and Q Perloza fine resins

The performance of these SP resins (A, B and D) was superior to that found for SP7F (Figure 4.3). The major difference between these resins was ligand density (lower for SP7F). Little difference was found between crosslinked and uncrosslinked (10% AB) SP resins (B and D) and the performance was similar to that of the neutral (BME) resin of Figure 4.3. The performance of the quaternary amine resin (C) was similar to that of SP7F. Bed heights were compressed from 29.5 to 25.2 cm (A), 29.4 to 26 cm (B), 29.2 to 24.5 cm (C) and 29.3 to 26.4 cm (D).

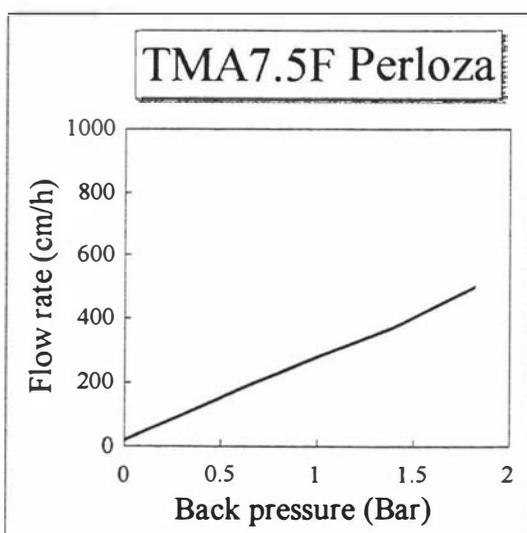
A



B



C



D

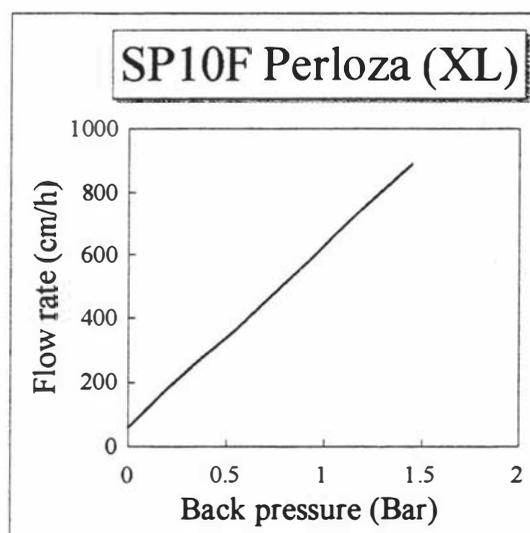
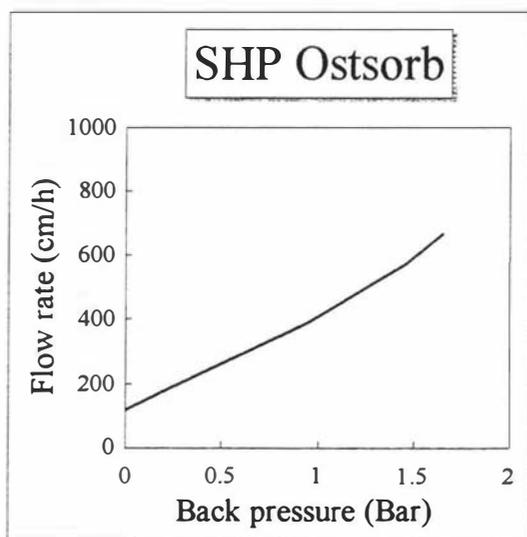


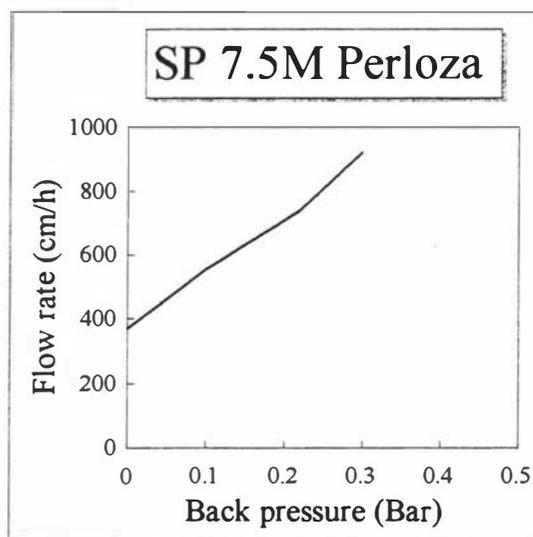
Figure 4.5 : Flow properties of SP and Q Perloza and Otsorb (medium grades)

Sulphohydroxypropyl (SHP) Otsorb performance (A) was surprisingly poor. However, minimal back pressure was found with the other resins, up to the maximum pump rate. This was expected for the coarser resin grade. The erratic lines for these resins are caused by the difficulty of obtaining accurate values for back pressures below 0.2 Bar. Bed height compressions were 30 to 25.4 cm (A), 29.1 to 27.5 cm (B), 29.5 to 29.3 cm (C) and 29.4 to 27.9 cm (D). The compression was a lot less than that found for the fine resins, except for SHP Otsorb, reflecting the lower pressure build-up.

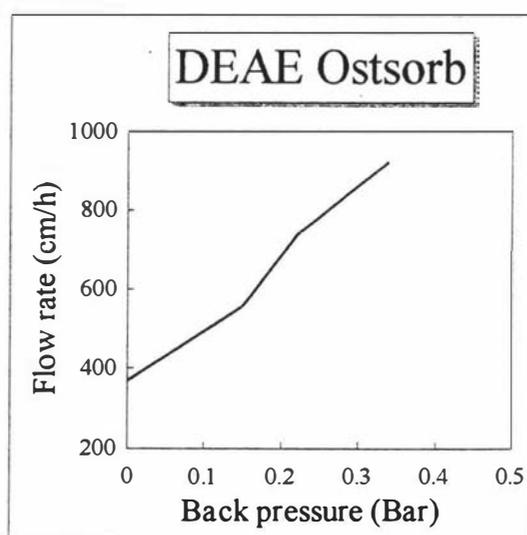
A



B



C



D

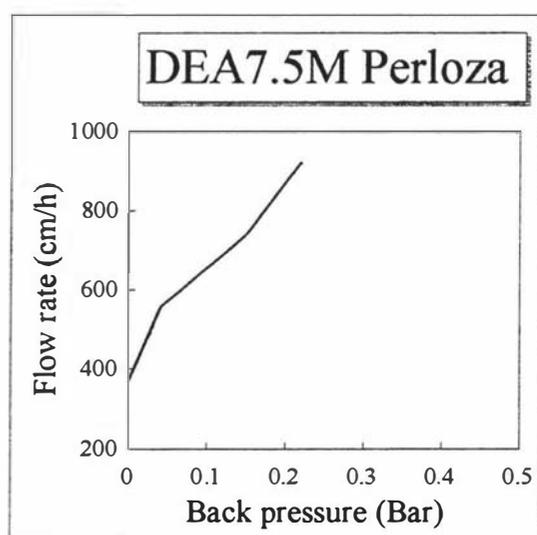
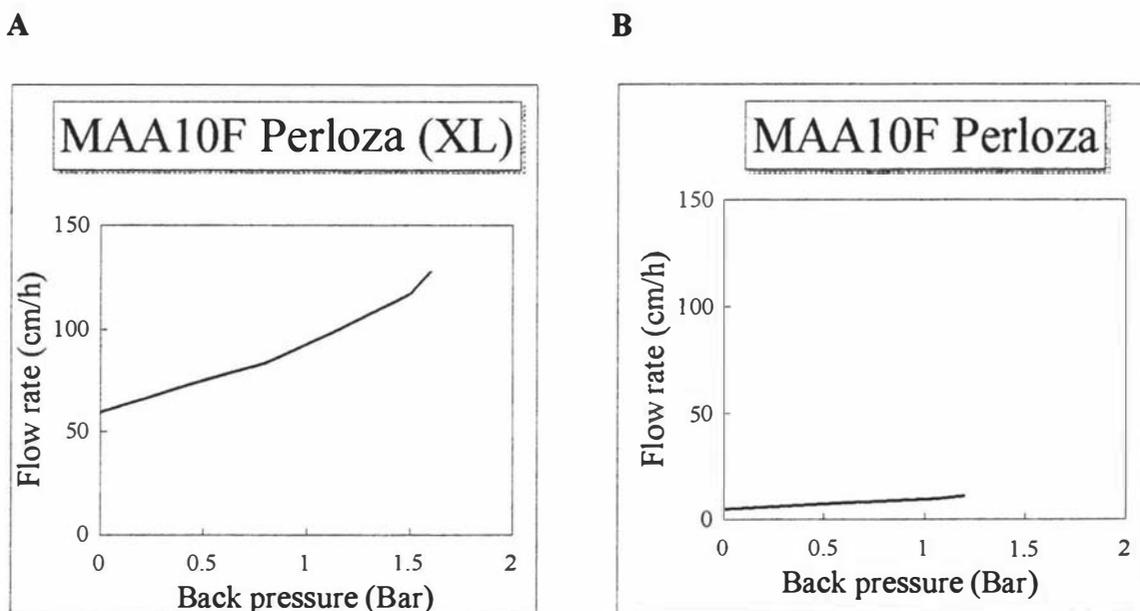


Figure 4.6 : Flow properties of (A) crosslinked and (B) uncrosslinked MAA Perloza

Bed compression (30 cm down to 24.8 and 26.1 cm respectively) and back pressure developed much more rapidly with the MAA resins.



A CM Perloza (2.4/3.8/0.85 F, Table 2.7, Chapter 2) was tested by these methods and had a somewhat better performance (maximum flow rate of 250 cm/h at 1.55 Bar). A medium grade CM Perloza (2.8/4/1 M) had a maximum flow rate of 670 cm/h, at 1.65 Bar. Comparison of these results with those for SP Perloza samples (Figures 4.3 to 4.5) indicate that the performance of the carboxylate was poorer than the sulphonate ion exchange resins, regardless of the chemistry used.

Nevertheless, the flow properties of SP, TMA and DEA Perloza resins (especially the medium grades) were very good. This suggested these resins would be suitable for large scale use, providing chromatographic properties were comparable to commercial resins.

Protein chromatography and capacities on SP and TMA Perloza resins

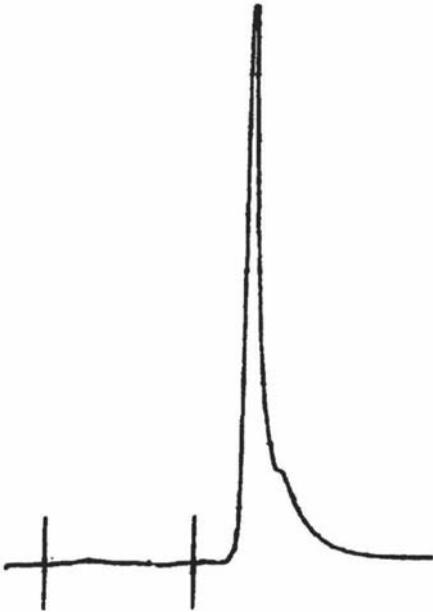
Adsorption and elution of BSA

Absorbance (280 nm) traces are shown in Figure 4.7. No significant differences of adsorption (near quantitative) or elution peak size or shape were apparent between Perloza (fine) and Sepharose resins. The absorbance of NaOH washes was very small, indicating that protein recovery at elution had been very efficient.

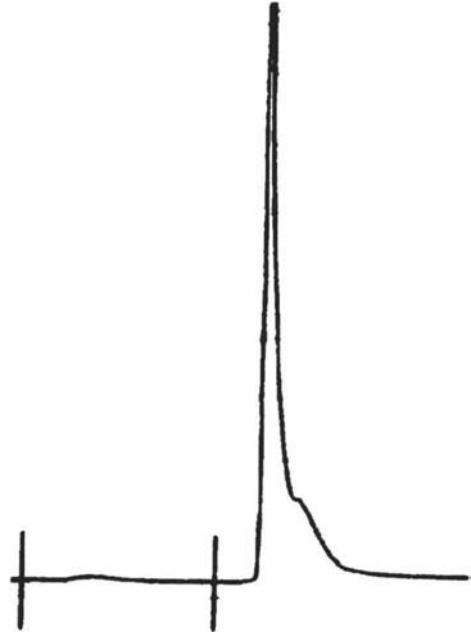
Figure 4.7 : Absorbance traces for BSA chromatography on (A) SP Perloza, (B) SP Sepharose, (C) TMA Perloza and (D) Q Sepharose.

Load and change to elution buffer (+ 0.5 M NaCl) are marked by vertical lines.

A



B



C



D



Anion exchange resin capacities

Batch capacities of Perloza resins for β -lactoglobulin and ovalbumin were high (100-130 mg/ml), while that of Sepharose was somewhat lower (Table 4.2). Otsorb resins also had high capacity for β -lactoglobulin. However, the BSA capacities of the Perloza resins was poorer than those of Sepharose and Otsorb.

Table 4.2 : Batch capacity data for Perloza, Otsorb and Sepharose resins

TMA10F* was prepared by reaction of BHP Perloza with TMA at pH 11 to minimise possible crosslinking reactions.

Resin	Capacity (mg/ml)		
	β -lactoglobulin	ovalbumin	BSA
TMA7F	130	n.d.	48
TMA10F	129	102	36
TMA10F*	n.d.	101	47
Q Sepharose	85	65	91
Q Otsorb	121	n.d.	65
DEA7F	n.d.	n.d.	41
DEA7.5M	106	n.d.	48
DEAE Otsorb	n.d.	n.d.	67
SP7F	n.d.	n.d.	93
SP10F	n.d.	n.d.	96
SP7.5M	n.d.	n.d.	69
SP Sepharose	n.d.	n.d.	122

BSA adsorption onto TMA7F Perloza was much slower than for β -lactoglobulin, but slow adsorption was also found for Q Sepharose (Table 4.3). Crosslinking due to the high pH of ligand attachment, might have restricted the capacity for the larger (BSA) protein. However, the BSA capacity of TMA10F* Perloza (prepared at a pH 11), was still low (Table 4.2). Whether these results were a peculiarity of BSA or indicated steric restrictions for larger proteins was uncertain. Perloza resins with comparatively low ligand densities, had higher BSA capacities than TMA10F. This might indicate "crowding" effects at high ligand densities. BSA capacities were still acceptable and the capacity for smaller proteins (e.g. ovalbumin, 45 kDa) was very good.

Table 4.3 : Batch adsorption time versus capacity

QFF = Q Sepharose Fast Flow, β -lac. = β -lactoglobulin. The 30 minute capacity of β -lactoglobulin was 84% of the final value. Equilibrium was approached much more slowly with BSA.

Sample time (minutes)	Capacity (mg/ml)			
	TMA7F, β -lac.	TMA7F, BSA	DEA7.5M, BSA	QFF, BSA
15	n.d.	27	25	56
30	109	28	30	66
60	125	39	39	80
120	130	48	48	91

Cation exchange resin capacities

The (batch) BSA capacities of SP Perloza resins were much higher (> 90 mg/ml) than those obtained on the anion exchange resins, although the SP Sepharose capacity was still higher (Table 4.2). The capacity of a medium grade SP Perloza was lower, as expected, but still very good. Column capacity results of SP7F Perloza were high (90-144 mg/ml) for small to medium sized proteins. The capacity for immunoglobulin G was lower (39 mg/ml) but this was still a good result for a large protein. This figure might also have been improved if greater time was allowed for equilibration (e.g. batch adsorption). These column capacities are summarised in Table 4.4.

Table 4.4 : SP7F column capacities for various proteins

Approximate molecular weight values were obtained from Sigma (1986) and Lehninger (1987)

Protein	Molecular weight (Da)	Capacity
Ribonuclease	12,640	144
Lysozyme	13,930	130
Haemoglobin	64,000	130
BSA	66,000	89
Immunoglobulin G	150,000	39

With the exception of BSA/anion exchange Perloza derivatives, capacities were very high, indicating that the porosity of Perloza had not been noticeably compromised by ligand attachment chemistries.

Effect of unreacted allyl and BHP groups on chromatography

The similarity of BSA chromatography on Perloza and Sepharose Fast Flow resins suggested that if unreacted groups were interacting with proteins, it did not significantly affect adsorption or desorption. Although failure to detect differences in this example cannot be assumed to apply to other proteins, it does seem unlikely that a protein will react/interact with groups when the small, reactive ligand groups have failed to do so. Resins produced by both addition and substitution methods could also be "blocked" by reaction with mercaptoethanol (BME). The BME thiolate ion is an excellent nucleophile for substitution, while the thiol adds very efficiently to allyl groups (Chapter 3).

SUMMARY

Ion exchange resins, produced by the allyl chemistry methods of Chapter 3, were analysed by cost, and physical and chromatographic performance. The (reagent) cost of preparation was very low, which combined with simple methods suggested economic viability. Residual allyl or BHP groups were not expected to interfere with protein chromatography because of steric inaccessibility. Volume stability and flow properties of original Perloza 100 were fairly well maintained except for MAA derivatives. The high efficiency of MAA addition may have contributed to the relatively swollen products and their slow flow rates. The other ion exchange derivatives (SP, DEA and TMA) were apparently suitable (physically) for large scale use.

Protein chromatographic properties were only tested very briefly. Normal ion exchange adsorption and elution of BSA occurred (at low and high ionic strength). The size and shape of elution peaks of Perloza fine and Sepharose resins were very similar. The capacities of SP Perloza resins were high (typically 90-140 mg/ml). Capacities of the anion exchange resins were high for β -lactoglobulin and ovalbumin (100-130 mg/ml) but much lower for BSA (35-50 mg/ml). The poorer results for BSA may have been due to the greater molecular weight of BSA (50%) compared to ovalbumin, but high BSA capacities were found on the SP resins. Unintentional crosslinking might reduce capacity, and some improvement was found using a new TMA Perloza prepared at lower pH (crosslinking unlikely). The BSA capacity was higher for resins with low ligand densities and still lower ligand density may be preferable for BSA adsorption.

SP Perloza resins performed well in all aspects. This indicated that addition chemistry should be useful for general resin production. The flow properties of uncharged resin derivatives were expected to be as good as the SP resins, based on the performance of a mercaptoethanol AB Perloza resin. Greater control of the MAA addition reaction was required to produce a more desirable ion exchange resin. Except for their BSA capacities, the anion exchange resins performed as well as the SP resins, indicating that the NBS bromination and ligand substitution methods also resulted in useful products. Better results might yet be obtained if resin production methods were altered to optimise resulting protein adsorption characteristics.

Having demonstrated the usefulness of these methods for preparation of conventional resins, these methods and those described in Chapter 2 are applied to the production of new resin forms. The following chapters describe various new forms of mixed mode (HIC) chromatography and their application to specific purification requirements.

CHAPTER 5 PURIFICATION OF CHYMOSIN BY MIXED MODE CHROMATOGRAPHY

INTRODUCTION

Chymosin is an aspartyl protease produced in the abomasum of young ruminants. It is the preferred milk clotting protease for cheese manufacture. Its physical and enzymatic properties have been reviewed by Foltmann (1970, 1971). The isoelectric point is 4.6 and chymosin stability is greatest at about pH 2 and between pH 5 and 6.5. Activity is lost rapidly between pH 3 and 4 and above pH 7. Various values have been reported for molecular weight (30-34 kDa).

Chymosin catalyses specific cleavage of the PHE¹⁰⁵ - MET¹⁰⁶ bond of κ -casein. This destabilises the casein micelle and caseins are precipitated. The initial translation product, prochymosin, contains a 42 amino acid N terminal sequence which is cleaved (at acid pH) to yield mature chymosin (Foltmann, 1971). Commercial demand has led to development of recombinant prochymosin (Emtage et al., 1983; Goff et al., 1984) and chymosin (Harkki et al., 1989; Dunn-Coleman et al., 1991).

Several purification methods have been established for chymosin and prochymosin. Traditional methods have been based on selective precipitation (Foltmann, 1971; Clarke, 1976). The main contaminants of natural chymosin prepared by precipitation methods are mucoproteins. These are readily removed by DEAE cellulose anion exchange chromatography to produce highly pure natural chymosin (Clarke, 1976). Ion exchange chromatography has also been used to purify recombinant prochymosin (Marston et al., 1984; Goff et al., 1984) and chymosin (Pitts et al., 1991). Dye ligand chromatography has been used to separate chymosin and pepsin (Subramanian, 1987). Crude chymosin extracts have also been purified by affinity chromatography on pepstatin Sepharose (Kobayashi and Murakami, 1978), histidyl Sepharose (Amourache and Vijayalakshmi, 1984) and a peptide inhibitor Sepharose (Strop et al., 1990).

Chymosin has been non-covalently immobilised by hydrophobic interactions (Voutsinas and Nakai, 1983). The strong binding suggested that similar matrices could be useful for purification by HIC. Purification of recombinant (*A. awamori*) chymosin has been described by Heinsohn and Murphy (1989), using Phenyl Sepharose. Affinity interactions were implicated at low pH. The specificity of this method allowed production of food grade quality chymosin in one step. However the protein capacity

was low (5 mg/ml) and the matrix considered too soft and too expensive for large scale use (Heinsohn, 1988). Salt fractionation and anion exchange chromatography were insufficient for the required product purity (Heinsohn and Murphy, 1989).

Economic recovery of *A. awamori* chymosin by chromatography, required new resins and/or methods. Desired attributes were similar specificity to Phenyl Sepharose, but greater capacity, using a robust, cheap matrix. Highly substituted "phenyl" Perloza resins could satisfy these requirements, if affinity interactions were found. If high ligand densities were used, inclusion of charged groups (i.e. mixed mode HIC) would probably be necessary for efficient elution.

The synthesis of mixed mode bead cellulose and agarose resins, using simple hydrophobic amine and/or alkylcarboxylic acid ligands is reported here. Different attachment chemistries were compared and optimised, and the resins analysed by titration. **One step** methods for **high capacity** adsorption and **rapid elution** of chymosin are described, using these mixed mode resins. Adsorption to mixed mode resins was at **low or high ionic strength**. Therefore no pretreatment (salt addition or desalting) of crude chymosin broths was required before chromatography. These highlighted features and optimal resin attributes of **cost**, **physical performance** and **longevity** were the major requirements, providing adequate chymosin purity was obtained. Ion exchange chromatography on CM Perloza is also described. The different methods are compared by enzyme purity, resin capacity, fouling and cost.

MATERIALS AND METHODS

Reagents and equipment

4-(2-Aminoethyl)benzenesulphonamide and tyramine were from Aldrich-Chemie, Steinheim, Germany; phenylethylamine from Hopkin and Williams, Essex, England; and (1s,2s)-(+)-2-amino-1-phenyl-1,3-propanediol, hexylamine, tryptamine, phenylalaninol, phenylalanine, (-)-phenylpropanolamine, 1-cyclohexyl-3-(2-morpholinoethyl)carbodiimide 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide and bicinechonic acid solution from Sigma Chemical Co., St. Louis, MO, USA. Calf chymosin was from NZ Rennet Co., Eltham, NZ; and recombinant (*A. awamori*) chymosin (crude and purified samples) from Genencor International, Sth. San Francisco, CA, USA. Other reagents were described before or were analytical grade. N-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline (EEDQ) was prepared by the method of Belleau (1969). Sepharose 6B and CM Sepharose CL6B were from Pharmacia, Uppsala, Sweden. ECH activated Sepharose 6B was prepared by a proprietary method. CDI activated Sepharose CL6B and Perloza 100 fine and medium matrices and their aminobutyric, aminovaleric and aminocaproic acid derivatives, were prepared by the methods described in Chapter 2. ECH Perloza and CM Perloza medium (1.4 mMoles/g dry) and fine (1.33 mMoles/g dry) were prepared by the methods of Chapter 2. Preparation of mercaptobutyric and mercaptohexanoic acid AB Perloza and AGE NBS Perloza was described in Chapter 3.

Reaction mixing, absorbance measurement and titration methods and equipment were the same as those described before. Amine containing resins were titrated (down) to pH 4 and carboxyl resins (up) to pH 8. Chromatography was at 280 nm using a Pharmacia UV-1 path monitor, or Biorad Econosystem.

Ligand attachment

Ligand attachment to ECH resins and allyl (BHP) Perloza

The activation level of the ECH Sepharose was 1-1.3 mMoles/g dry, titrated by the sulphonation method of Chapter 2. The ligands used were phenylalaninol (PALOL), aminophenylpropanediol (APP), phenylpropanolamine (PPA), phenylethylamine (PEA), aminoethylbenzenesulphonamide (AEBS), tyramine, tryptamine, phenylalanine and hexylamine. Amine hydrochlorides were dissolved in a minimal volume of water and converted to pH 11 with addition of 5 M NaOH. Water insoluble amines (unprotonated form) were solubilised by addition of DMSO and water soluble amines used without modification. A 5 Molar excess of ligand solution was mixed with activated Sepharose,

for 48 hours at room temperature or 16 hours at 37°C. After the reaction was complete, the resin was washed with 0.1 M HCl and water to remove unreacted ligand. APP was reacted likewise with NBS brominated AGE Perloza (0.204 mMoles/g) and ECH Perloza (0.5 mMoles/g dry).

Ligand coupling to CDI-aminocaproic acid and CM matrices

Perloza fine CDI ACA (1.55, 1.7 or 1.78 mMoles/g dry), Sepharose CDI ACA (2.15 mMoles/g dry) and CM Sepharose (2.05 mMoles/g dry) resins were used. The ligands used were PALOL, PPA, APP, PEA and AEBS. They were attached to the carboxyl groups of the matrices using water soluble carbodiimides or EEDQ. Equimolar amounts of ligand and coupling agent were used. The efficiency of coupling was monitored by titration of residual carboxylic acid groups.

(a) Ethyldimethylaminopropylcarbodiimide (EDC) catalysed reactions : The ligand was dissolved in water (amine hydrochlorides) or 3 M HCl and water (amines), adjusting the pH to 7, using the minimum amount of water necessary to maintain small reaction volumes. The carboxylate matrix was adjusted to pH 4.7 and mixed with the ligand. EDC was dissolved in a small volume of water (≤ 1 ml/g), adjusted to pH 4.7 and added to the resin/ligand mixture. The pH of some mixtures (ACA matrices) decreased during the reaction. Therefore pH was maintained at the optimum of 4.7 (Cuatrecasas, 1970) during the first 1-2 hours of the reaction by dropwise addition of 1 M HCl or NaOH as appropriate. The reaction was then mixed overnight. Reaction volumes were typically 2-4 ml/g (the less soluble PALOL providing the upper limit). For 95-100% substitution of matrix carboxyl groups, a 3-5 molar excess of EDC and of ligand was used. For partial substitution (50-80%) a 1-2 molar excess of reagents was used

(b) Cyclohexylmorpolinoethylcarbodiimide (CMC) catalysed reactions : A 5 molar excess of CMC and ligand was used. A greater volume (> 10 , typically 15 ml/g) of water was required to dissolve CMC. The weight of carbodiimide used was higher due to the higher molecular weight of CMC (424 g/Mole), compared to EDC (192 g/Mole), and the greater molar excess used. Therefore significantly higher reaction volumes (up to 10 ml/g matrix) were used. The reaction mixing and pH control methods described for EDC were used.

(c) EEDQ: Ligand attachment was by an adaptation of the method of Boschetti (1978). A 3 molar excess was used for EEDQ coupling. The ligand was dissolved in water and pH adjusted to between 5 and 8 using 1 M HCl (for free amines) or 0.5 M NaOH (for amine hydrochlorides). The carboxylate matrix was washed with 100 mM acetate buffer

(pH 6), water and 50% ethanol (5 volumes each), prior to mixing with the ligand solution. EEDQ was dissolved in absolute ethanol (10 ml/g) and mixed with the matrix and ligand at room temperature for 6 hours. Reaction volumes were greater than for EDC, but pH monitoring was not required.

Chromatography

Buffers and conditions

Resins produced using epoxide or allyl chemistries (secondary amine linkage) were equilibrated with 10 mM citrate + 0, 0.5 or 2 M NaCl, pH 5.5. These buffers were also used for dissolution of lyophilised chymosin samples, loading, washing out unbound material (load wash) and intermediate washing steps. Chymosin was eluted with 50 mM HCl/KCl, pH 2.0.

CM (ion exchange) resins were equilibrated with 10 mM citrate, pH 4.4. The same buffer was used for the load wash. Chymosin was eluted with 10 mM citrate + 0.5 M NaCl, pH 4.4 or 20 mM citrate, pH 6.2. The above pH 4.4 buffers were used for equilibration, load and intermediate washes of mixed mode carboxylate resins. Chymosin was eluted with the pH 6.2 citrate buffer.

All resins were regenerated with 0.1 M NaOH. Chromatography was at room temperature, using 2 ml columns (50x7 mm). Flow rate was 0.4 ml/minute, chart recorder speed 0.5 mm/minute and full scale absorbance (AUFS) 0.2.

Sample preparation

A. awamori chymosin broths had a conductivity similar to that of 2 M NaCl and were stored at pH 5.5. Samples to be loaded at low ionic strength were diluted with water (1:100) or dialysed against the load buffer (3 changes). Samples to be loaded at high ionic strength were adjusted (if required) to the correct pH by addition of HCl. Lyophilised chymosin samples were dissolved in the appropriate load buffer.

Assay procedures

Chromatography fractions were assayed qualitatively for chymosin activity by clotting of fresh whole milk. A 200 µl chymosin sample was incubated with 4 ml of milk at 37°C for up to 1 hour. Milk clotting was assessed visually. Recovery of activity was determined by comparison of clotting time for 150 µl original crude chymosin and elution fractions (150 µl x elution volume/load volume). Total protein was assayed by the bicinchoninic acid method of Smith et al. (1985) using BSA as the standard.

Capacity testing

Capacity testing was by batch operation using lyophilised, recombinant (purified) or natural chymosin. Load buffer equilibrated matrix (0.1-0.16g) was weighed into a 25 ml vial. Another sample (2-8g) was weighed into a 10 ml measuring cylinder, suspended in load buffer, and the settled matrix volume recorded after 48 hours (to determine the weight : volume ratio). Chymosin (25 mg) and load buffer (10 ml) were added to the vial, which was sealed and mixed for 2 hours on a rotating wheel. The contents of the vial were quantitatively transferred to a 2 ml column, by washing with load buffer. The resin was washed with load buffer until the effluent absorbance at 280 nm returned to zero. Samples prepared at pH 4.4, low ionic strength, were cloudy due to low solubility of chymosin close to its isoelectric point. Any insoluble material retained after mixing passed through the column frits. The matrix was stirred to ensure complete removal of insoluble chymosin. Chymosin was eluted into a 10 ml volumetric flask (and a 25 ml vial, if required) and the absorbance at 280 nm measured. The extinction coefficient was determined, as described previously, using natural and purified *A. awamori* chymosin (dialysed and lyophilised to constant weight), prepared as a 0.05% solution in buffer G.

Crude chymosin capacities were obtained similarly, but chymosin eluted was measured by the bicinchoninic acid assay, rather than absorbance. Crude chymosin solution (22 ml containing approximately 0.3 mg of chymosin/ml), adjusted to the same pH as the load buffer, was used instead of lyophilised chymosin and load buffer.

Analytical methods

RP-HPLC

Samples were analysed using a Pharmacia Smart system and a C18 (3 μ m spherical silica) Smart column (30x3.2 mm). The column was equilibrated with buffer 1, 90% A (0.05 M formic acid + 1% NaCl), 10% B (acetonitrile) and the chromatogram developed by a 20 minute gradient to 100% buffer 2 (40% A, 60% B). The flow rate was 0.1 ml/minute and elution was monitored at 280 nm.

Electrophoresis

A Pharmacia Phast automatic system was used. SDS-PAGE was performed using 20% homogeneous gels (separation file 111). Protein bands were stained by the sensitive silver method (development file 210), except that formaldehyde content of the fixer was reduced from 0.04 to 0.025%. Molecular weight markers (lysozyme, carbonic anhydrase and BSA) were from Sigma Chemical Co.

RESULTS AND DISCUSSION

Epoxide activated matrices

Ligand selection and inhibition studies

Ligands of similar structure to the aromatic amino acids were investigated because of the enzymes specificity for the PHE¹⁰⁵ - MET¹⁰⁶ bond. Chymosin was adsorbed to a phenylalaninol (PALOL) ECH Sepharose resin at pH 5.5, high or low ionic strength. The ligand density was not recorded but on the basis of other results is estimated to have been 0.6 - 0.8 mMoles/g dry. It was not adsorbed to phenylalanine Sepharose at the same pH, at either ionic strength. A reduction in pH (to 2) was required for elution from PALOL Sepharose. This strength of interaction suggested that chymosin might have a special affinity for PALOL. PALOL (20 mM) was shown to be a weak inhibitor of chymosin, increasing clotting time by 80%. Attachment of cheaper aromatic ligands, phenylethylamine (PEA), phenylpropanolamine (PPA) and aminophenylpropanediol (APP), to ECH Sepharose produced resins with similar chymosin adsorption properties. The substitution level of the APP resin was 0.81 mMoles/g dry. These ligands (20 mM) were still weaker inhibitors of chymosin than PALOL, increasing the clotting time 0-20%). These "millimolar" levels of inhibition were considered too low for affinity binding (Turkova, 1993). Hydrophobic and/or ionic interactions were considered more probable causes of strong chymosin binding.

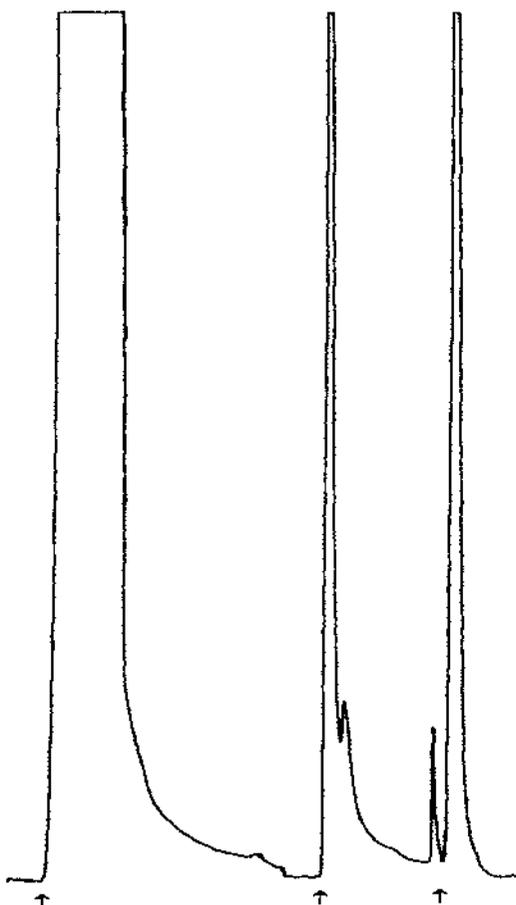
Other ligands

Because affinity interactions with phenyl groups were not considered important, several other hydrophobic amine ligands were attached to the above ECH Sepharose. Tyramine, tryptamine and AEBS Sepharose bound chymosin in identical fashion to PALOL, PEA, PPA and APP Sepharose. Hexylamine attached to ECH Sepharose (1.28 mMoles/g dry), behaved likewise. Recorded ligand densities were 0.76 and 0.81 mMoles/g dry (AEBS and tryptamine) and 0.99 mMoles/g dry (hexylamine). The common feature of the strong binding matrices was a high substitution (0.7-1 mMoles/g dry) of a hydrophobic amine which did not contain any anionic groups (unlike phenylalanine), at pH 5.5. The ligand was attached to the matrix through a secondary amine group, which is positively charged at pH 5.5. The net surface charge of chymosin is negative at pH 5.5 (and positive at pH 2). Therefore favourable ionic and hydrophobic interactions (mixed mode HIC) could occur between the resin and chymosin. Strong binding allowed the matrix to be washed with low and high ionic strength solutions. Therefore chymosin could be adsorbed without the requirement for prior salt addition or removal. This binding strength could have been due to the favourable combination of interactions, or to

high ligand density. The pH change (to 2) resulted in charge repulsion between chymosin and the matrix and hence chymosin was eluted rapidly and apparently quantitatively (Figure 5.1). These resins and methods also provided a substantial purification of crude chymosin in one step.

Figure 5.1 : Chymosin chromatography on APP ECH Sepharose 6B

Load (pH 5.5, 0.5 M NaCl), elution (pH 2) and 0.1 M NaOH steps are marked with arrows.



Mixed mode resins produced from CDI activated, aminocaproic acid Perloza

Neutral linkage

CDI activation produces an uncharged urethane linkage with amine ligands (Bethell et al, 1979). High substitution levels of the spacer arm, aminocaproic acid (ACA), can be obtained (Chapter 2). If the aminocaproic acid carboxyl groups are fully substituted with ligand, the resulting resin will be uncharged.

The ligands initially tested were PALOL and PEA, coupled to ACA Sepharose and Perloza. They were selected on the basis of the original ECH Sepharose results, before a wider range of hydrophobic amines had been successfully tested. These resins were tested to determine whether uncharged matrices would be more specific for chymosin, and hence give superior capacity and resolution. These resins were prepared using CMC catalysed condensation. The reaction was assumed to approach completion, due to the use of a 5 molar excess of CMC and ligand. The resins bound chymosin at pH 2 and 4.4, but not at pH 5.5 used for ECH resins. Therefore chymosin was easily eluted at pH 5.5-6.5. When CMC was replaced by EDC (also a 5 molar excess), chymosin eluted incompletely or not at all at pH 6. Subsequent back titration of CMC resins showed that 50% of carboxyl groups were unreacted, whereas $\leq 15\%$ were unreacted for EDC resins. If the carboxyl groups of ACA resins was largely substituted with a hydrophobic amine ligand (95 % +), elution required harsh, enzyme denaturing conditions (0.1 M NaOH). A resin with PEA attached directly to CDI also bound chymosin at pH 2 and 4.4, but 0.1 M NaOH was again required for elution.

Incomplete substitution of spacer arm carboxyl groups was necessary to allow rapid and complete elution of chymosin at pH 6. The best results were obtained when 40-50 % of the original carboxyl groups were unreacted. This method was another form of mixed mode HIC. Unlike the ECH resins, the matrix charge was negative and hence elution was by an increase in pH. Other differences were that the spatial arrangement of charge and ligand was more random, higher ligand substitution levels were obtained, a hydrophobic spacer arm was included and the ratio of charged groups to ligand was not fixed at 1:1 by the attachment chemistry.

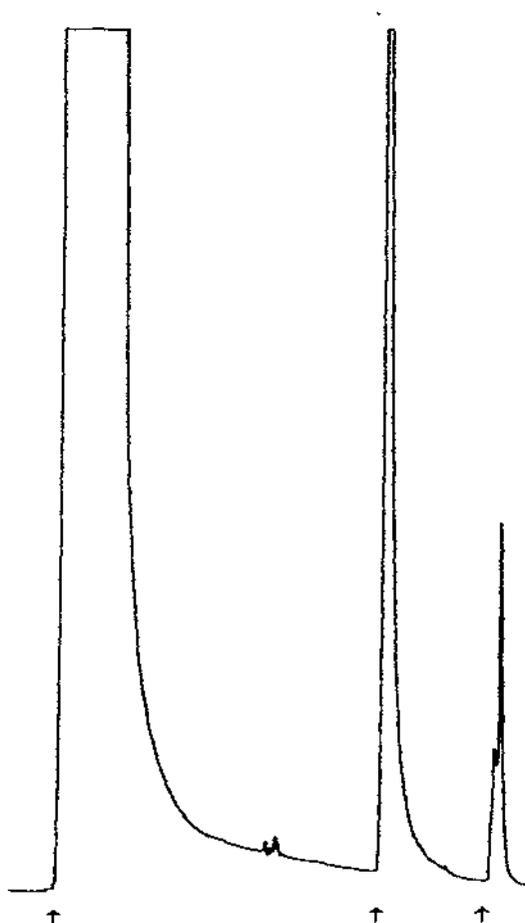
The efficiency of the condensation reaction was affected by the choice of carbodiimide (EDC was more efficient than CMC), ligand solubility (PPA, APP and PEA are more soluble than PALOL and react more efficiently) and the molar excess of carbodiimide and ligand used. Using a 5 molar excess of CMC and ligand only 40-60% carboxyl group substitution resulted. Using a 3-5 molar excess of EDC 85-100% substitution resulted. For PEA, PPA and APP with EDC a 1.5 molar excess was required to produce approximately 60% substituted (40% carboxyl group) matrices. For PALOL with EDC a 2 molar excess was required for 60% substitution. Because this incomplete substitution was acceptable, and indeed required, the cost of matrix synthesis was substantially reduced. The substitution efficiency using EEDQ was lower than for EDC (use of a 3 molar excess resulted in the desired substitution range). The modified EDC method was used henceforth. PALOL, PEA, PPA and AEBS were attached accordingly to an ACA Perloza resin (1.70 mMoles/g dry). The back titrations of these resins were 0.38, 0.41,

0.69 and 0.53 mMoles/g dry (22, 24, 41 and 31% unreacted carboxyl groups) respectively. APP was reacted similarly with ACA Perloza (1.78 mMoles/g dry) with a back titration of 0.59 mMoles/g dry (33%).

Chymosin was adsorbed to these resins at pH 4.4. It was not eluted by increasing the ionic strength but was rapidly eluted at pH 6, due to charge repulsion (Figure 5.2). Therefore, as before, samples could be loaded without adjustment of ionic strength. Elution, by pH adjustment to 6 was rapid and apparently quantitative. Load and elution fractions had identical milk clotting times once they were adjusted to the same volume and pH. There was no significant chromatographic difference between the ligands used. Adsorption was again independent of ionic strength and elution by a pH change. Substantially purified chymosin was again obtained in one step. Equivalent results were obtained with Sepharose ACA (2.15 mMoles/g dry) substituted with PALOL (60%).

Figure 5.2 : Chymosin chromatography on Perloza-CDI-aminocaproic-PPA (67%).

Load (pH 4.4, 0.5 M NaCl), elution (pH 6.2) and 0.1 M NaOH steps are marked with arrows.



Other carboxylate resins

Ligand substituted CM matrices

The CDI-aminocaproic acid activation method had three disadvantages. Solvent exchange was required for CDI activation, the ligand is attached through urethane and amide bonds and the carbodiimide ligand attachment chemistry was expensive. Urethane and amide bonds are slowly hydrolysed at high pH (Bethell et al., 1981b; McMurray, 1988). Therefore sanitation of a matrix using 0.1 M NaOH could result in ligand leaching. Carboxymethyl (CM) resins could be used for ligand attachment, instead of CDI-aminocaproic acid activated matrices. CM matrices should be cheaper to produce than their CDI based counterparts. CM carboxyl groups are attached to the matrix through stable ether bonds, although ligand attachment would still require expensive carbodiimide chemistry. Moreover, a spacer arm is not included which could influence protein binding strength and resin capacity (O'Carra et al., 1973).

Ligand substituted CM Sepharose and CM Perloza resins bound chymosin weakly (it was eluted at high ionic strength), except for one CM Sepharose APP resin, containing only 20% unreacted carboxyl groups. The requirement for higher ligand substitution was not surprising because a hydrophobic spacer arm was not included (unlike CDI ACA resins). Thus most CM derivatives appeared to adsorb chymosin predominantly by ionic interactions. The requirement of higher ligand percentages for strong binding made this option less economic (extra carbodiimide expense) and it was not pursued further.

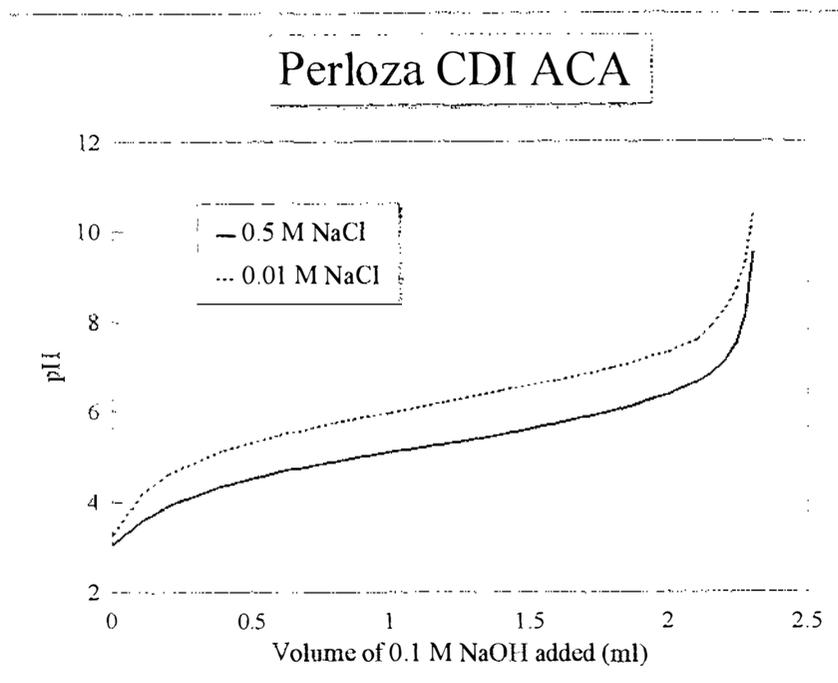
CM ion exchange chromatography

Most contaminants of the crude *A. awamori* chymosin washed through CDI ACA ligand resins with the low ionic strength buffer. The peak eluted with 0.5 M NaCl addition was very small by comparison with the load and elution peaks. Therefore it was thought that an unmodified CM resin might produce a similar level of enzyme purification under these low ionic strength conditions. CM resins did bind chymosin at pH 4.4 (10 mM citrate), despite being very close to the isoelectric point. It could be eluted by increasing ionic strength or pH but the latter was used routinely because elution profile and chymosin stability were superior. Adsorption was very sensitive to ionic strength. Dilution (fifty fold), dialysis or another desalting step was required before crude chymosin solutions could be applied to the resin. This extra step and/or large increase in sample volume was not desirable for large scale use.

Chymosin adsorption to Perloza ACA resins

The importance of the ACA spacer arm to strong binding (at high ionic strength) was indicated by results with ligand modified CM resins. The sensitivity of the CM based resins to small levels of unreacted carboxyl groups contrasted with the consistent performance of CDI resins containing 30-60% unreacted carboxyl groups. Titration curves of ACA resins at low and high ionic strength (Figure 5.3) revealed that only a small proportion of carboxyl groups ($\leq 25\%$) were ionised at pH 4.4. The level of CM ionisation is much higher for, due to a lower pKa (Scopes, 1987). Because of the consistent performance of variably substituted ACA resins, the chymosin adsorption properties of unmodified resins was tested.

Figure 5.3 : Titration curves of Perloza CDI ACA, at high and low ionic strength



It was found that unsubstituted CDI ACA resins also adsorbed chymosin at both high and low ionic strengths, at pH 4.4. Fouling of these unsubstituted resins by crude chymosin broths was less severe than for the substituted resins. Because ligands and expensive carbodiimides were not required, cost was significantly reduced. An aminovaleric acid (AVA) CDI Perloza also bound chymosin at high ionic strength, but aminobutyric acid resins only retarded chymosin. ACA was preferred because it is much cheaper than AVA. The major cost became the CDI activation step (in particular solvent exchange). The lability of the urethane linkage remained a weakness of these resins.

Purification performance comparison

Crude chymosin was purified significantly by chromatography on ECH Sepharose derivatives, in one step. Chymosin could be loaded without prior salt addition or removal, although a high salt wash was required for optimal purity. Purification was much lower if only the low ionic strength buffer was used (Table 5.1), presumably due to non-specific ion exchange adsorption of contaminants. Electrophoresis, using the sensitive silver stain (Figure 5.4), and reversed phase HPLC (Figure 5.6) confirmed purity was very good.

Chymosin of high purity was obtained in one step using partially (ligand) derivatised Sepharose and Perloza ACA resins. Buffer ionic strength did not affect purity, unlike the ECH Sepharose resins. This was attributed to the low proportion of ionised carboxyl groups at adsorption pH (4.4), low ionic strength (Figure 5.3). The purity of eluted chymosin, by protein assay, was slightly lower than that (optimal) obtained from the Sepharose ECH APP resin (Table 5.1). Electrophoresis (Figure 5.4) and HPLC (Figure 5.6) again indicated a high level of purification.

The level of chymosin purification obtained by CM ion exchange chromatography, determined by protein assay, was apparently lower (Table 5.1) and electrophoresis (Figure 5.5) showed the presence of a low molecular weight contaminant. However HPLC (Figure 5.7) showed that the level of purification was still very good. Purity was also superior to that obtained at low ionic strength on Sepharose ECH APP (functioning as an anion exchange resin, Table 5.1).

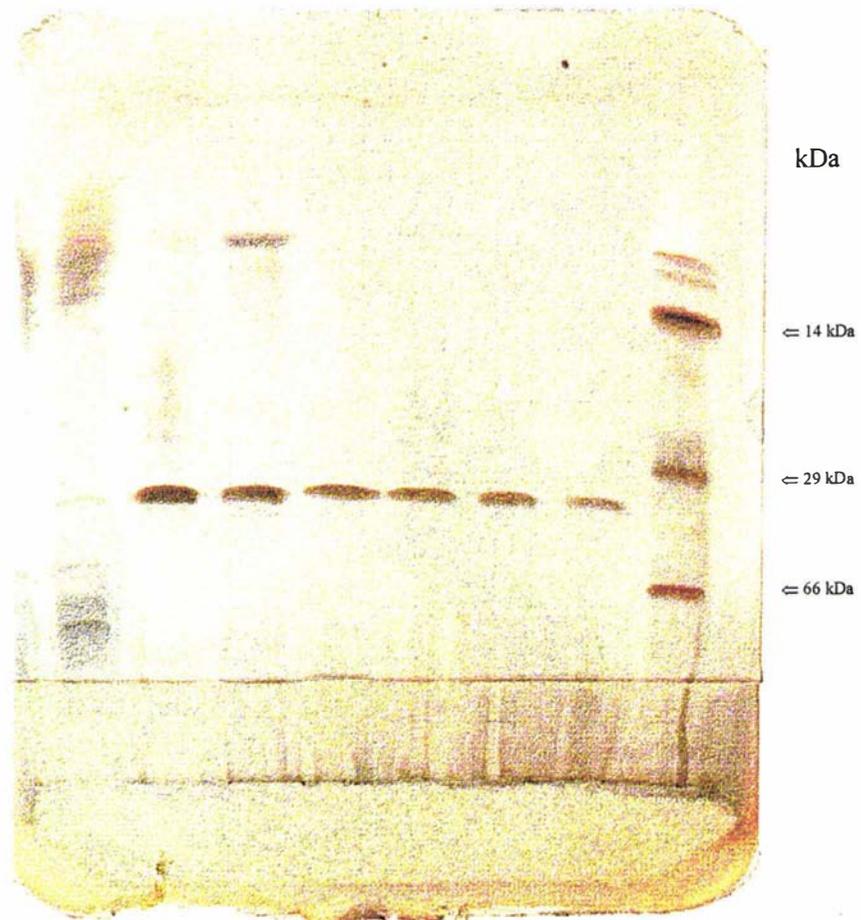
Similar purification levels (to those of the ligand substituted resins) were obtained using unsubstituted ACA resins (Table 5.1, Figures 5.4 and 5.7). This had been anticipated, because chymosin was adsorbed and eluted under the same conditions. One elution sample (Figure 5.4) has a low molecular weight contaminant (or breakdown product). This was obtained without an intermediate wash at high ionic strength and may thus be due to a low level of non-specific ion exchange interactions. It was not considered as significant as the CM contaminant, because other measures of purity were not affected. To emphasise this, the CM elution sample, described above, was chromatographed at high ionic strength on a Perloza CDI ACA resin. The low molecular weight contaminant found in the CM elution was not adsorbed to the ACA resin (Figure 5.5).

Table 5.1 : Comparison of chymosin purification by different methods

All resins were tested with crude filtered *A awamori* chymosin. The "low IS" (low ionic strength) sample used undialysed broth, diluted 1:100 with water. No chymosin activity was found in (load) flowthrough samples. Clotting assays of elution samples indicated 95-100% recovery of chymosin activity. Therefore it was assumed that the total activity of elution samples and original sample was equivalent. Purification was calculated by the reduction in total protein (original+elution). Perloza (P) and Sepharose (S) matrices were used. Epichlorohydrin (E) and CDI aminocaproic acid (CC) chemistries were used. Resins are identified by ligand and where appropriate percentage (of carboxyl groups reacted with ligand). # represents data for crude chymosin before chromatography

Resin	Chymosin	Total protein (mg)	Purification
#	undialysed (8 ml)	68.8	
SE AEBS	undialysed (8 ml)	2.8	25
SE APP	undialysed (8 ml)	2.5	28
PCC APP 67%	undialysed (8 ml)	3.0	23
PCC	undialysed (8 ml)	3.0	23
#	undialysed (6 ml)	51.6	
SE hexylamine	undialysed (6 ml)	2.1	25
SE tryptamine	undialysed (6 ml)	6.4	8
SE APP (low IS)	undialysed (6 ml)	9.3	6
#	dialysed (8 ml)	12.8	
SE APP	dialysed (8 ml)	1.2	11
PCC APP 67%	dialysed (8 ml)	1.1	12
SCC PALOL 60%	dialysed (8 ml)	1.0	13
CM Perloza fine	dialysed (8 ml)	1.4	9

Figure 5.4 : SDS PAGE of chymosin elution samples



Lane 1 : original chymosin broth

Lane 2 : elution from Perloza ACA APP (67%)

Lane 3 : elution from Perloza ACA

Lane 4 : elution from Sepharose APP

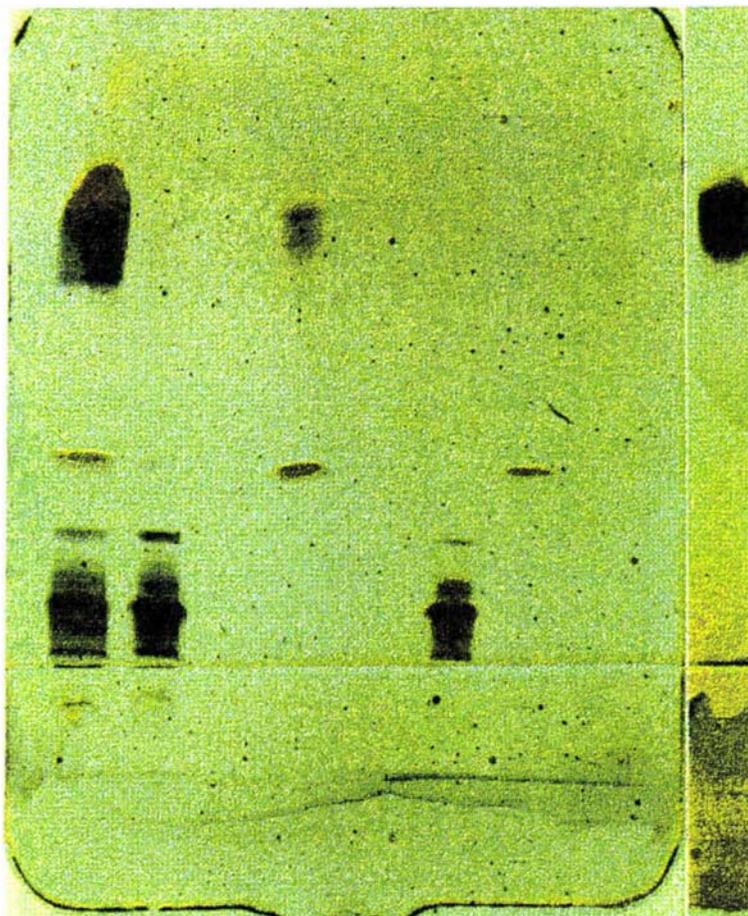
Lane 5 : elution from Sepharose AEBS

Lane 6 : dilution (1/2) of lane 2

Lane 7 : dilution (1/2) of lane 3

Lane 8 : molecular weight markers (α -lactalbumin, carbonic anhydrase and BSA)

Figure 5.5 : SDS PAGE of CM and ACA chymosin load and elution samples



Lane 1 : original chymosin broth

Lane 2 : load wash from CM Perloza

Lane 3 : empty

Lane 4 : elution from CM Perloza

Lane 5 : empty

Lane 6 : load wash from Perloza ACA

Lane 7 : elution from Perloza ACA

Lane 8 : load wash from Perloza ACA (using CM Perloza elution sample)

Figure 5.6 : RP-HPLC of chymosin : Perloza and Sepharose (APP) elution samples

Original broth, Perloza ACA APP (67%) and Sepharose APP elution samples. Chymosin elution is marked with an arrow.

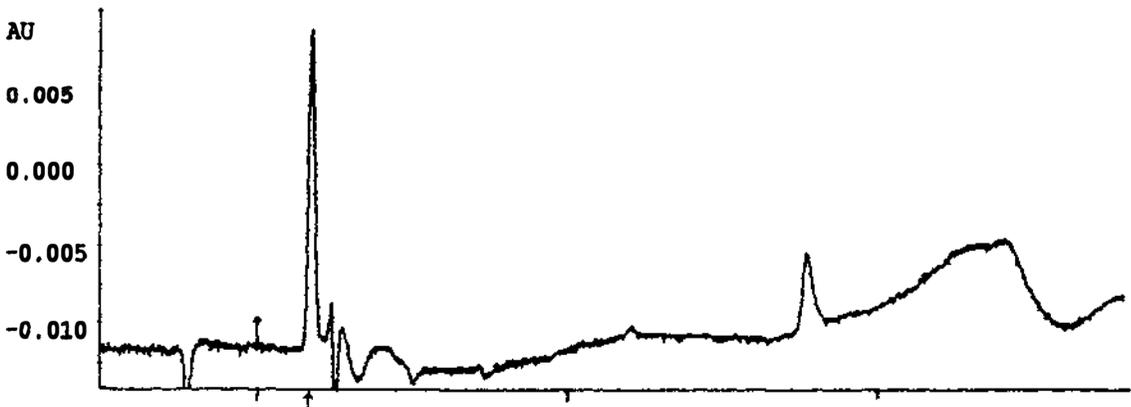
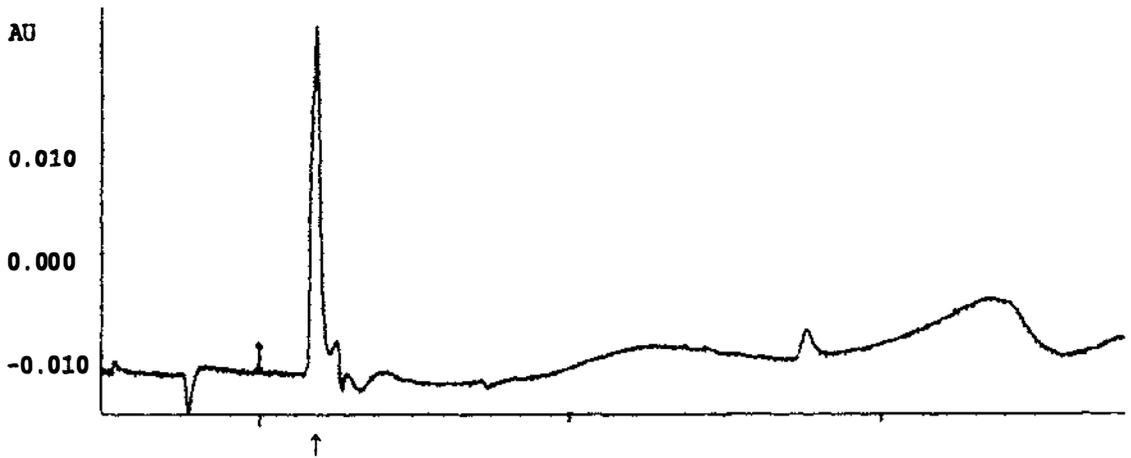
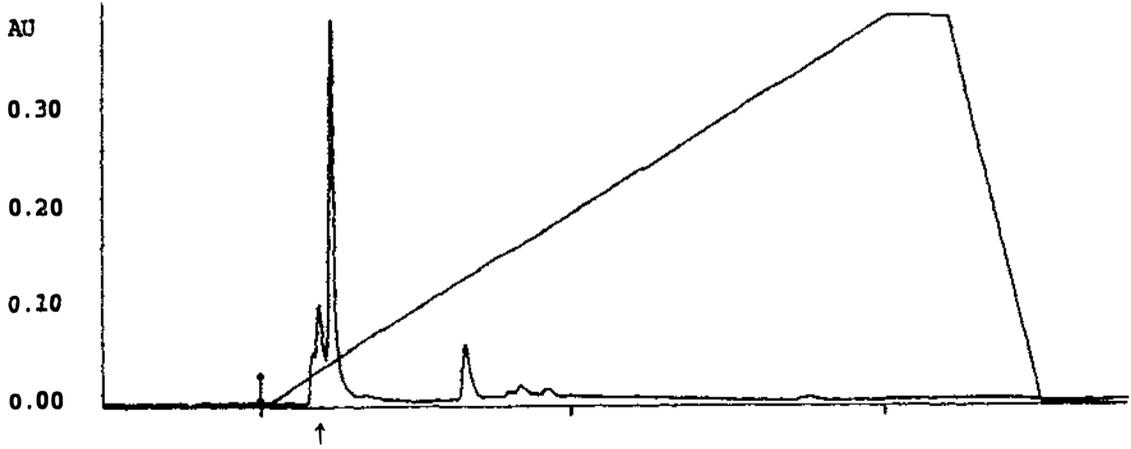
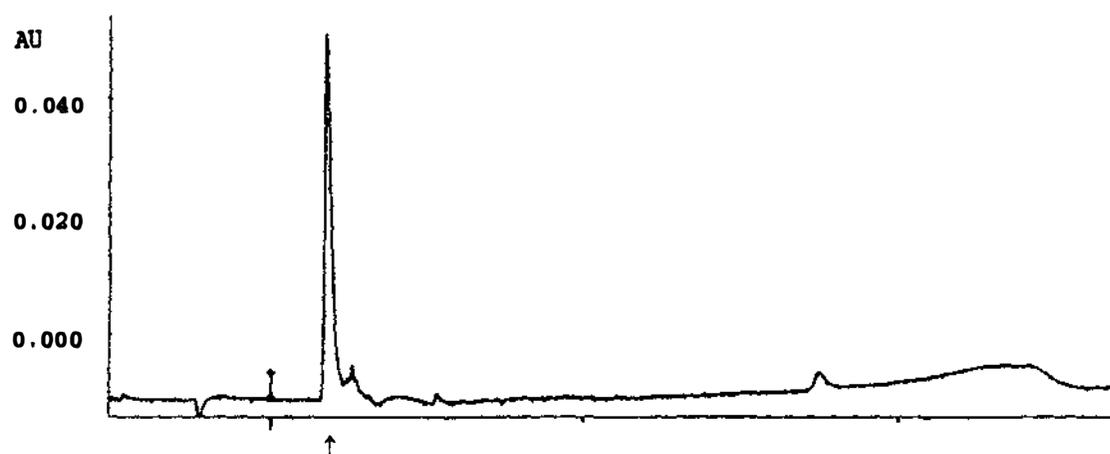
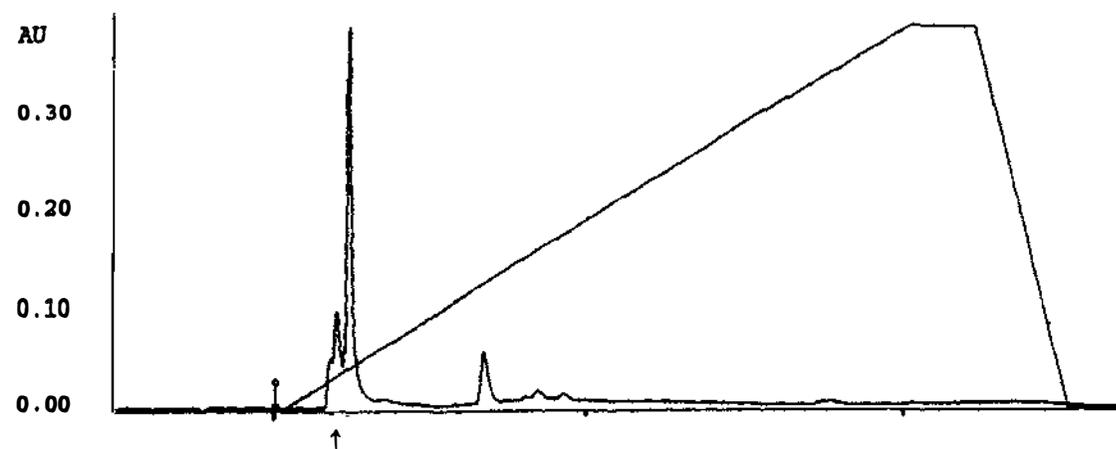


Figure 5.7 : RP-HPLC of chymosin : CM and Perloza ACA elution samples

Original broth, CM Perloza and Perloza ACA elution samples. Chymosin elution is marked with an arrow.



Capacity testing

The average absorbance of five 0.05% solutions of natural chymosin in buffer G was 0.67 (range of 0.64-0.69). This equated to an extinction coefficient (1%) of 13.4, which was used for capacity measurements. A similar value (13) was obtained using purified *A. awamori* chymosin. High capacities were found for all mixed mode resins tested at low ionic strength. The highest capacities were obtained using Sepharose and Perloza 100 fine matrices. Capacities on CM resins were also high (58-70 mg/ml), but lower than on the CDI and CM based mixed mode matrices. No significant capacity difference was found between natural and purified recombinant chymosin. The capacity of Sepharose ECH resins at high ionic strength varied depending on the ligand used. The less hydrophobic ligands had lower capacity except for APP, which was surprisingly high. This might be due to a contribution of affinity interactions to adsorption. The capacity of ACA resins was similar to that of ligand substituted resins. The lower capacity results for crude chymosin were attributed to lower chymosin concentration in the batch mixture and competition by foulants for binding sites. These results are summarised in Table 5.2.

Resin fouling

Fouling of unsubstituted carboxylate resins was apparently insignificant (no coloured material retained) after elution at pH 6.2. Very little material was removed by the 0.1 M NaOH regeneration wash (by protein assay or absorbance at 280 nm). With increasing ligand substitution of these carboxylate resins greater colour fouling was evident after chymosin elution, but this was mostly removed by the regeneration wash. The fouling of mixed mode amine resins, after chymosin elution at pH 2, was much greater. Some of this (colour) was removed by the regeneration wash, but much was retained. The effect of fouling on resin longevity was not studied, but the regeneration performance of the carboxylate resins suggested their performance would be better in this regard.

Low molecular weight coloured contaminants of elution samples

Mixed mode resin eluates from crude chymosin chromatography were contaminated with a brown coloured material, especially using the carboxyl resins. This was evidently of low molecular weight (removed by dialysis) and hydrophobic (retained on mixed mode but not CM resins at pH 4.4). Samples could be cleaned by dialysis or a second chromatography step. It did not appear to interfere significantly with the BCA protein assay (little difference after dialysis), but did affect the absorbance at 280 nm. It was not apparently stained by the silver method (electrophoresis).

Table 5.2 : Comparison of chymosin capacity for mixed mode and CM resins

Chymosin was recombinant (*A. awamori*), except for CM capacity tests and one mixed mode test, as indicated. Capacity for purified chymosin was determined by absorbance at 280 nm, using an extinction coefficient of 13.4. Low molecular weight (dialysable) contaminants interfered with the spectrophotometric assay for crude elution samples. Crude chymosin capacity was assumed to be equivalent to total protein capacity because of the degree of purity found at electrophoresis. Total protein was determined by bicinchoninic acid assay (Smith et al., 1985). Little difference was found between dialysed (values recorded here) and undialysed samples. Low IS = low ionic strength (10mM load buffer) used. Resin identification follows the conventions of Table 5.1.

Resin	Chymosin source	Capacity (mg/ml)
SE AEBS	purified	10
SE hexylamine	purified	22
SE APP	purified	93 (low IS)
SE APP	purified	32
SE APP	crude	20
SCC PALOL 60%	purified	52
SCC PALOL 60%	purified	69 (low IS)
SCC PALOL 60%	natural	71 (low IS)
PCC PALOL 78%	purified	80
PCC AEBS 69%	purified	74
PCC PPA 59%	purified	68
PCC APP 67%	purified	80 (low IS)
PCC APP 67%	purified	71
PCC APP 67%	crude	35
PCC	purified	91 (low IS)
PCC	purified	68
PCC	crude	37
CM Sepharose CL6B	natural	69 (low IS)
CM Perloza (fine)	natural	65 (low IS)
CM Perloza (medium)	natural	58 (low IS)

Resins produced using allyl chemistry

A Perloza ECH APP resin (mMoles/g dry) adsorbed little chymosin (6 mg/ml, crude chymosin capacity), whereas Perloza AGE APP (0.157 mMoles/g) adsorbed strongly (40 mg/ml, crude capacity). Fouling of the highly substituted AGE resin was more severe however. Chymosin purity was not tested but an intermediate ligand density might be more useful. Other mixed mode carboxyl resins were obtained by reaction of (NBS) AB and AGE Perloza with mercaptobutyric acid (MBA) and mercaptohexanoic acid (MHA), described in Chapter 3. Chymosin adsorption to (at high and low ionic strength, pH 4.4) and elution from (at pH 6.2) these resins was analogous to earlier results with ACA and AVA derivatives of CDI Perloza. Unlike ACA resins, a strong thioether linkage was used. The linkage was presumed to be more hydrophobic, because MBA derivatives bound chymosin strongly, whereas aminobutyric acid CDI Perloza did not. However, MBA and MHA are not commercially available.

Preferred method

Perloza substituted with ACA (or a mercaptoacid such as MBA) was preferred because it has high capacity, rapid elution at a favourable pH and good regeneration properties (low fouling). These resins are also easy to prepare, although the CDI activation required for ACA is relatively expensive. If MBA can be obtained at a low price, this would be preferred. The only drawback of these resins is that the elution fractions contain more coloured contaminants than those obtained by the other methods. These contaminants can be removed by chromatography (ion exchange) or dialysis. Although chymosin purity might be slightly better, using a secondary amine APP resin, this advantage is offset by the more severe fouling found with these resins. A pretreatment step to remove foulants would be required if these resins were to be used over a large number of cycles.

Comparison with previous mixed mode chromatography examples

Previous use of mixed mode resins for protein chromatography has not focussed on resin simplicity, ligand density, adsorption independent of ionic strength and elution by a pH change. Mixed mode resins described by Bischoff and McLaughlin (1984) were only used for HPLC of nucleic acids, typically with isocratic elution. Strong, salt independent adsorption and protein use were not reported. Histidyl resins, used by Amourache and Vijayalakshmi (1984) for chymosin purification, had charged and aromatic groups but very weak adsorption properties (low ionic strength required). Weak protein adsorption

was typically found at high ionic strength by Hofstee (1973 a/b) and Jost et al. (1974). Low ligand densities were used. Complex, very hydrophobic ligands were used by Yon (1972 and 1974) and Yon and Simmonds (1975) to obtain strong adsorption properties. Kasche et al. (1990) reported adsorption of penicillin amidase to a phenylbutylamine resin at high ionic strength. The resin was only partially charged at the adsorption pH. The preferred matrix, Eupergit C is very expensive. Phenylbutylamine would be more hydrophobic than most of the ligands used here and could lead to greater elution contamination (as was found using tryptamine Sepharose for chymosin purification, Table 5.1). Furthermore, the severe fouling of secondary amine resins, found with crude chymosin broth, would also be expected for the phenylbutylamine resin. Use of such resins early in a purification process is therefore likely to be problematic.

Sasaki et al. (1979 and 1982) reported strong adsorption properties and elution by a pH increase. Adsorption is to an unmodified matrix backbone rather than an immobilised ligand. The Amberlite resin used has a very high density of carboxyl groups and therefore requires about tenfold more buffering to effect a pH change (for equilibration, elution or regeneration) than the carboxylate resins described here, e.g. ACA Perloza. Therefore consumable costs would be much higher and have a significant impact on large scale economics. The useful pH range for adsorption was narrow and acidic (4-4.5). While this might be useful for chymosin purification, it would be much less desirable for the majority of enzymes. Adsorption of other enzymes to Perloza mixed mode carboxylate resins, at higher pH's is described in Chapter 7.

SUMMARY

Mixed mode Sepharose and Perloza resins were prepared using chemistries described before. These contained hydrophobic (aliphatic and/or aromatic) and ionic (carboxylate or alkylamine) groups. Recombinant chymosin was adsorbed to these resins at high and low ionic strengths, reducing sample preparation requirements. Highly pure chymosin (nearly homogeneous by electrophoresis) was recovered simply, by a pH change. Chymosin was also purified using CM Sepharose and CM Perloza. Although capacity and stability were good, low ionic strength was essential for adsorption and the level of purification was inferior to that of the mixed mode resins.

Hydrophobic amine ligands were attached to ECH activated Sepharose ("mixed mode amine" resins). Chymosin was adsorbed to these resins at pH 5.5 (+/- 0.5 M NaCl) and eluted by a pH decrease (to 2). Hexylamine, aminophenylpropanediol (APP) and phenylethylamine were the preferred ligands, on the basis of cost and performance. The capacity of an APP ECH Perloza was much lower, presumably due to the low activation level of this method. However, use of the alternative allyl chemistry (Chapter 3) allowed production of analogous resins with higher ligand densities. The capacity of one such (APP) resin was higher than its ECH Sepharose equivalent.

Other mixed mode resins were produced by incomplete attachment (50-80%) of the same amine ligands to carboxylate matrices. The ligand was coupled most economically, using a low molar excess of a condensation reagent (EDC or EEDQ). The best performance was obtained using aminocaproic acid (ACA) modified matrices. Subsequently it was shown that ACA matrices behaved as mixed mode resins, without requirement for any extra ligand. Chymosin was adsorbed to these "mixed mode carboxyl" resins at pH 4.4 (+/- 0.5 M NaCl) and eluted by a pH increase (to 6.2). High substitution levels (of ACA) were readily obtained on Sepharose and Perloza and the capacity performance of the latter was again superior.

The methods described were simple, efficient, inexpensive and provided very good resolution of chymosin from a crude recombinant source. Fouling was most apparent for mixed mode amine resins, especially for highly substituted derivatives. The "mixed mode carboxyl" resins had the highest chymosin capacities and good regeneration properties. Therefore the latter resins were preferred for chymosin recovery. The performance of Sepharose and Perloza resins was apparently similar, if adequate substitution levels were obtained but higher capacities were found using Perloza. Perloza is cheaper and more rigid than Sepharose and thus should be better suited to large scale chymosin recovery.

CHAPTER 6 PREPARATION AND USE OF HYDROPHOBIC IONISABLE RESINS

INTRODUCTION

Traditional HIC resins are relatively lowly substituted (e.g. Phenyl Sepharose CL4B contains 40 μ moles/ml of phenyl ligand) to maximise protein recovery by decreasing salt gradients. Nevertheless desorption can require the use of ethylene glycol (Pharmacia, 1980; Zhuo et al., 1993) or harsher procedures (reported in Chapter 1). At higher ligand densities, such desorption difficulties would be more commonplace.

The potential advantages of high ligand densities have been demonstrated for chymosin separation (Chapter 5). A combination of high ligand density and charged (amine or carboxyl) groups allowed simple recovery of active chymosin. The high ligand density enabled (high capacity) adsorption without use of lyotropic salts (e.g. ammonium sulphate). In particular chymosin was adsorbed strongly at both high and low ionic strengths. Therefore a pre-chromatography step of salt addition, or removal by dialysis, dilution or ultrafiltration was not required. The carboxyl or amine groups, which were essential to facilitate elution, were partially ionised at the pH of adsorption. Resins containing secondary amine linkages were expected to be substantially ionised at pH 9 and below. The carboxyl groups of aminocaproic acid resins and their derivatives were substantially ionised above pH 4-4.5. Therefore some ionised groups will always be present on these resins in the pH 4-10 range and resins will only be uncharged at pH extremes.

This could result in non-specific electrostatic adsorption, which in turn could result in low product purity and resin fouling problems. This was demonstrated by the fouling of resins containing protonated amine groups by crude chymosin samples. Complete removal of foulants was difficult from these resins, although non-specific binding did not appear to adversely affect chymosin purity. However, if poorer selectivity were found for another protein separation, purity and capacity would be compromised.

If weaker acid or base groups were used instead, resins could be operated in an uncharged form at adsorption, within the pH 4-10 range. Ideally partially or fully charged forms would be obtainable within the same range. This would allow protein interaction with two distinct resin surfaces without the use of extreme pH. Attachment of ligands containing imidazole or other weakly ionisable groups to suitably hydrophobic matrices (e.g. ACA CDI Perloza) should allow hydrophobic and ionic modes to be used

on the same resin, but separately. Adsorption would not be complicated by electrostatic or mixed mode interactions. High ligand densities could still be used, because of the "ionisable potential" which could be released by a suitable pH change. The use of high ligand densities should again lead to adsorption independent of ionic strength and high protein capacity. This might reduce the level of purification, depending upon the application, but further resolution may be obtained by using pH steps for elution, exploiting surface charge differences of proteins.

Equally, lower ligand densities might be used for proteins which bind very strongly to conventional HIC resins. In contrast to HIC, efficient elution might be obtained, by a pH change, without using polarity reducing agents, solvents or detergents.

Many ligands which appear to satisfy the criteria are commercially available. Most of these are aromatic (e.g. pyridyl) and expected to contribute to hydrophobic interactions, especially in the uncharged form. Pyridyl (silica and alumina) resins have been used previously for HPLC of various organic compounds (Knox and Pryde, 1975) and of nucleic acids (Bischoff and McLaughlin, 1983). Strong adsorption was not found. Resins were used in reversed phase and ion exchange modes, with isocratic elution. Other pyridyl (Sephacrose) resins were used for purification of immunoglobulins by salt promoted chromatography (Porath and Oscarsson, 1988; Oscarsson and Porath, 1990; Oscarsson et al., 1995). The adsorption was attributed to thiophilic interactions, required ammonium sulphate and did not exploit the ionisable nature of the pyridine group.

In this chapter, the preparation and titration properties of weakly ionisable hydrophobic resins of varying ligand density is described. The (salt promoted) chromatographic properties of low ligand density resins were compared with those of a commercial HIC resin (Phenyl Sepharose CL4B), using a variety of "model" proteins. The same proteins were used to study the adsorption properties of medium to high ligand density resins at high and low ionic strength. High ligand density ($> 100 \mu\text{Moles/ml}$) resins were also used for the purification of crude recombinant chymosin and their performance compared with the mixed mode resins of Chapter 5.

MATERIALS AND METHODS

Reagents and equipment

4-(3-Aminopropyl)morpholine, 3-(aminomethyl)pyridine, 4-(aminomethyl)pyridine, 1-(3-aminopropyl)imidazole, 2-(aminomethyl)benzimidazole HCl, 4-hydroxyphenylacetic acid, 2-mercaptobenzimidazole, tyramine HCl and 3,5-dichlorosalicylic acid were from Aldrich-Chemie, Steinheim, Germany; 4-hydroxy-3-nitrobenzoic acid, 3-nitrotyrosine, 3-chloro-4-hydroxyphenylacetic acid, 2-(aminomethyl)pyridine and 2-mercaptopyridine from Janssen Chimica, Geel, Belgium; 4-hydroxybenzoic acid from Hopkin and Williams, Essex, England; and L-histidine, histamine and 2-mercapto-1-methylimidazole from Sigma Chemical Company, St. Louis, MO, USA.

3-,5-Dibromotyramine was prepared (by Dr D. Harding) from tyramine by the method of Zeynek (1922). Other reagents were analytical grade or described previously.

Conductivity (for small ion titration of resin carboxyl groups) was monitored by a Biorad Econosystem conductivity meter. A Pharmacia FPLC was used for salt gradient chromatography of myoglobin, ribonuclease, chymotrysinogen and lysozyme. Packed columns were 45-50x5 mm. Columns were equilibrated with buffer A (20 mM HEPES + 1 M ammonium sulphate, pH 7.5). Lyophilised protein standards were prepared by dissolution in buffer A (1% solutions) and 100 μ l loaded (60 μ l for myoglobin). Chromatograms were developed by a 40 minute gradient to 100% B (20 mM HEPES, pH 7.5), followed by 15 minutes of 100% B. The flow rate and chart recorder speeds were 0.4 ml/minute, and 2 mm/minute respectively. Some resins were also washed for 15 minutes with 50 mM acetate, pH 5. Full scale absorbance was 0.5 except for myoglobin (1). Other equipment and methods for chromatography, titration and electrophoresis were the same as described previously.

Ligand attachment

Amine ligand substitution of CDI activated resins

A 5 molar excess of ligand was used. Liquid amines were mixed directly with CDI activated Perloza (dioxan solvated). Aminomethylbenzimidazole HCl (AMB) was converted to a free base by dissolution in water and pH adjustment to 11.5 with 7.5 M NaOH. Water was removed by rotary evaporation leaving NaCl crystals and a viscous liquid (AMB), easily separated with a pipette. Tyramine and 3-,5-dibromotyramine were prepared likewise, but were insoluble at pH 11.5. The solids obtained after evaporation were not separated from NaCl. They were dissolved in 75:25 DMSO/water (8% solutions). Nitrotyrosine and histidine were prepared by dissolution in water and pH adjustment to 11 with 7.5 M NaOH (final concentration 20 and 30% respectively).

Amine ligand condensation

The 100% EDC method of Chapter 5 was used for ligand attachment to ACA resins. After reaction, the resin was washed with 0.1 M HCl and water to remove excess ligand. Butylamine or ethanolamine were used to "cap" nitrotyrosine and histidine resins (again by the 100% EDC method).

Thiol ligand substitution

Initially, ligands were attached to ECH Sepharose at room temperature and to allyl NBS Perloza at 60°C. The activated matrix (5-10g) was suspended in 1 M phosphate buffer, pH 7.2, and NaBH₄ (1 ml and 10 mg/g matrix respectively). Ligands (5 molar excess) were mixed directly (mercaptobenzimidazole was pre-dissolved in ethanol or DMSO).

Subsequently, allyl NBS matrices were reacted at pH 10-11, as described for mercaptoethylpyridine in Chapter 3.

Carboxyl ligands

Amine resins were prepared by attachment of diaminohexane (Bethell et al., 1979) to CDI activated Perloza (1.24 mMoles/g dry). Dichlorosalicylic acid was dissolved in ethanol (10% solution) and attached by reaction with EEDQ (5 molar excess of ligand and reagent). All other ligands were attached using a 5 molar excess of EDC. Hydroxyphenylacetic acid (HPA) and chlorohydroxyphenylacetic acid (CHPA) were dissolved in water and 7.5 M NaOH to pH 5 (12.5% solutions). Nitrohydroxybenzoic acid (NHBA) was prepared likewise but as a 10% solution at pH 6.4. The attachment reactions were inefficient (compared to analogous attachment of amine ligands) and therefore were repeated. Unreacted amine groups remaining after the second reaction were capped by (EDC catalysed) reaction with 1 M acetate.

Small ion titration of unreacted spacer arm carboxyl or amine groups

The carboxylate resin derivative (approximately 1 ml in a 2 ml column) was washed with 0.1 M NaOH to convert weak base groups to their unprotonated form and residual carboxyl groups to the Na⁺ form. Unbound Na⁺ was removed by washing with up to 50 bed volumes of water, or until baseline was reached. Na⁺ retained on the resin by carboxyl groups was detected as a peak of conductivity (sodium acetate) when the resin was washed with 0.1 M acetic acid. Control resins (Perloza CDI 4AMP and Perloza CDI ACA PPA, 95% substituted) containing a known proportion of carboxyl groups (0 and 0.011 mMoles/g respectively) were tested likewise.

The same method was used to measure the completeness of amine reaction. The resin was washed with 0.1 M HCl, converting amine groups to their hydrochloride and weak acid groups to their protonated form. Excess Cl⁻ was removed with water as before. HCl retained by amine groups was displaced by 0.1 M Tris (free base).

RESULTS AND DISCUSSION

Ligand options

Ionisable groups

Bases with pKa values of 4-7 and acids with pKa values of 7-10 were preferred, because it was expected that uncharged forms could be obtained without going to extremely high or low pH values. The pKa values of common weak organic acids and bases (CRC, 1971) are listed in Tables 6.1 and 6.2. The ethylamine value (Table 6.1) suggested that a typical aliphatic amine ligand would be predominantly charged below pH 10. In contrast, ligands containing base groups such as imidazole and pyridine were expected to remain uncharged at much lower pH values. Several weak base group options were available, with pKa values regularly spaced across the range of 4.5-8.5.

Table 6.1 : Dissociation constants of some common weak organic bases

Base	pKa
ethylamine	10.81
morpholine	8.21
imidazole	6.95
picoline (methylpyridine : α , β and γ)	5.97, 5.68 and 6.02
benzimidazole	5.53
pyridine	5.25
aniline	4.63

Table 6.2 : Dissociation constants of some common weak organic acids

Acid	pKa
acetic acid	4.75
dinitrophenol (2,4- and 3,6-)	3.96 and 5.15
trichlorophenol	6.00
2,3-dichlorophenol	7.44
nitrophenol (ortho, meta and para)	7.17, 8.28 and 7.15
chlorophenol (ortho, meta and para)	8.49, 8.85 and 9.18
phenol	9.89

Similarly, the acetic acid pKa value (Table 6.2) suggested carboxyl groups would be predominantly charged at pH values above 5 (as found with MAA Perloza, Chapter 4). The only simple, weak organic acid groups to select from were phenol and its derivatives. Except for dinitrophenol, the pKa values of these groups were well spread across the pH range (6-10).

Of greatest interest were ligands containing acid or base groups, which would exist as an uncharged species, within the range pH 5-9, and could be (at least partially) titrated to the charged form, within the same range. It was apparent that these requirements were satisfied by several base groups (Table 6.1), but only by phenol and its derivatives of the acid groups (Table 6.2). The thiol group was not considered because of its reactivity.

Ligands were selected from commercial catalogues, based on price and suitable ionisable and "attachment" (thiol, amine or carboxyl) groups. For example, although both were tested, aminopropylimidazole (APIMID) was preferred over histamine, because of lower cost and toxicity. Ortho, meta and para aminomethylpyridines (2AMP, 3AMP and 4AMP), aminomethylbenzimidazole (AMB) and aminopropylmorpholine (APM) were other ligands chosen to cover the range of weak bases. Aniline ligands were avoided, because of their carcinogenicity/toxicity risk. Diethylaminopropylamine (DEAPA) was used to represent a "typical" tertiary amine group. Thiol ligands containing imidazole (mercaptomethylimidazole, MIM) and benzimidazole (mercaptobenzimidazole, MB) were also available at a suitable price. Ortho and para mercaptopyridine (2MP and 4MP) ligands were also tested, although the cost of 4MP was much greater.

Several carboxylate ligands (hydroxyphenylacetic acid, chlorohydroxyphenylacetic acid, hydroxybenzoic acid, nitrohydroxybenzoic acid, and dichlorosalicylic acid) were commercially available, although the cost was generally higher than for the weak base ligands. Amine ligand (commercial) options were limited to tyramine, tyrosine and nitrotyrosine. The latter two would require an extra reaction step ("capping" of the carboxyl group with an amine). Therefore tyramine was preferred, although it was not cheap. The only suitable thiol ligand, hydroxythiophenol, was not cheap either. Nitro and halo derivatives of tyramine and hydroxythiophenol were not commercially available.

Prices of commercially available ligands are included in Appendix 1. The commercial ligands were supplemented by 4-mercaptoethylpyridine (MEP, synthesis and resin attachment described in Chapter 3) and dibromotyramine. The former ligand was prepared, because the cheap starting materials and simple methods indicated that it would be much cheaper than 4MP. 2-Mercaptoethylpyridine could also be prepared, using 2-vinylpyridine, if required. Dibromotyramine was prepared as an alternative to the carboxylate ligand dichlorosalicylic acid. Structures of all ligands used are shown in Appendix 2.

Ligand attachment

Amine ligands

Ligand attachment to CDI activated matrices was simple, because the linkage is neutral and near complete reaction of matrix imidazolyl groups with the amine ligand was expected in non-aqueous media. Any groups which did not react should be removed by hydrolysis. The only ionisable group on the resulting resins should be the desired ligand moiety. The exception to this was attachment of amino acid spacer arms (e.g. nitrotyrosine). Carboxylate as well as phenolic groups were attached. The carboxylate groups were then blocked with an amine (condensation reaction producing a neutral amide).

A mixture of ionisable groups also occurred, if ligand attachment to carboxylate matrices (e.g. ACA CDI Perloza), by carbodiimide catalysed condensation, was incomplete. This could not be quantified by the usual acid base titration methods, because the presence of carboxyl groups was obscured by the much greater proportion of ligand ionisable groups. However if the ligand ionisable group was a base (e.g. imidazole) it could be distinguished by a form of small ion titration.

Small ion titration of resin carboxyl groups

The resin was prepared in the base form, by washing with NaOH. After excess base was removed, the presence of carboxyl groups was indicated by the release of their counterion (Na^+), when washed with dilute acetic acid. This release was monitored as a conductivity peak. The validity of this approach was demonstrated by analysis of a control resin with a known percentage of unreacted carboxyl groups. This ACA resin derivatised with phenylpropanolamine (a neutral matrix-ligand product) was found to contain 0.011 mMoles/g unreacted carboxyl groups by base titration. The conductivity titration clearly demonstrated the presence of anionic groups. This conductivity peak was not found for an aminomethylpyridine CDI Perloza resin, indicating the (expected) absence of anionic groups. These titrations and examples of carboxylate matrix derivatives are depicted in Figure 6.1. For the products of the carbodiimide reaction, a peak height $\leq 20\%$ of that for the control resin was required. This was considered equivalent to 99% or greater reaction of carboxyl groups.

Thiol ligands

The attachment was simple but a side reaction occurred with pyridyl ligands before reaction conditions were optimised. The side reaction was attributed to nucleophilic substitution by the pyridyl rather than the thiol group (Figure 3.7, Chapter 3). This was most noticeable when MEP or 4MP was reacted at pH 7.5, 60°C. The level of side reaction when MEP was attached at pH 10-11 (room temperature or 60°C) was very low

Figure 6.1 : Small ion titration of residual carboxyl groups after ligand attachment

A is a control with no carboxyl groups, B a control with a known amount (0.011 mMoles/g) of carboxyl groups and C and D are two ionisable resins prepared from CM Perloza. Comparison of "elution spikes", marked \uparrow , shows that the 4AMP resin (C) is virtually 100% modified, whereas the APIMID resin (D) retains about 0.01 mMoles/g of carboxyl groups.

A 4AMP CDI Perloza



B PPA (95%) ACA CDI Perloza



C APIMID CM Perloza



D 4AMP CM Perloza



or undetectable. This was presumably due to the much higher concentration, at this pH, of the very reactive thiolate ion. No evidence of a side reaction was found with MIM or MB ligands.

Carboxyl ligands

The carboxylate group was less desirable for ligand attachment, because condensation chemistry was required. This required expensive reagents and careful control to ensure reaction completeness. Reaction completeness was harder to obtain than for amine ligand attachment to carboxylate resins. This may have been due to the poorer solubility of the carboxyl ligands (at pH 4.7). An extra reaction step (attachment of amine groups to the activated matrix) was also required. By contrast amine and thiol ligands could be attached simply to activated (CDI or allyl/NBS) Perloza. Unreacted amine groups were monitored by small ion titration, similar to that described above for carboxyl groups, except that the counterion detected was Cl⁻. The performance was apparently similar to that described before.

Examples of amine, thiol and carboxyl ligand resins are depicted in Figure 6.2.

Titration curve analysis of resins

Titration data are summarised in Table 6.3. Weak base resins were titrated with 0.1 M HCl despite the fact that clear endpoints could not be determined for some resins without correction for acid dilution effects (by subtracting the titration values found for an unmodified Perloza control). It was found that when acid washed pyridyl resins were rinsed with water the resin pH was between 6 and 7. No evidence of base titratable groups was found, indicating that hydrochloride had been washed out.

Titration of the weak acid resins with NaOH gave poor results (a slow pH drift) above pH 9, limiting its usefulness to the nitrophenol resins. Therefore titration curves were obtained by acid titration of resins adjusted to a start pH of 12 with 1 M NaOH. The values for "start" pH (the point at which ionisation of the neutral form begins) of the weak acid resins were still obtained by washing with 0.1 M HCl and water (and suspension in 0.5 M NaCl). The start pH is important because it gives the upper or lower (acid and base resins respectively) pH limit of the neutral resin form.

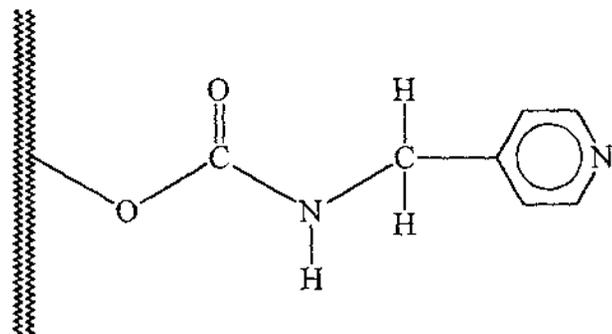
Titration curves of several weak acid and base resins are depicted in Figures 6.3, 6.4 and 6.5.

A variety of weak acid ligands were attached to Perloza to demonstrate titration properties, although none of these were as simple or cheap to prepare as the weak base resins. Therefore the chromatographic assessment was focussed on the latter group.

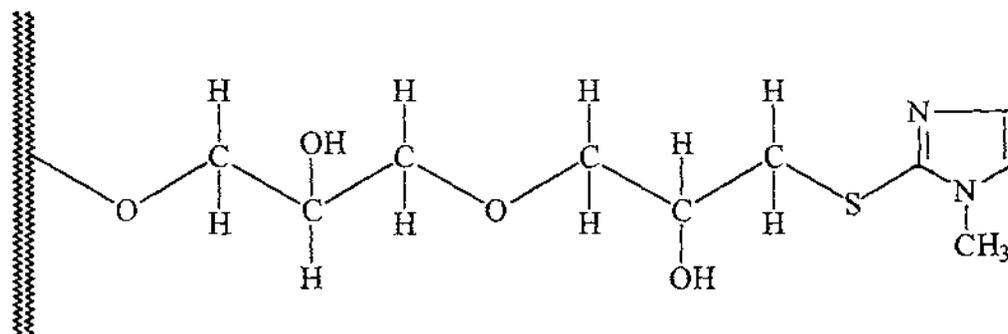
Figure 6.2 : Structures for some matrix/ionisable ligand products

A is produced by reaction of 4-aminomethylpyridine with CDI Perloza (urethane linkage). B is produced by reaction of mercaptomethylimidazole with AGE/NBS Perloza (ether and thioether linkages). C is produced by a condensation reaction between diamino-hexane (CDI) Perloza and hydroxybenzoic acid (urethane and amide linkages).

A



B



C

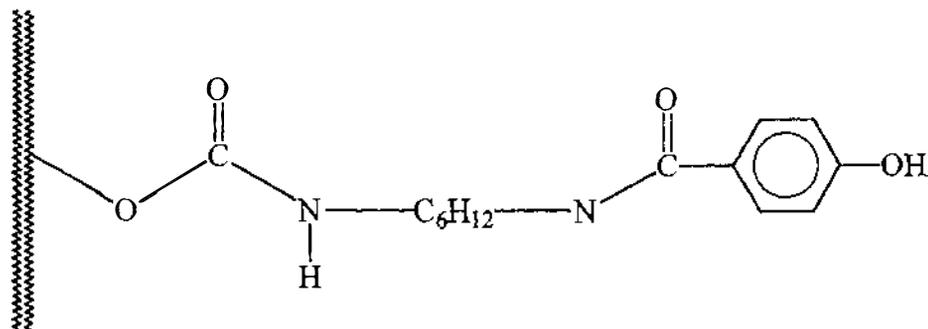


Table 6.3 : Titration data for weak ionisable resins

Unless otherwise stated, resins (1g) were titrated in the presence of 0.5 M NaCl (7 ml).

Resin	Start pH	pKa	pH 90% titrated
DEAPA	10.5	9.3	8.4
aminopropylmorpholine	8.9	7.1	6.2
aminopropylmorpholine (10mM)	8.05	5.8	4.8
aminopropylimidazole	8.0	6.25	5.2
histamine	8.4	6.85	5.6
aminomethylbenzimidazole	6.75	4.8	4.0
4-aminomethylpyridine	7.2	4.7	3.7
3-aminomethylpyridine	6.7	4.2	3.3
2-aminomethylpyridine	6.1	4	3.0
mercaptomethylimidazole	6.9	5.3	3.8
4-mercaptopyridine	7.2	4.8	3.7
2-mercaptopyridine	5.4	2.8*	1.9*
4-mercaptoethylpyridine	7.0	4.8	3.7
2-mercaptobenzimidazole	6.2	4.1	3.0
aminocaproic acid	3.0	5.2	6.5
aminocaproic acid (10mM)	3.3	6.1	7.5
nitrohydroxybenzoic acid	4	6.4	7.5
nitrotyrosine/ethanolamine	5	7.2	9.0
dichlorosalicylic acid	5	7.2	9.3
dibromotyramine	5	7.7	9.3
chlorohydroxyphenylacetic acid	6.5	9.8	10.8
hydroxyphenylacetic acid	7.5	10.7	11.2
tyramine	7.5	10.7	11.3

* the accuracy of these values is likely to be lower due to the relatively large correction for dilution required at very acidic pH values

Figure 6.3 : Titration curves of Perloza CDI (A) diethylaminopropylamine, (B) aminopropylmorpholine, (C) aminopropylimidazole and (D) aminomethylbenzimidazole.

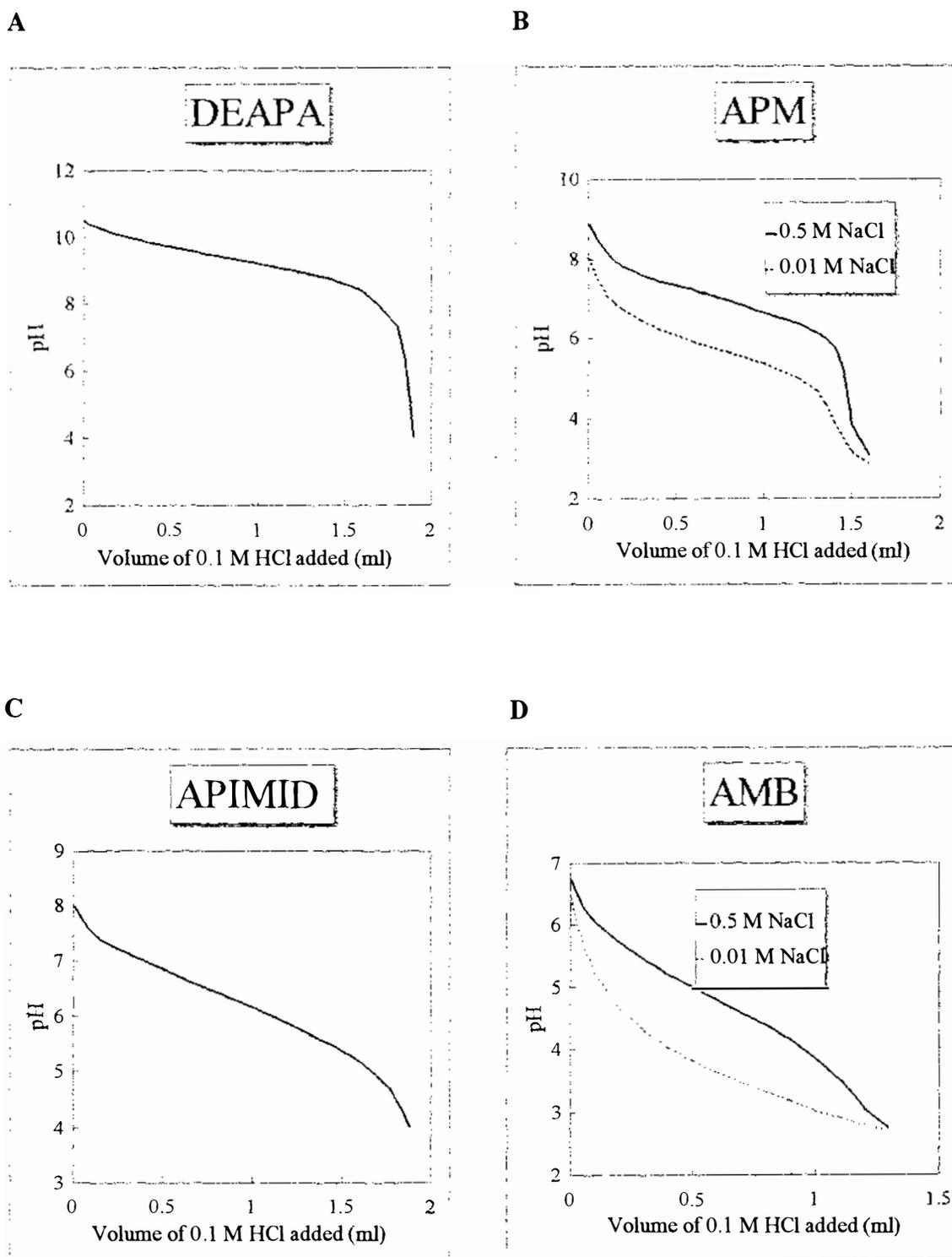


Figure 6.4 : Titration curves of Perloza CDI (A) 4-AMP and (B) 3-AMP; and Perloza AGE/NBS (C) MEP and (D) MIM

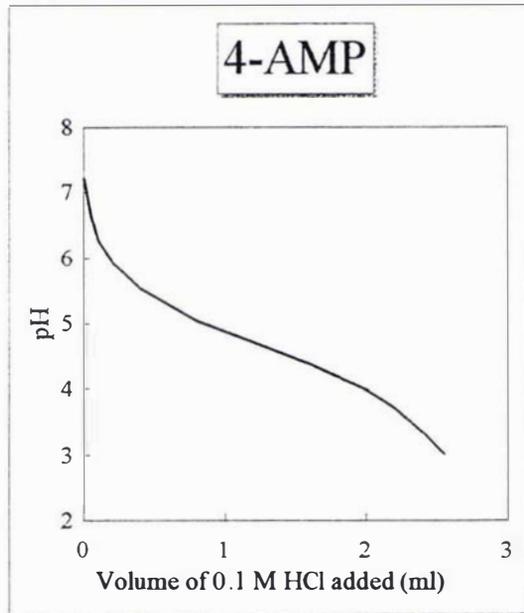
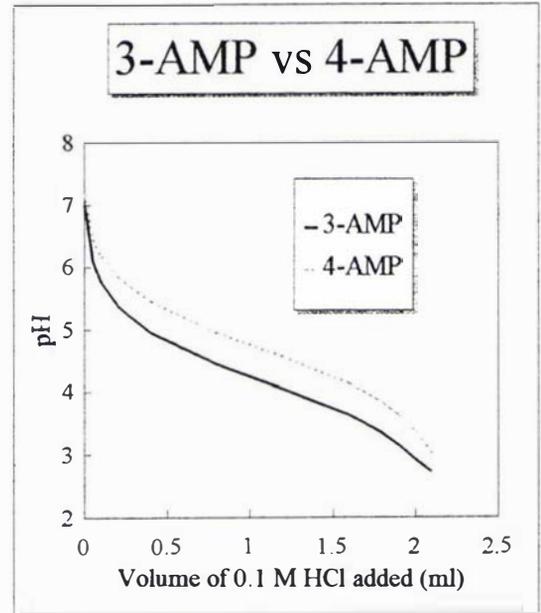
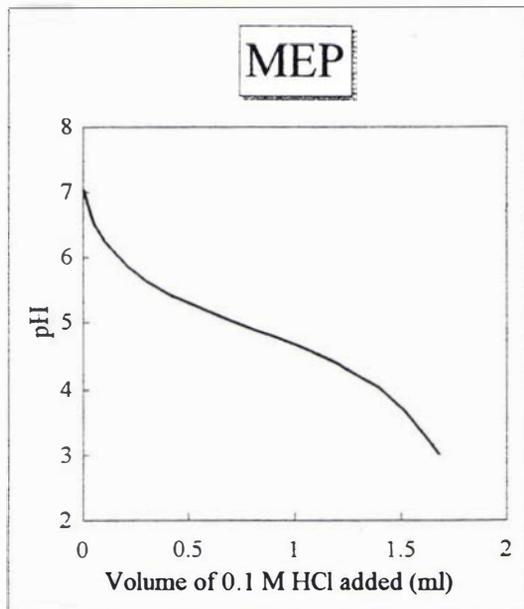
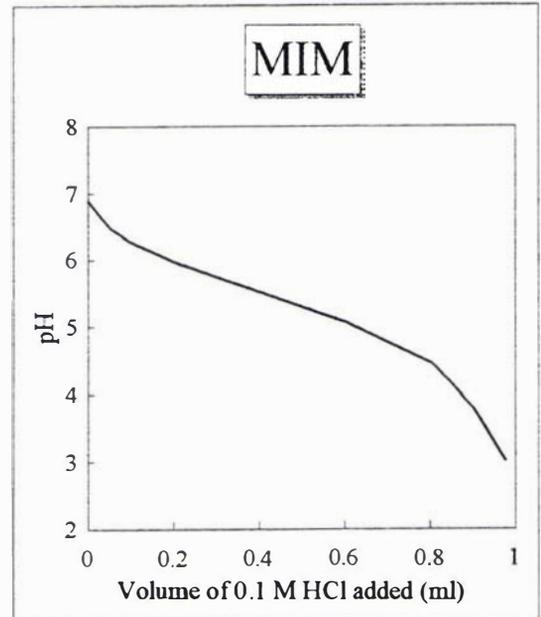
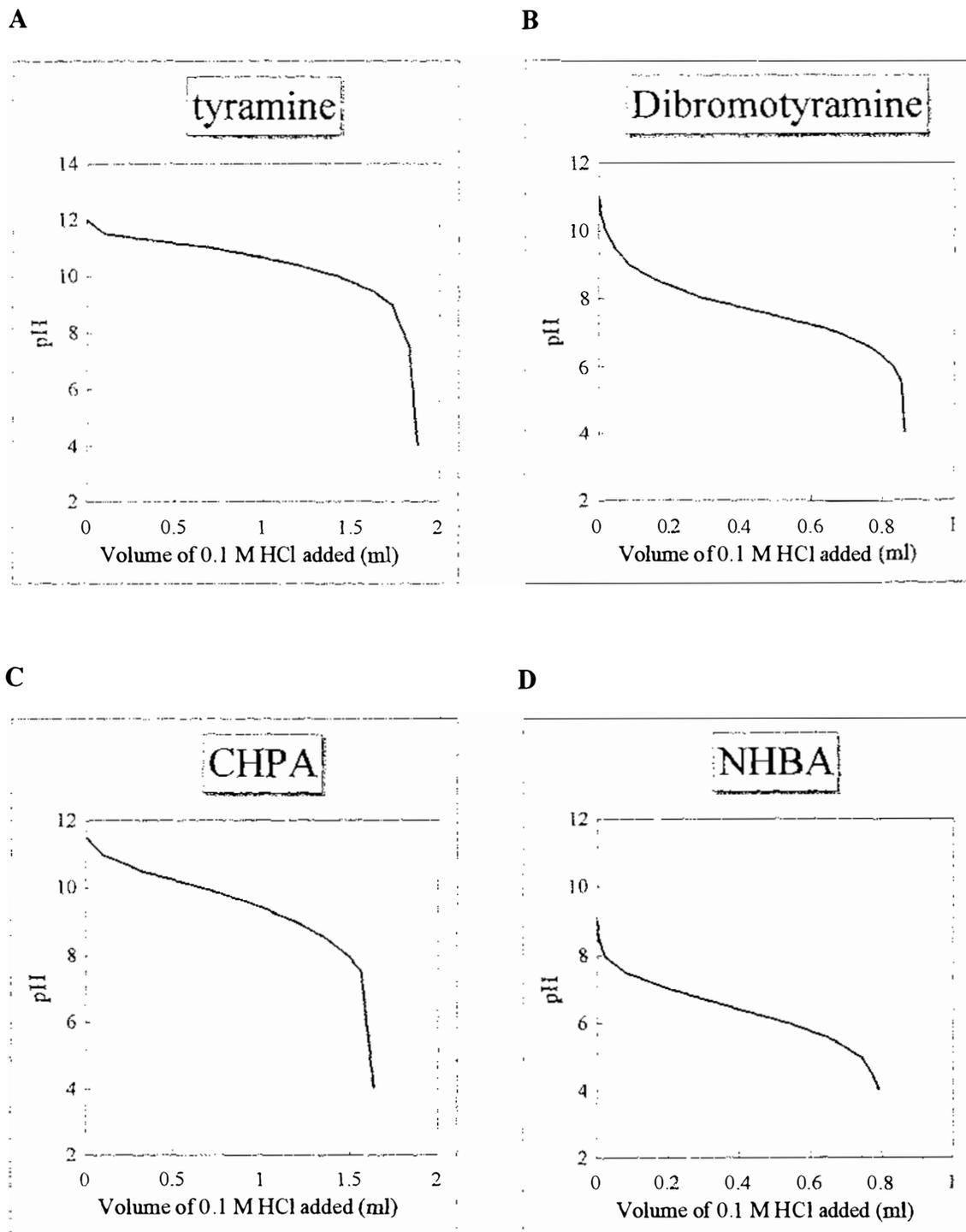
A**B****C****D**

Figure 6.5 : Titration curves of Perloza CDI (A) dibromotyramine, (B) tyramine, (C) DAH nitrohydroxybenzoic acid and (D) DAH chlorohydroxyphenylacetic acid.

DAH = diaminohexane spacer arm.



Model protein chromatography

Phenyl Sepharose CL4B was compared with two hydrophobic ionisable resins (4-AMP CDI and MEP AB Perloza) of similar ligand density (40-50 $\mu\text{Moles/ml}$) for salt (ammonium sulphate) promoted chromatography of myoglobin, ribonuclease, lysozyme and chymotrypsinogen at pH 7.5. Some variations were found in elution patterns, but the order of elution was unchanged (Figures 6.6-6.9). Although peak shape differences were found with myoglobin and ribonuclease, these were eluted in the void volume or very early in the gradient. Packing as well as matrix differences could therefore have a significant influence on peak shape. The irregular peak found for chymotrypsinogen on Phenyl Sepharose, may have been due to a mixture of chymotrypsinogen and chymotrypsin. The separation might be due to affinity interactions with the phenyl ligand. At the equivalent stage a single peak was eluted from AMP Perloza. The split peak found for lysozyme on Phenyl Sepharose may be due to overloading, because the lysozyme used is nearly homogeneous (electrophoresis). Lysozyme and chymotrypsinogen were retained longer on MEP Perloza than on AMP Perloza or Phenyl Sepharose. The MEP ligand is expected to be more hydrophobic due to a combination of aliphatic (ethyl-hydroxypropyl-thioether) and aromatic interactions. The MEP resin was washed with 50 mM acetate buffer, pH 5.5 to complete lysozyme elution.

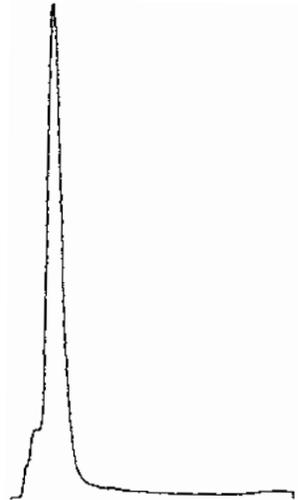
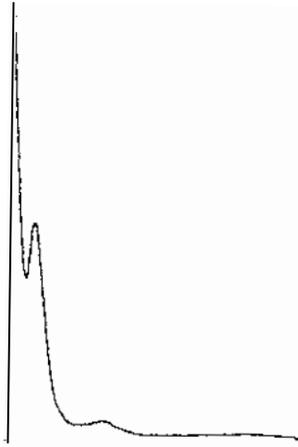
The order of elution from a more highly substituted (65 $\mu\text{Moles/ml}$) 4-AMP CDI Perloza resin was unchanged. Retention of the ribonuclease, lysozyme and chymotrypsinogen was somewhat longer. Desorption of lysozyme at low ionic strength was incomplete. A pH change (to 5.5) resulted in rapid, complete elution. The greater retention was expected, due to an increase in multi-point interactions (Jennissen, 1978). At high ligand densities chymotrypsinogen and lysozyme were adsorbed at low and high (0.5 M NaCl) ionic strengths, at pH 7.5. Rapid elution was again obtained by a pH change to 5.5 (data not shown here).

Although some differences have been recorded, these results were consistent with hydrophobic adsorption. However, differences between salt promoted adsorption patterns (for some proteins) on 2-mercaptopyridine and Phenyl Sepharoses were reported by Porath and Oscarsson (1988), Oscarsson and Porath (1990) and Oscarsson et al. (1995). The differences were attributed to thiophilic interactions. The thioether linkage was considered important for thiophilic adsorption. No obvious difference in adsorption properties of urethane and thioether linked pyridine resins is shown here, albeit using a limited number of test proteins.

Figure 6.6 : Salt promoted chromatography of myoglobin

A : Phenyl Sepharose

B : AMP Perloza (45 μ Moles/ml)



C : MEP Perloza (50 μ Moles/ml)

D : AMP Perloza (65 μ Moles/ml)

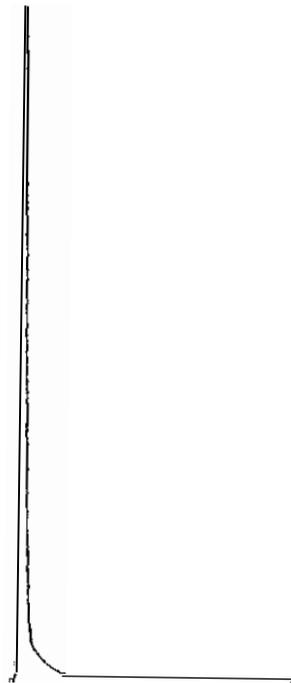
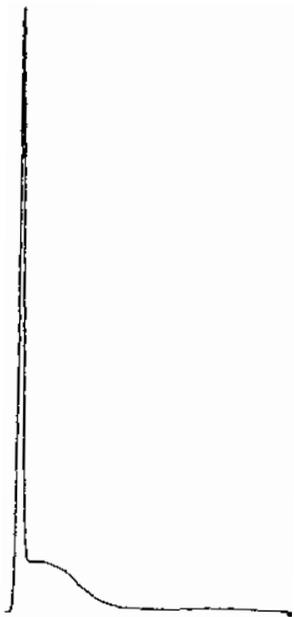


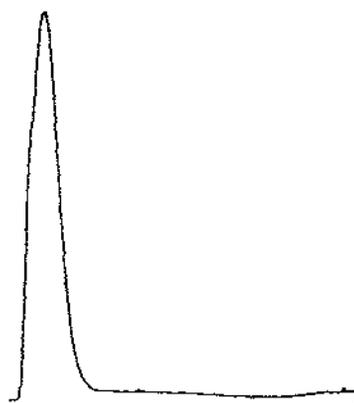
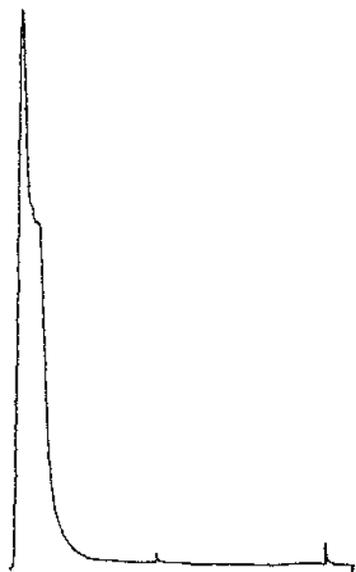
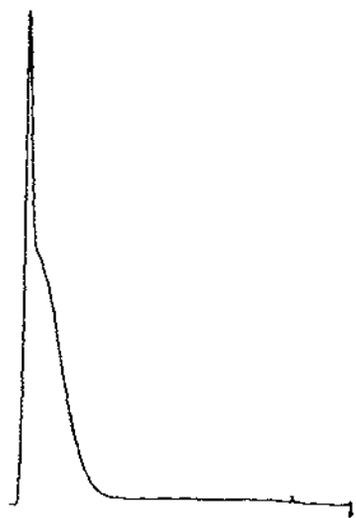
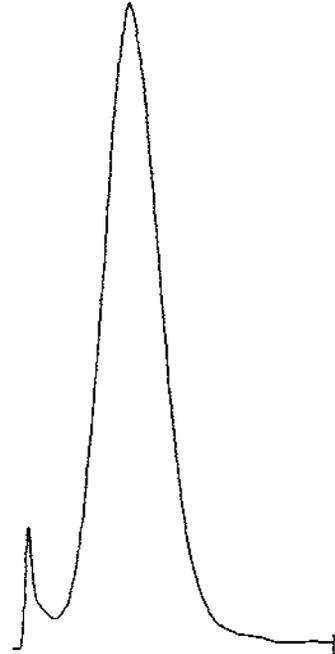
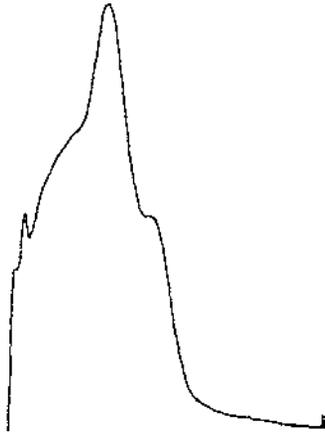
Figure 6.7 : Salt promoted chromatography of ribonuclease**A : Phenyl Sepharose****B : AMP Perloza (45 μ Moles/ml)****C : MEP Perloza (50 μ Moles/ml)****D : AMP Perloza (65 μ Moles/ml)**

Figure 6.8 : Salt promoted chromatography of chymotrypsinogen

Further elution with 50 mM acetate, pH 5.5, is marked with an arrow (D).

A : Phenyl Sepharose

B : AMP Perloza (45 μ Moles/ml)



C : MEP Perloza (50 μ Moles/ml)

D : AMP Perloza (65 μ Moles/ml)

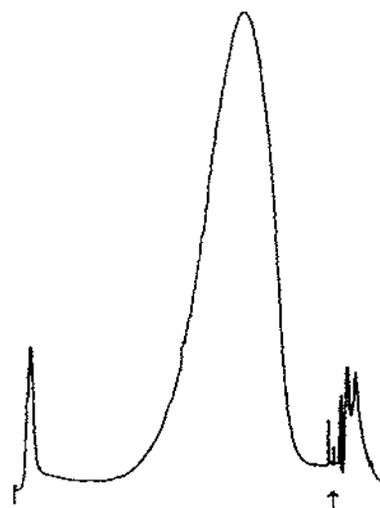
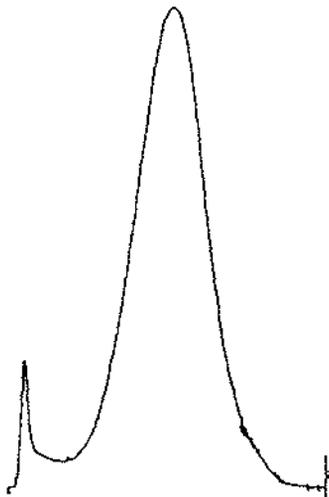
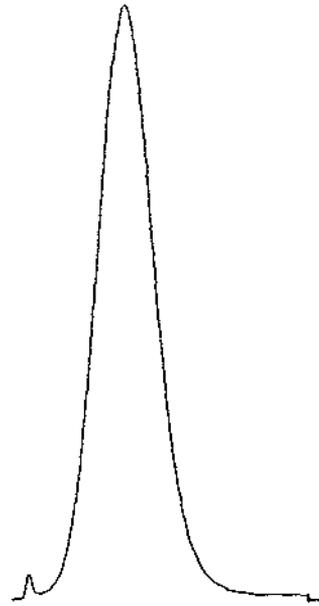
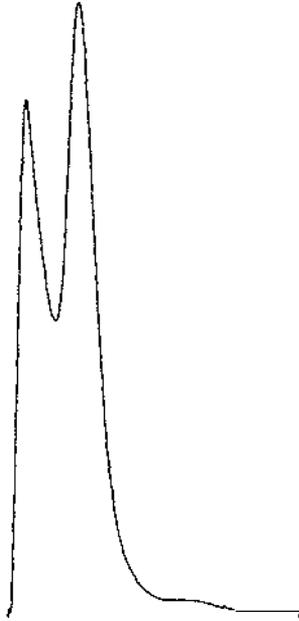


Figure 6.9 : Salt promoted chromatography of lysozyme

Further elution with 50 mM acetate, pH 5.5, is marked with an arrow (C and D).

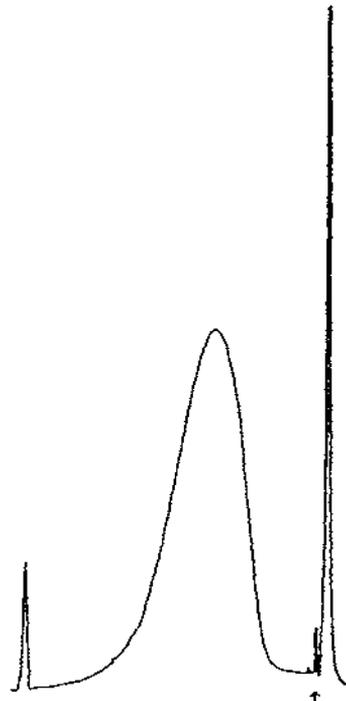
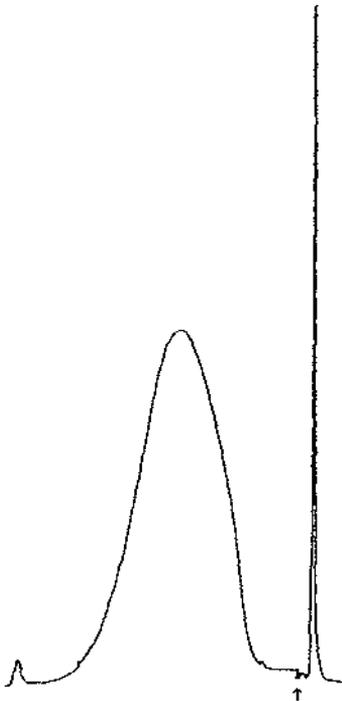
A : Phenyl Sepharose

B : AMP Perloza (45 μ Moles/ml)



C : MEP Perloza (50 μ Moles/ml)

D : AMP Perloza (65 μ Moles/ml)



Potential advantages of hydrophobic ionisable resins

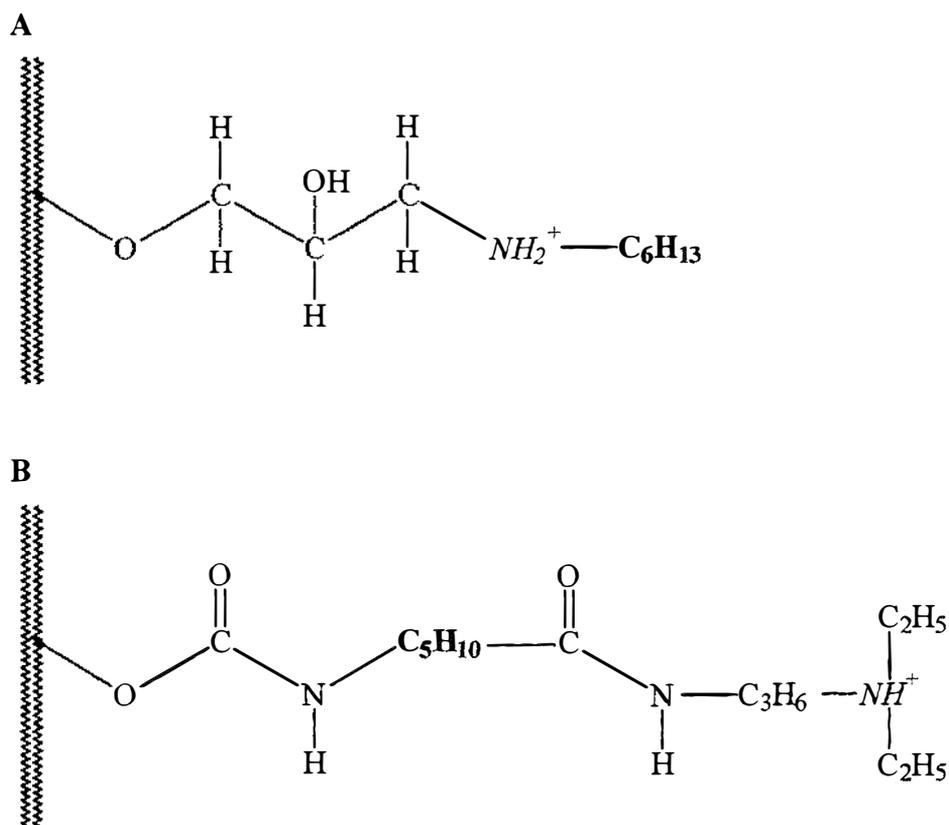
Proteins which were not completely desorbed by the decreasing salt gradient were easily recovered using aqueous buffers (50 mM acetate, pH 5.5 and/or 100 mM acetate, pH 4). In these examples, proteins of weak to moderate hydrophobicity were used and were recoverable from the commercial HIC resin by the decreasing salt gradient. This ease of recovery would not be expected if the ligand density were higher. Other, more hydrophobic, proteins are more difficult or impossible to recover from HIC resins with aqueous buffers (Zhuo et al., 1993). In these situations, the use of ionisable resins should allow comparatively simple, rapid protein recovery. In most cases, resins of low ligand density are expected to have similar adsorption properties to commercial HIC resins and be especially useful for applications where very strong adsorption (protein or foulant) is found. Moreover, resins of high ligand density could be used in the manner described for mixed mode resins in Chapter 5 (adsorption at low and high ionic strength). The main difference and potential advantage over mixed mode is the elimination of non specific ionic interactions from adsorption.

Chymosin adsorption to weak base resins

Chymosin was previously adsorbed to ACA Perloza resins at both high and low ionic strength (pH 4.4). It was also adsorbed to hexylamine ECH Sepharose, irrespective of ionic strength, at pH 5.5. The different adsorption pH's were a consequence of the opposite nature of the charged groups of these resins (and the isoelectric point of chymosin, 4.6). The adsorption at high ionic strength was ascribed to hydrophobic interactions between chymosin and resin hexyl groups. Therefore it was thought that substitution of Perloza ACA carboxyl groups with a basic group would produce a resin with similar adsorption properties to the hexylamine resin. Diethylaminopropylamine (DEAPA) was attached to an ACA Perloza (1.57 mMoles/g dry) by EDC condensation. The caproic carboxyl group was converted to an amide by reaction with the primary amine of DEAPA, leaving the tertiary amine as the sole ionisable group (Figure 6.10b).

However, the resulting resin behaved differently, only adsorbing chymosin at low ionic strength (pH 5.5). The difference from the hexylamine resin was ascribed to the location of the charged group at the surface rather than buried (Figure 6.10). The carboxyl group of ACA resins is also located at the surface but it is largely uncharged at the adsorption pH of 4.4 (Figure 5.1, Chapter 5). The amine groups of the DEAPA resin are largely charged at pH 5.5 (Figure 6.3a). Replacement of DEAPA with aminopropylimidazole (APIMID) did not alter the weak adsorption (low ionic strength only) of chymosin. APIMID ACA Perloza has a lower proportion of ionised groups than the DEAPA resin at pH 5.5 (Figure 6.3b) but it is still greater than 50% (Table 6.3).

Figure 6.10 : Structures of (A) hexylamine ECH and (B) DEAPA ACA resins



If a ligand with a lower pKa value were used, a predominantly uncharged resin (at pH 5.5) would be produced. A 4-aminomethylpyridine (4AMP) derivative of ACA Perloza did adsorb chymosin at high and low ionic strength (pH 5.5), in similar fashion to the hexylamine resin. The percentage of ionised pyridyl groups at this pH (and high ionic strength) was estimated at 20% (Figure 6.3). This suggested that above a certain threshold of surface charged groups, hydrophobic interactions were not strong enough to retain chymosin. The expected greater hydrophobicity of the pyridyl ligand (than imidazolyl) could also contribute to the difference. Indeed, resins with 4AMP attached to CDI Perloza (1.54 mMoles/g dry), also bound chymosin at high ionic strength. Other pyridyl (2AMP, 3AMP and MEP) and benzimidazolyl (AMB) resins, of similar ligand density, also adsorbed chymosin at high and low ionic strength. Adsorption at pH 6-7 was preferred to minimise non-specific ionic adsorption. The pyridyl ligands with lowest pKa values (2AMP, 3AMP or 2MP) were preferred because their low titration range suited the pH requirements of chymosin. Desorption of chymosin was achieved by a pH decrease to 2, analogous to elution from the hexylamine resin. The elution fraction was colourless. Resin fouling from a crude chymosin broth was less severe, especially using a 2AMP CDI Perloza resin. After regeneration of the latter resin with 0.1 M NaOH no (coloured) foulants were detected on the resin. It was concluded that less non-specific

adsorption occurred (as expected) and hence the level of resin fouling and elution contamination was lower.

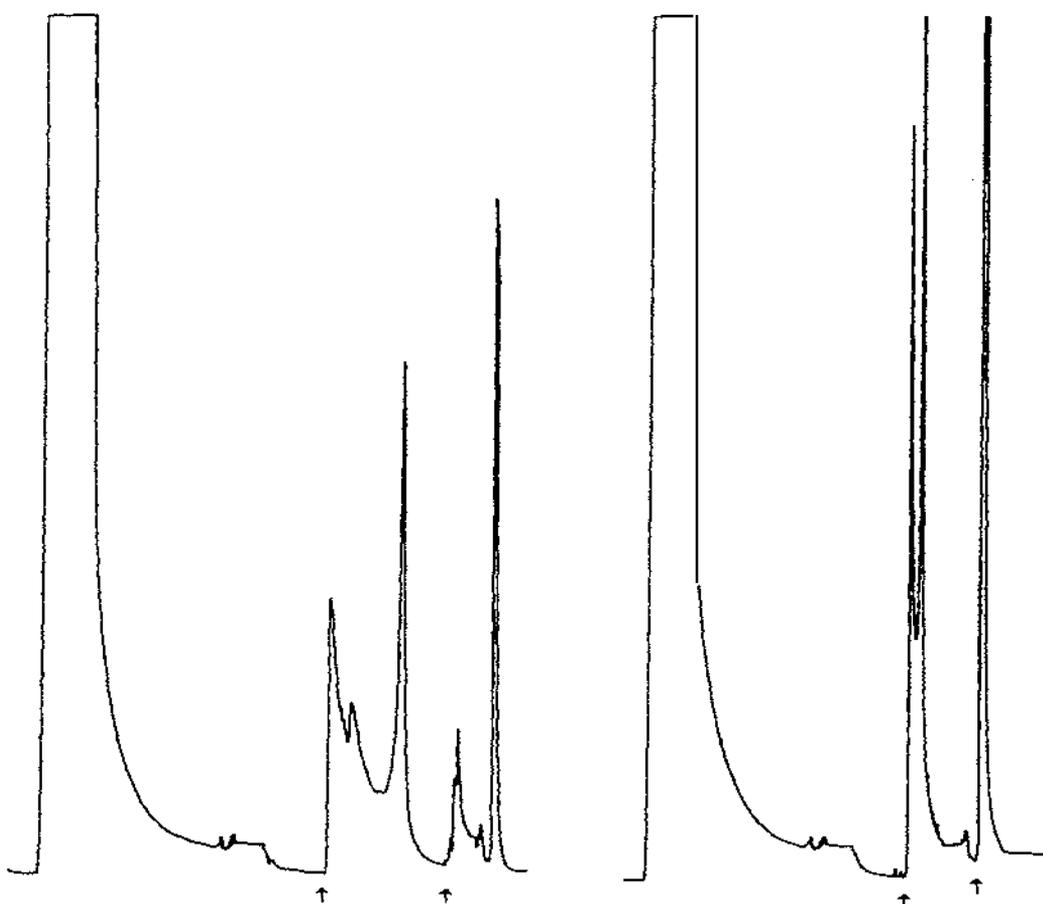
The elution principle was the same as that used with the hexylamine resin (charge repulsion at a pH below the isoelectric point). However, the time between buffer change and elution was comparatively long if low molarity buffers or those with limited buffering potential below pH 4 were used. Chymosin elution with 0.2 M acetic acid, pH adjusted to 2 with 0.1 M HCl, is shown in Figure 6.10a. Elution was slow (but peak resolution was good) because the ligand groups buffered the pH change (the hexylamine resin was already predominantly ionised and did not buffer significantly below pH 5). This effect was countered by using an organic acid buffer with a lower pKa value (100 mM malonic acid) for elution (Figure 6.10b).

Figure 6.11 : Elution of chymosin from 2AMP Perloza

Elution (pH 2) and regeneration washes are marked with arrows.

A : Elution with 200mM acetic acid + HCl

B : Elution with 100 mM malonic acid



Ligand selection

These results clearly demonstrated the effect of ligand selection on adsorption. The first requirement was to have a ligand with a neutral form between pH 5 and 7, to fit the optimum range for chymosin stability. The other requirement was that the ligand have an ionised or partly ionised form at a pH below the isoelectric point to provide charge repulsion effects. It was apparent that the pyridyl and benzimidazolyl ligands fitted these requirements, but other ligands (e.g. APIMID) did not because a higher pH (approximately 8) was required for the ligand to be substantially uncharged. The preferred pH conditions of chymosin are comparatively acidic. The reverse attributes were used for chymosin adsorption to ACA resins (Chapter 5). The neutral resin form occurred at pH 2, whereas the charged form was in the pH 5.5-7 range. When chymosin was adsorbed at pH 2, the ACA resin was used as an **hydrophobic ionisable** rather than true **mixed mode** resin. This resin was only uncharged at very low pH values. The pyridyl resins have been used in an uncharged form much closer to neutral pH. The pyridyl ligands were sufficiently hydrophobic to adsorb chymosin strongly without use of an aliphatic spacer arm. This allowed simple resin preparation methods to be used. Resin hydrophobicity could be manipulated by variation of ligand type or density.

Other proteins

The requirements of proteins other than chymosin will vary greatly (e.g. preferred pH range closer to neutral). However, the range of ligands available (especially the weak bases) should allow a rational selection for operation near neutral pH, if the stability range is wide enough (e.g. ≥ 2 pH units) to allow adequate ligand titration. Selection would be based on the protein's isoelectric point and stability requirements.

Although the 4-pyridyl ligands adsorbed chymosin when partially charged (pH 5.5), the charge interactions between the resin and protein were not repulsive. A protein with a higher isoelectric point (lysozyme) was adsorbed to the neutral form of a 4-pyridyl resin at pH 7.5 (low ionic strength) and eluted rapidly from at pH 5.5 by electrostatic repulsion. This indicated that complete titration of ligand groups was not required for elution if electrostatic repulsion occurred. Therefore the method should also be operable over a relatively narrow pH "window" (e.g. 1-2 pH units). This also demonstrated that 4-pyridyl resin use need not be restricted to a very acidic (elution) range.

The proportion of ionised groups was smaller at low ionic strength than at high ionic strength (Table 6.3; Figure 6.1b and d). Adsorption (at a given pH) at both low and high ionic strengths is a desired attribute of this method. If a protein is to be eluted at low ionic strength, the required pH change may therefore include up to one pH unit, simply to reach the starting point of titration. Therefore an intermediate to high ionic strength elution buffer, may be preferred in some cases, to narrow the pH change window.

SUMMARY

A new form of mixed mode chromatography has been described, which incorporates favourable features of HIC and mixed mode chromatography. Adsorption is by hydrophobic interactions (with neutral and/or ionisable hydrophobic groups), in the absence of non-specific ionic interactions. Desorption is due to a weakening of hydrophobicity and ionic interactions, induced by a pH change

Ligands were selected on the basis of the pKa data of simple functional groups and the catalogue prices. As well as an ionisable moiety, ligands required an attachment group, preferably thiol or amine. The ligands were attached by neutral (thioether, urethane and/or amide) linkages. The range of commercially available thiol and amine weak base ligands, supplemented by mercaptoethylpyridine, allowed preparation of resins covering a range of hydrophobicity. The selection of weak acid ligands was more limited.

Titration curves were obtained for these resins at high (0.5 M NaCl) and low (10 mM NaCl) ionic strength and significant pKa differences (up to 1 pH unit) found at the two salt concentrations. The resin pKa values, the pH at which the neutral form began to titrate, and the pH at which 90% of ligand groups were ionised, were determined for each resin. These values can be used to guide the selection of a resin for a particular protein's requirements. These resins have different pH ranges (windows) for conversion from the uncharged to the ionised form. Therefore it should be possible to choose an ionisable ligand on the basis of titration data, to match the pH requirements of a protein.

At low ligand densities, the salt promoted protein elution characteristics of pyridyl resins were similar to those of Phenyl Sepharose, suggesting a similar mode of interaction. At higher ligand densities, adsorption strength was increased until chymotrypsinogen and lysozyme were no longer eluted by changing ionic strength. The advantages of this adsorption at low or high ionic strength were described in Chapter 5. However it causes a problem of effective protein recovery from conventional HIC resins. These new resins contained ionisable groups, which could be converted to a charged form by a simple pH change. Rapid elution resulted, even using high ligand densities, because hydrophobic interactions were weakened and electrostatic repulsion effects exploited.

These resins were used to purify chymosin. The pH requirements of this enzyme dictated use of resins with a low pKa (pyridyl). Strong adsorption did not occur if there were a large number of surface charged groups (imidazolyl resins). Significantly less fouling occurred than with analogous mixed mode resins. This demonstrated that the resin could be adapted (by ligand selection) to fit the preferred pH range of the protein, rather than the pH being chosen to suit resin (adsorption) requirements.

CHAPTER 7 APPLICATIONS OF MIXED MODE AND HYDROPHOBIC IONISABLE RESINS

INTRODUCTION

The mixed mode and hydrophobic ionisable resins (and methods) described in the last two chapters were applied to the purification of chymosin. The application of these resins to the purification of subtilisin, α -amylase and catalase is reported here.

Subtilisin is an alkaline protease of bacterial (usually *Bacillus* species) origin (Markland Jr. and Smith, 1971; Ottesen and Svendsen, 1970). It is produced on a very large scale, principally for laundry detergent use (Yamane, 1989). It is a serine protease of broad specificity, with a molecular weight of approximately 26 kDa. Isoelectric point values between 7.8 and 9.4 have been reported (Markland Jr. and Smith, 1971). It is most stable between pH 5 and 7, moderately stable above this range and rapidly denatured below. Subtilisin has been purified by ion exchange (Chicz and Regnier, 1989a), IMAC and hydroxyapatite (Chicz and Regnier, 1989b) and HIC (Chicz and Regnier, 1990). It has also been purified by affinity chromatography (Fujiwara et al., 1975; Chandrasekaran and Dhar, 1985) using CBZ-Phe and aminophenylazophenylarsonic acid ligands respectively. The separations described by Chicz and Regnier were analytical HPLC methods and impractical for large scale recovery of an industrial enzyme. The usefulness of the affinity methods is expected to be limited by economic considerations (ligand and chemistry cost), capacity and stability.

Bacterial α -amylase is a starch hydrolysing (α ,1-4 glycosidase) enzyme used in the brewing industry to supplement malt amylases, and in the baking and textile industries (Debabov, 1982; Yamane, 1989). An isoelectric point of 5.4 was reported by Fischer and Stein (1960) for the *B. subtilis* enzyme. It is reported to be stable between pH 4.8-8.5, although 5-7 is the preferred range (Schmidt et al., 1994). Molecular weight values in the range of 45-51 kDa have been reported for monomeric forms (Fischer and Stein, 1960). Previous chromatographic purification methods include IEC (Fischer and Stein, 1960), HIC (Sada et al., 1985), thiophilic and HIC (Pazos et al., 1994) and affinity (Li and Geng, 1992; Somers et al., 1993). Both thiophilic and HIC were salt promoted, although malt α -amylases were eluted late in the gradient or ethylene glycol was required. Schmidt et al. (1994) reported that "Amylase has a comparatively high surface hydrophobicity, as evaluated by hydrophobic interaction chromatography. Virtually all other supernatant proteins of *B. subtilis* culture did not bind to the Phenyl Superose column at 1.0 M $(\text{NH}_4)_2\text{SO}_4$, whereas subtilisin eluted at ca. 0.57 M and α -amylase at

only 0.28 M $(\text{NH}_4)_2\text{SO}_4$ ". It therefore appeared to be a good candidate for application of the hydrophobic ionisable and mixed mode chromatography. Amylase has also been purified by aqueous two phase separation (Stredansky et al., 1993; Schmidt et al., 1994).

Catalase is an oxidoreductase enzyme. It catalyses the removal of peroxides (by conversion of H_2O_2 to water). The isoelectric point is reported to be 5.5-6 (mammalian). The molecular weight is 225-250 kDa (4 subunits of approximately 60 kDa). Its stability is dependent on maintenance of the oligomeric structure. However, dissociation generally requires drastic conditions, causing irreversible denaturation : pH below 3 or above 10 and/or detergents (Schonbaum and Chance, 1976). Therefore chromatography between pH 4 and 10 should be practical. Catalase has been purified by crystallisation (Dounce and Mourtzikos, 1981), precipitation (Miyahara et al., 1978) and ion exchange chromatography (Saha et al., 1964).

Subtilisin and amylase can be eluted at low ionic strength from Phenyl Sepharose and were studied to determine the requirements of ligand type and density for strong adsorption. Amylase and subtilisin constitute the bulk of the industrial enzyme market. The annual sales of this market were reported to be \$700-800 million (Arbige et al., 1993). Therefore new purification methods which are economic could be extremely valuable.

Unlike subtilisin and amylase, catalase was not found to elute from Phenyl (or Octyl) Sepharose at low ionic strength (Pharmacia, 1980). It was chosen to compare elution of a strongly adsorbed protein from a traditional HIC resin with that from hydrophobic ionisable resins.

MATERIALS AND METHODS

Reagents and equipment

Catalase type C 10 was from Sigma Chemical Co., St. Louis, MO, USA. Subtilisin, amylase and N-succinyl Ala-Ala-Pro-Phe *p*-nitroanilide were from Genencor International Inc., Sth. San Francisco, CA, USA. Subtilisin was from *B. subtilis*, molecular weight 26,600, isoelectric point 8-8.5 (Genencor, 1993). Amylase was from *B. licheniformis*, molecular weight 48,000, isoelectric point 6.7-6.8 (Genencor, 1994). *p*-Nitrophenyl-maltoheptaoside was a kind gift from Megazyme, N.S.W., Australia.

Absorbance was monitored as described before, except a Hitachi U-1100 spectrophotometer was used for measuring absorbance at 240 nm. Electrophoresis equipment and methods were the same as described before except a Pharmacia low molecular weight standard was used and the formalin content of the developer was reduced to 0.013%. Homogeneous 12.5% or 20% Phast gels (and their recommended separation methods) were used.

All resins used were described in Chapters 5 and 6 except the 67% APP, 33% APIMID resin. This was prepared from a 66% APP (33% unsubstituted ACA) Perloza and APIMID by the "100%" EDC catalysed reaction described in Chapter 5.

Assays

The subtilisin and amylase assays (Becker and Steele, 1993) were based on the methods of Estell et al. (1985) and McCleary and Sheehan (1987) respectively. The chromogenic substrates were N-succinyl Ala-Ala-Pro-Phe *p*-nitroanilide and *p*-Nitrophenyl-maltoheptaoside and the release of nitroaniline and nitrophenol was monitored at 410 nm. Catalase was assayed by the Sigma catalogue method (1994), except that the assay was at room temperature (approximately 18°C) and the assay buffer was 20 mM HEPES, pH 7.5. The rate of absorbance change between 0.45 and 0.4 (at 240 nm) was recorded. Total protein was assayed by the bicinchoninic acid assay, using a BSA standard.

Amylase and subtilisin chromatography

Chromatographic equipment and monitoring was the same as described in Chapter 5, except the full scale absorbance was 2. Amylase chromatography was at room temperature. Quantitative subtilisin activity recovery experiments were at 4°C and qualitative binding and elution experiments were at room temperature, using buffers chilled to 4-10°C. The column was 2 ml (50x7 mm). The load samples were 1 ml of subtilisin (2.4 mg/ml enzyme) or 0.3 ml of amylase (35 mg/ml), adjusted to the load pH. Calcium chloride (10 mM) was initially included in subtilisin load and elution buffers.

Subtilisin load wash fractions (10-20 ml) were assayed without dilution. Samples with an absorbance change of 0.02-0.05/minute was described as slightly active. Samples with an absorbance change of 0.1/minute or greater were described as active. Original subtilisin, diluted 500 fold produced an absorbance change of approximately 0.1/minute.

Perloza ACA (and derivatives), MPA, MBA and MHA

Buffers used for load and equilibration were : 20 mM acetate +/- 0.25 or 0.5 M NaCl, pH 5.2. If used, the intermediate wash buffer (subtilisin only) was 20 mM Tris, pH 8. Subtilisin was eluted with 20 mM Tris + 0.5 M NaCl, pH 9 or 0.1 M NaOH (collected into 2 M acetate, pH 5.2). Amylase was eluted with 20 mM HEPES or 100 mM phosphate, pH 7.5. Regeneration was with 0.1 M NaOH (to zero absorbance) followed by 0.5 column volumes of 2 M acetate, pH 5.2.

Secondary amine and weak base resins

Buffers used for load and equilibration were : 20 mM HEPES, pH 7 or 7.5; 20 mM Tris, pH 8, 8.5 or 9; 20 mM ornithine, pH 10.5; (all +/- 0.5 M NaCl). Elution was with 20 mM acetate, pH 5.2 or 100 mM acetate, pH 4.5. Regeneration was with 0.1 M HCl and 0.1 M NaOH (both to zero absorbance), followed by 10 column volumes of load buffer.

Activity recovery

Buffers chilled to 4°C were used. Subtilisin (1 ml) was loaded onto a 1.5-2 ml column and the first 20 ml of the load wash collected. The resin was washed with a further 20 ml of load buffer followed by elution (10 ml). This was pH adjusted, if required, and diluted 100 fold (at 4°C) before assay.

DEAE guard column use for subtilisin

DEAE Otsorb (10 ml column) was equilibrated with the same load buffer to be used for the adsorption resins. Subtilisin (10 ml), adjusted to the same pH was loaded and the flowthrough fraction of peak absorbance collected (20 ml).

Subtilisin capacity

Subtilisin activity recovery and capacity tests were performed at 4°C. Capacity testing was by batch adsorption. Crude broth (10 ml, adjusted to load pH) was mixed with 0.1-0.12 g resin for 30 minutes. The resin was transferred to a 2 ml column and washed with load buffer to remove unbound material. Elution was initiated with 200 µl of 2 M acetate, pH 5.2 (weak base resins) or 1 M Tris, pH 9 (Perloza ACA). The use of high molarity buffers ensured rapid pH adjustment. Capacity was determined by total protein (bicinchoninic acid assay), absorbance at 280 nm and protease activity using a proprietary conversion factor.

Catalase chromatography

Phenyl Sepharose, 4-AMP CDI Perloza (45 µMoles/ml) and MEP AB Perloza (50 µMoles/ml) were tested. The latter two resins were pre-equilibrated with 0.1 M NaOH and water, to the uncharged form. The column (2 ml) was equilibrated with 20 mM HEPES + 1 M ammonium sulphate, pH 7.5 (buffer A). Catalase (6.5 mg) was dissolved in buffer A (1 ml) loaded (gravity feed) onto the column and the resin washed with 4 ml of buffer A. The columns were eluted at 0.4 ml/min and fractions (2 ml) were collected every 5 minutes. A 40 minute gradient from 100% buffer A to 100% buffer B (20 mM HEPES, pH 7.5) was followed by a 20 minute wash with buffer B and 30 minute steps to buffer C (100 mM acetate, pH 4) and buffer D (50:50 buffer B and ethylene glycol). Chromatography was at room temperature.

RESULTS AND DISCUSSION

Subtilisin

Perloza ACA, MBA and MHA mixed mode resins

Chymosin was adsorbed to these resins at pH 4.4. This was unsuitable for subtilisin because it is rapidly denatured below pH 5. Therefore the adsorption pH chosen for subtilisin was 5.2. At this pH, a much greater percentage (approximately 50%) of ACA carboxyl groups are ionised (Figure 5.1, Chapter 5), which might interfere with hydrophobic interactions. However, electrostatic interactions were expected to be favourable for adsorption at this pH, because the isoelectric point of subtilisin is higher (8-8.5) than that of chymosin (4.6).

Subtilisin was adsorbed strongly at low ionic strength (20 mM acetate), with no activity detected in the flowthrough fraction. Subtilisin was only retarded at high ionic strength (20 mM acetate + 0.5 M NaCl). Retention increased in the order MBA < ACA < MHA (Figure 7.1a-c). Subtilisin was adsorbed to the latter two resins at an intermediate ionic strength (250 mM NaCl), similar to that of subtilisin broths.

Elution by a pH increase (up to 10) was not very effective compared to that described for chymosin. A reasonable elution profile from MHA Perloza was obtained with the inclusion of 0.5 M NaCl at pH 9 (Figure 7.1d). A similar elution pattern was found from a CM Perloza cation exchange resin, although the pH was increased above the isoelectric point, and electrostatic repulsion was expected.

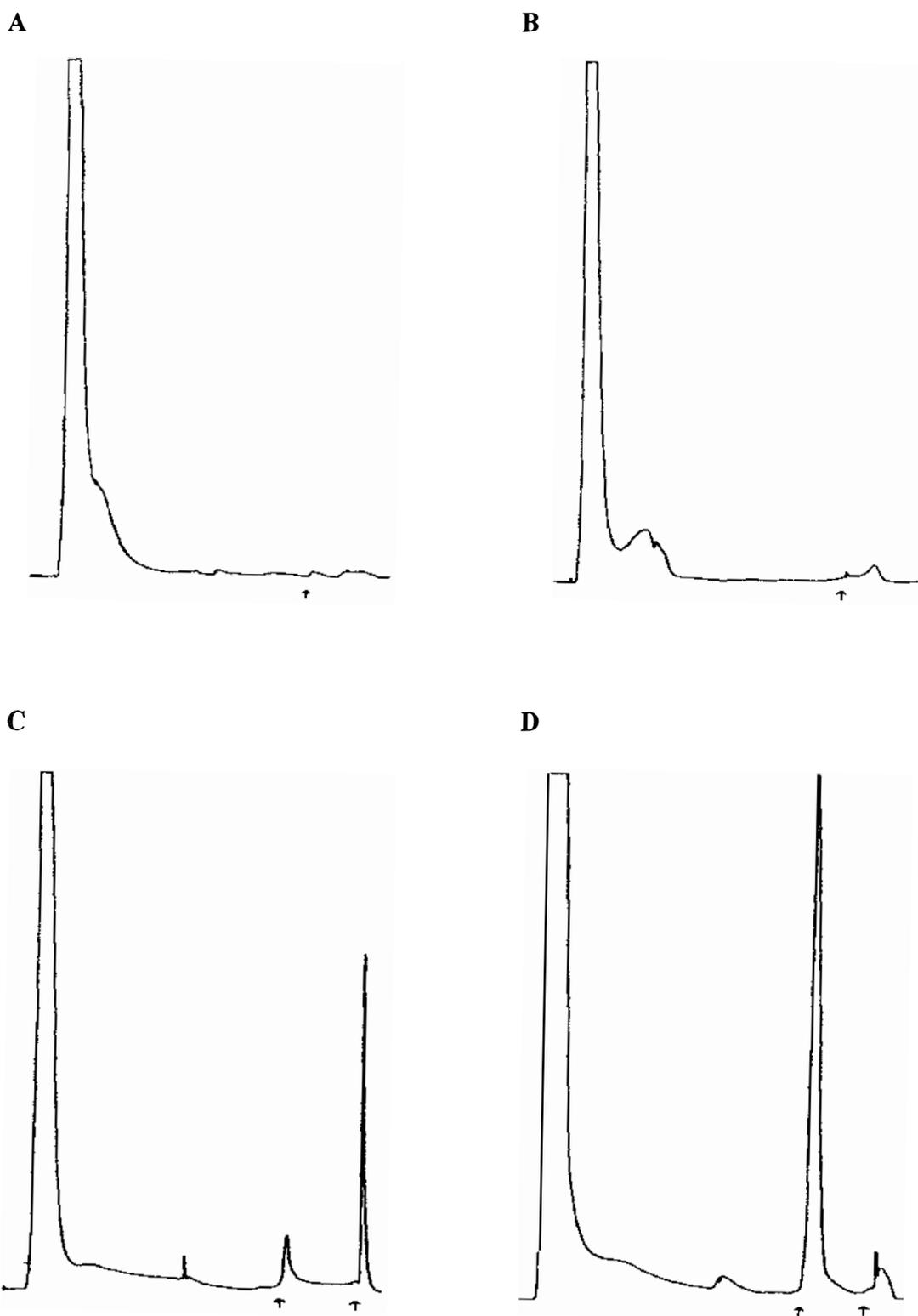
Subtilisin was not adsorbed to or retarded by the weak acid Amberlite CG50 resin (at high ionic strength, pH 5.2). This was consistent with the weak adsorption properties of this resin outside the pH range 4-4.5 (Sasaki et al., 1979). The useful mixed mode adsorption (pH) range of this resin is clearly limited by comparison with the ACA and MHA Perloza resins.

Partially substituted ACA Perloza resins

Adsorption to the Perloza ACA derivatives (APP and PPA) described in Chapter 5, was strong at both low and high ionic strength (no activity detected in the flowthrough). However, elution of subtilisin was even more difficult than from Perloza ACA (pH 9 + 0.5 M NaCl was ineffective). Slow elution was found with 20 mM ornithine (pH 11) or 20 mM ornithine + 0.5 M NaCl (pH 10.5). The latter elution was somewhat faster if 20% ethylene glycol were included. Rapid elution did occur using 10-50 mM NaOH and

Figure 7.1 : Subtilisin chromatography on carboxylate resins

The adsorption buffers were 20 mM acetate + 0.5 M NaCl (A-C) and + 0.25 M NaCl (D). Elution (20 mM Tris + 0.5 M NaCl) and regeneration (0.1 M NaOH) steps are marked with arrows.



the protease remained active if the eluate was adjusted rapidly to pH 5-6 (with 2 M acetate, pH 5.2). However the extreme pH requirement is not ideal, especially if the process were scaled up. The loss of activity due to denaturation is likely to increase due to greater time requirements for elution and hence adjustment to a lower pH.

Sepharose ECH hydrophobic amine resins

Of those ECH resins used in Chapter 5, only tryptamine Sepharose adsorbed subtilisin strongly at high and low ionic strengths. Moreover, this adsorption was only found at a high pH (10.5), at which pH the resins were expected to be largely uncharged. At pH 9 subtilisin was not adsorbed to any of these resins. Although the enzyme was not adsorbed, considerable (colour) fouling of the resins occurred. The extreme pH required for adsorption was not ideal for maintenance of subtilisin activity.

The binding of subtilisin to carboxylate resins at high and low ionic strength showed that subtilisin could be adsorbed strongly by hydrophobic interactions. Non-adsorption in the presence of positively charged groups suggested these could be exploited for elution more readily than carboxylate groups. These features indicated that the weak base resins described in Chapter 6 would be useful subtilisin adsorbents. Adsorption to the uncharged resin form would not be hindered by electrostatic effects, but these could be exploited for elution. Moreover, adsorption at or near neutral pH would be possible.

Perloza ACA APIMID

The first weak base resin tested for subtilisin adsorption was the APIMID derivative of Perloza CDI ACA (0.224 mMoles/g, 1.57 mMoles/g dry). Subtilisin was adsorbed at pH 8.5, 9 and 10.5. The imidazole groups would be uncharged at each of these values. Adsorption was strong at both high and low ionic strength, equivalent to that of the Perloza CDI ACA resins partially derivatised with PPA or APP. The elution pH of 5.2 (20 mM acetate), resulted in electrostatic repulsion effects, without compromising subtilisin stability. Subtilisin was eluted rapidly by this pH change, without addition of salt. This confirmed that resin positively charged groups were much more effective than the carboxyl groups for desorption of subtilisin (by charge repulsion)

Ligand and chemistry selection

Although the adsorption pH used for the APIMID resin was much lower than that used for tryptamine Sepharose, a lower pH was still considered more desirable. Also, resins prepared by methods other than carbodiimide catalysed condensation reactions were preferred on economic and practical grounds. An ACA Perloza was modified with APIMID (33%) and APP (67%) was used to adsorb subtilisin at pH 8. Elution at pH 5.2

was indistinguishable from that of the 100% APIMID resin, indicating that only a small proportion of ionised groups were required. However, preparation of this resin was still comparatively complicated and expensive. Two step methods (activation and ligand attachment), which did not require EDC, were preferred.

Aminomethylpyridine (3AMP or 4AMP) derivatives of ACA Perloza adsorbed subtilisin at pH 7 or 8 (high and low ionic strengths). Elution was again rapid at pH 5.2. Equivalent adsorption was found with these ligands and aminomethylbenzimidazole attached directly to CDI Perloza. However, Perloza CDI APIMID did not adsorb subtilisin strongly, unlike the APIMID resin containing the hydrophobic spacer arm (ACA). The load wash was active (the absorbance change was too rapid to measure without dilution). It was presumed that the imidazole ligand was less hydrophobic than the pyridyl and benzimidazole ligands. Therefore the alkyl spacer arm was needed to produce sufficient resin hydrophobicity for strong binding.

Thiol ligand resins

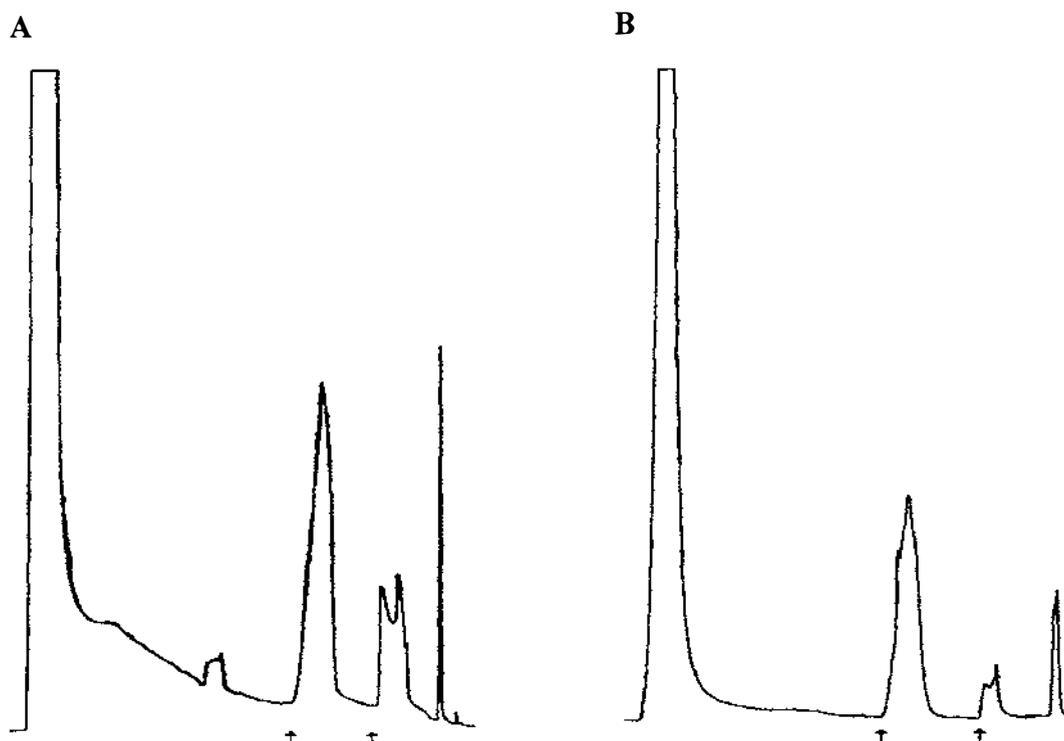
Perloza AGE 4MP (0.1-0.14 mMoles/g) adsorbed and eluted subtilisin in the same fashion as the 4AMP resins. Elution at pH 5.2 was also apparently equivalent. Subtilisin chromatography on MEP AB Perloza (0.22 mMoles/g) was also similar and is shown in Figure 7.2a. Mercaptomethylimidazole (MIM) derivatives of allyl Perloza (0.13-0.17 mMoles/g) were also used to adsorb subtilisin at pH 7.5 (Figure 7.2b). This adsorption pH was lower than for APIMID resins, reflecting the lower "start pH" of this resin (Table 6.3, Chapter 6). Adsorption was weaker than that of the pyridyl resins (evidenced by a "slight leak" of activity in the flowthrough). Subtilisin was strongly adsorbed by mercaptobenzimidazole AGE Perloza, but not eluted at pH 5.2. This resin has a lower titration range and few groups would be ionised at pH 5.2 (Table 6.3). The preference for the thioether ligand attachment has been described before. MEP was preferred over 4MP for cost reasons. Although subtilisin adsorption was weaker, MIM was also preferred to 4MP (again on economic grounds). MIM is likely to be even more useful for proteins of greater hydrophobicity.

Elution from weak base resins

Elution from weak base resins (by electrostatic repulsion) was much more effective than from weak acid resins, including ion exchangers. Despite a lower proportion of charged groups at pH 5.2 (Figures 6.2 and 6.3, Chapter 6), it appeared that elution from pyridyl (para) and aminomethylbenzimidazole resins was equally as effective as from the Perloza ACA APIMID resin. Resins of lower titration range than 4AMP Perloza were considered less suitable because enzyme recovery was poor (at a favourable pH).

Figure 7.2 Subtilisin chromatography on (A) MEP and (B) MIM AB Perloza

The adsorption buffer was 20 mM HEPES + 0.5 M NaCl, pH 7.5. Steps to elution buffer (20 mM acetate, pH 5.2) and regeneration (0.1 M HCl and NaOH) are marked with arrows.



Purification performance

Significant purification was found using carboxylate or weak base resins. The bulk of the material absorbing at 280 nm was not adsorbed to the resins (Figures 7.1-7.3). Subtilisin elution fractions were apparently colourless. However electrophoresis results were less satisfactory than those found for chymosin and capacity results (below) indicated a considerable proportion of inactive enzyme or contaminant proteins were bound. The electrophoresis results could reflect (apparently) poorer sensitivity of subtilisin to the silver stain, greater contaminant coelution or greater autolysis. The level of protease activity in crude samples appeared to be reduced significantly during shipping. It was also found to drop steadily during (prolonged) storage, even at 4°C. The contaminants found by SDS PAGE were all of lower molecular weight than subtilisin, and therefore could be autolysis products. Better electrophoresis results might be obtained if fresh subtilisin broth were used. Nevertheless, several important objectives were met : coloured material was removed; subtilisin could be eluted rapidly at an appropriate pH; salt addition or removal was not required; and capacity was good.

Activity recovery

Optimal activity recovery was less than 90% from a Perloza ACA (PPA) derivative (Table 7.1). Recovery from APIMID and 4AMP ACA Perloza resins was nearly quantitative, without any optimisation other than operation at 4°C. Low temperature was found to be essential for good recovery, whereas calcium had little effect over the short time involved with purification. The eluates from the latter resins did not require pH adjustment, because subtilisin is relatively stable at pH 5.2.

Table 7.1 : Subtilisin activity recovery from selected resins

Resin	Activity absorbance change x dilution	Percentage recovery
not chromatographed	54.4	n.a.
Perloza ACA PPA (67%)	47	86
Perloza ACA 4-AMP	53.8	99
Perloza ACA APIMID	51.7	95

Capacity

Batch protein capacities (at high ionic strength) were in a similar range to those found for chymosin with mixed mode resins. However the capacity deduced from activity assay was 3-4 times lower. The discrepancy could be due to non-specifically bound protein inactive protease. These results are summarised in Table 7.2.

Comparison to Chymosin

The major differences from chymosin purification were the pH requirements and the apparent level of purification. The weak base resins were readily adapted to use over a higher pH range. Both adsorption and elution performance was good. In contrast, the ACA Perloza resins adsorbed subtilisin well but elution was less satisfactory. This was apparently an unusual feature of the enzyme rather than a resin "failing", because the same effect was found with a CM resin. The apparently poorer level of purification may have been due to proteolysis effects and/or electrophoresis staining differences. Another difference between the subtilisin and chymosin, which may be of greater significance, is that the former is of bacterial origin while the latter is fungal. Therefore the potential contaminants (host proteins) will be different. The chymosin expression vector (*A. awamori*) may be particularly well suited to these purification methods.

Table 7.2 : Subtilisin capacity data

A proprietary conversion factor was used to obtain mass values from activity data. The adsorption pH was 5.2 for carboxylate resins and 7 or 8 (indicated in brackets after the resin) for weak base resins.

Resin	Capacity (mg/ml)	
	activity	protein assay
Perloza ACA	23.4	103
Perloza ACA PPA (67%)	18.5	63
Perloza ACA 3-AMP (7)	13	35
Perloza ACA 4-AMP (7)	15.2	50
Perloza ACA 4-AMP (8)	20	75
Perloza CDI 4-AMP (8)	18.2	67
Perloza APP/APIMID (8)	13.5	44

Fouling and use of a DEAE guard column

The weak base resins suffered greater fouling than the mixed mode carboxylate resins. Whereas foulants were removed from the latter using 0.1 M NaOH, some colour was retained by the former resins. This was found despite using a 0.1 M HCl wash after subtilisin elution, to fully titrate the weak base groups to their ionised form. This suggested that the foulants might be strongly retained by an ionised resin, at high ionic strength, while subtilisin would pass through. This was confirmed by using a DEAE Ostsorb "guard" column. crude broth was adjusted to pH 7.5 and passed through the column, pre-equilibrated with load buffer (20 mM HEPES + 0.5 M NaCl, pH 7.5). Subtilisin activity was not retained (or apparently retarded), but considerable fouling of the DEAE resin occurred. When the flowthrough sample was applied to a weak base resin, fouling was insignificant. No adjustment of pH or ionic strength was required between the guard column and the "subtilisin" column. Therefore they could be used in line" without overly complicating the purification process.

Amylase

Adsorption

Binding of amylase was expected to be stronger than that found for subtilisin, because of their relative retention times on Phenyl Sepharose (Schmidt et al., 1994). The resins used were MEP and MIM AB Perloza (0.15 and 0.13 mMoles/g respectively), at pH 7.5, and Perloza ACA, MPA, MBA and MHA weak acid resins (all approximately 0.2 mMoles/g), at pH 5.2. Each of these resins adsorbed amylase at high (MPA was only tested at an ionic strength equivalent to 0.25 M NaCl) and low ionic strength, with no evidence of activity in the flowthrough fractions. This contrasted with the "incomplete" adsorption of subtilisin by MIM and carboxylate Perloza resins and confirmed the expectation of stronger adsorption.

Elution

Elution from MIM and MEP Perloza (at pH 5) was rapid and the eluate was nearly colourless (Figure 7.3a/b). Elution from carboxylate resins was also efficient using 20 mM HEPES or 100 mM phosphate, pH 7.5 (Figure 7.3c/d). The range of the pH change (5-7.5) coincided with the maximum buffering of this resin, at low ionic strength (Figure 5.1, Chapter 5). Therefore elution with 100 mM phosphate was preferred. The eluates from these resins were slightly coloured. These results demonstrated that although strong adsorption occurred, it was easily countered by manipulation of ionic interactions.

Purity

Unlike subtilisin, amylase was strongly stained by the silver method. Autolysis was not a problem either, although any proteases in the crude broth could produce breakdown products. The electrophoretic purity of elution samples from MIM and carboxylate resins appeared similar to that found for chymosin, although the starting material was already enriched (Figure 7.4). This purity of the MEP elution was poorer than the other samples. Minor contaminants could be due to proteolysis.

Fouling

No evidence of (colour) fouling was found on the carboxylate or MIM resins. A slight discolouration was found with MEP resins. This was a considerable improvement over the fouling found with subtilisin broths.

Figure 7.3 : Amylase chromatography : (A) MIM, (B) MEP, (C) MBA and (D) MHA Perloza

Adsorption was at pH 7.5 (A and B) and pH 5.2 (C and D). Elution (pH 7.5 and 5.2 respectively) and regeneration (0.1 M NaOH) are marked with arrows.

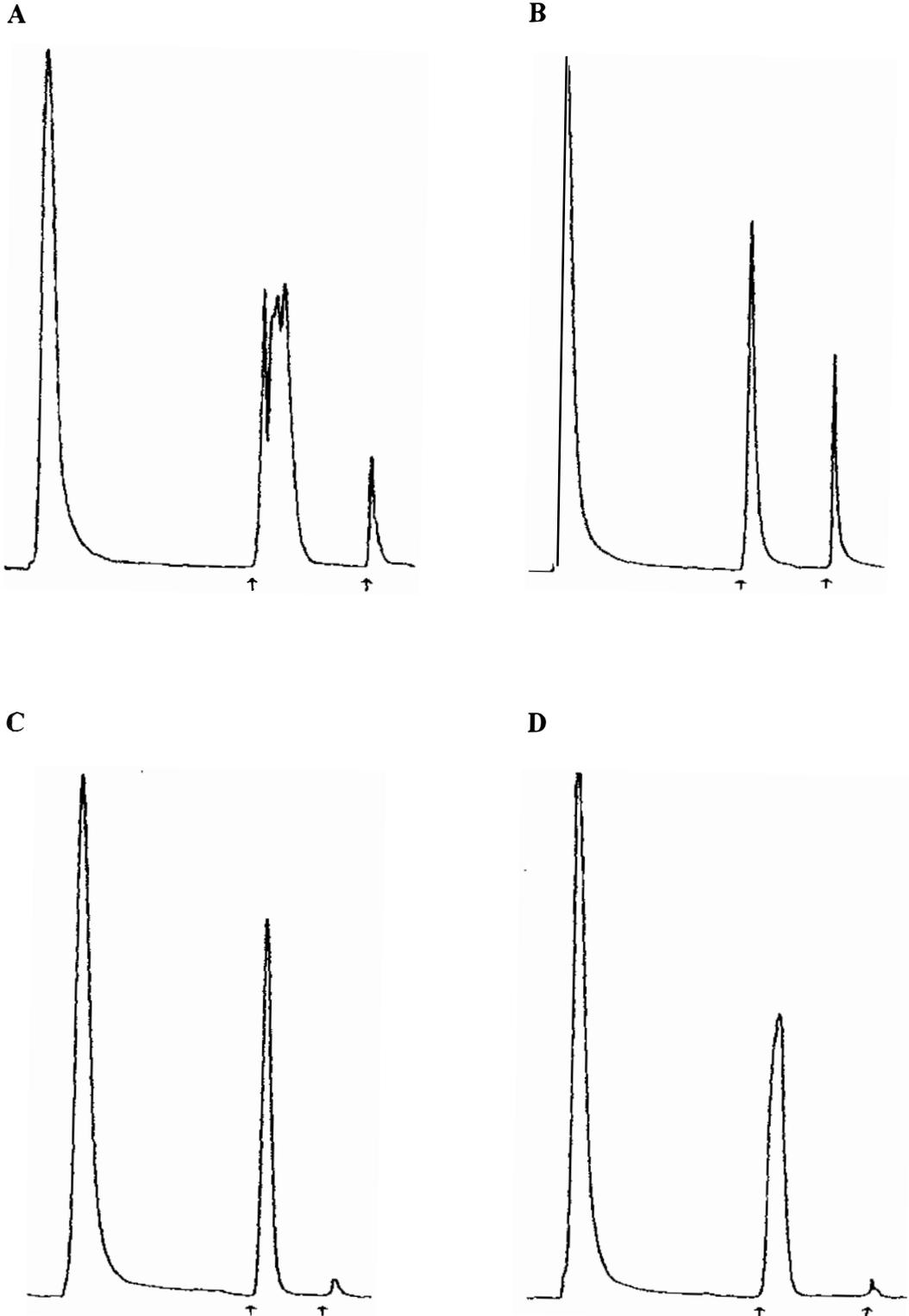
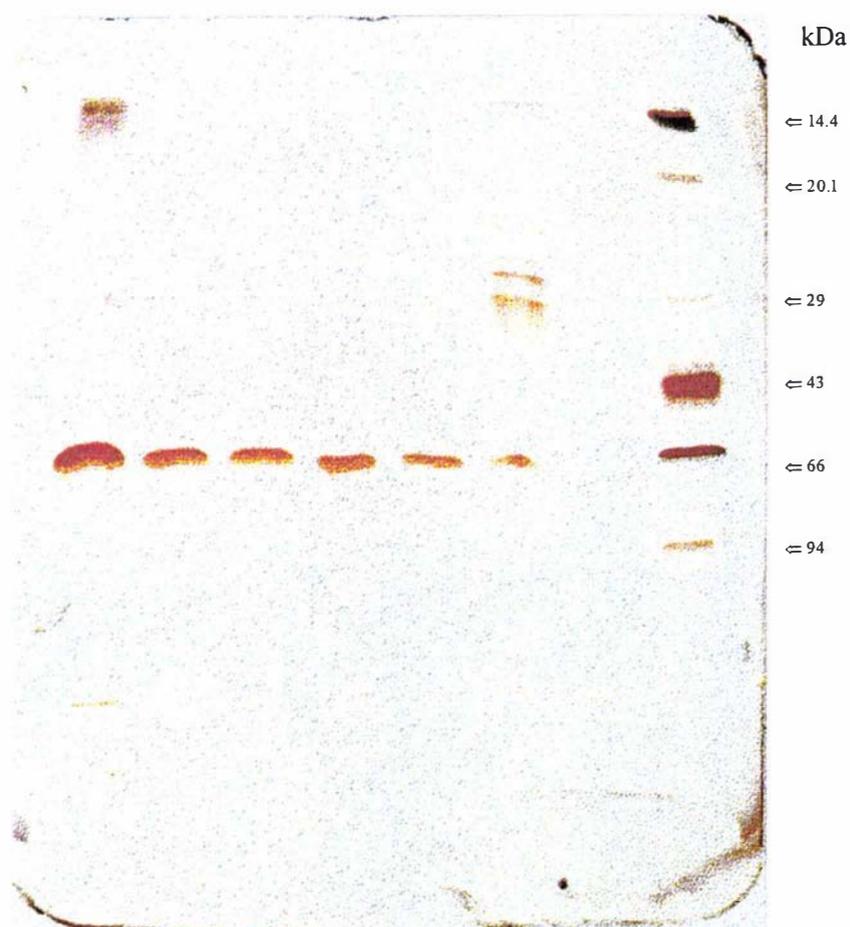


Figure 7.4 : SDS PAGE of amylase elution samples

Lane 1 : original amylase

Lane 2 : MPA Perloza elution

Lane 3 : MBA Perloza elution

Lane 4 : MHA Perloza elution

Lane 5 : MIM Perloza elution

Lane 6 : MEP Perloza elution

Lane 7 : empty

Lane 8 : molecular weight markers (Pharmacia LMW kit)

Catalase

Ammonium sulphate gradients on Phenyl Sepharose and hydrophobic ionisable resins

Catalase was adsorbed to all the resins using 1M ammonium sulphate. A small proportion (<10%) of catalase loaded onto Phenyl Sepharose was eluted at the end of the (decreasing) salt gradient (Figure 7.6a). No activity was eluted by decreasing the pH to 4 (100 mM acetate). The bulk of the catalase activity was recovered using 50% ethylene glycol. Except for the partial desorption at the end of the salt gradient, these results confirmed data reported elsewhere (Pharmacia, 1980). The top of the Phenyl Sepharose column remained discoloured (brown) after the ethylene glycol wash.

Desorption at pH 4 from 4AMP Perloza

The gradient elution pattern from a 45 μ Moles/ml 4AMP CDI Perloza was similar, except a greater proportion of the catalase activity was desorbed at the end. The remaining activity (%) was rapidly eluted at pH 4 (Figure 7.6b). No absorbance peak or activity was detected in the subsequent ethylene glycol wash, implying elution at pH 4 was complete. The top of the column was not fouled after the pH 4 wash. Evidently, catalase and a contaminant were desorbed from the resin at pH 4. Although this implied that the level of purification was less, this might be improved by using an intermediate pH for elution. Elution at pH 4 was rapid compared to the ethylene glycol elution from Phenyl Sepharose. The absence of elution, at low pH on Phenyl Sepharose, indicated that elution from the AMP resin was not due to a conformational change of catalase. The lower level of fouling should allow a greater column lifetime.

Similar results were obtained using MEP AGE Perloza (50 μ Moles/ml). However, the percentage of catalase activity eluted at the end of the salt gradient was minimal. This was consistent with the results of model protein chromatography (Chapter 6). Rapid and apparently complete elution was again obtained by pH adjustment to 4 (Figure 7.5c).

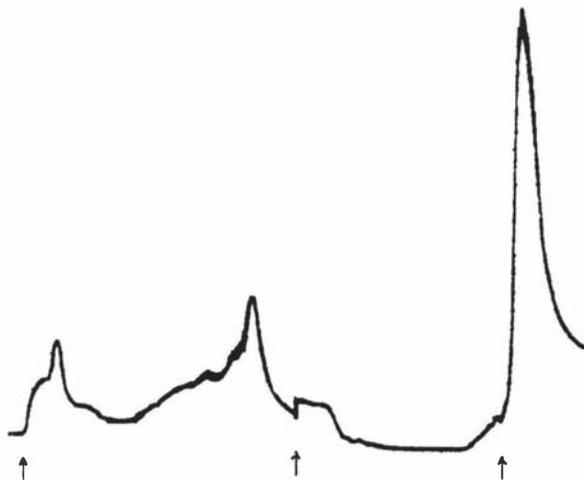
Advantages

These results demonstrated the potential for easy elution of a protein which was strongly adsorbed by hydrophobic interactions. Desorption was faster than that obtained from the conventional HIC resin, using ethylene glycol. The enzyme was recovered in a small volume of a low ionic strength, aqueous buffer. These results also suggest that catalase could be adsorbed to a weak base resin, without prior adjustment of ionic strength. Thus the salt gradient could be avoided as well as the ethylene glycol step. The ligand density required should not be high, given the minimal desorption from the lowly substituted MEP resin, during the salt gradient.

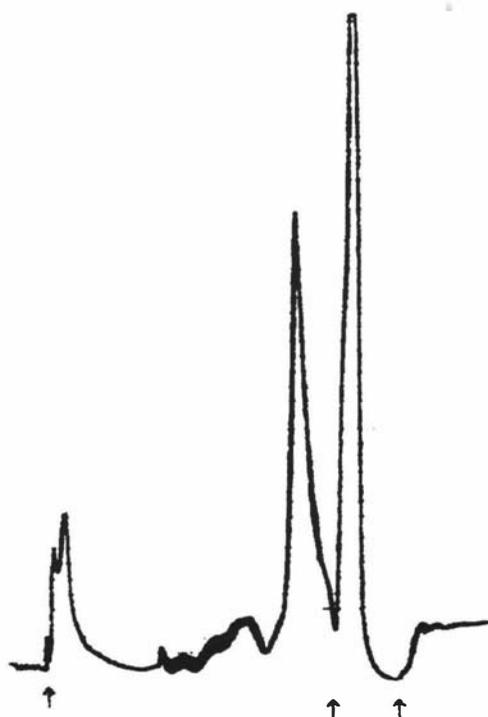
Figure 7.5 : Catalase chromatography on (A) Phenyl Sepharose, (B) 4AMP and (C) MEP Perloza

The adsorption buffer was + 1 M ammonium sulphate, pH 7.5. Arrows mark (1) the start of the decreasing ammonium sulphate gradient, and the steps to (2) pH 4 and (3) ethylene glycol respectively.

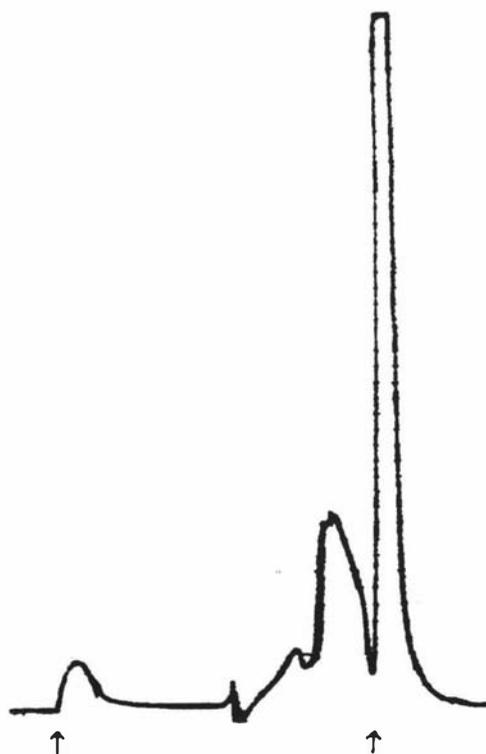
A



B



C



SUMMARY

The simple adsorption and elution methods used previously for chymosin purification have been applied to three other enzymes. These enzymes have been successfully adsorbed at high and low ionic strength, although their different hydrophobicity has led to different ligand type and density requirements. Elution of these enzymes was again induced by a pH change.

Subtilisin was expected to be the least hydrophobic of the three enzymes and this proved to be the case. At high ionic strength, it was retarded to varying degrees on carboxylate and imidazole resins but completely adsorbed to resins of greater hydrophobicity. Positively charged groups on the resin prevented adsorption and thus induced elution strongly. Subtilisin recovery from these resins was nearly quantitative. Pyridyl resins had the best combination of adsorption and elution properties but suffered greater fouling. Negatively charged groups apparently had a much weaker effect on adsorption and elution. Subtilisin recovery from these resins was therefore more difficult. This was the only example in which a simple pH change did not result in very rapid elution. The purification performance was apparently poorer than that found for chymosin, but elution samples were colourless and fouling much less significant.

Amylase was adsorbed more strongly than subtilisin. Complete adsorption of activity was found using carboxylate and imidazole resins. Despite the strong adsorption to these resins (and MEP Perloza), amylase was simply and rapidly recovered by a pH change. The pH range used for adsorption and elution was 5-7. This is also the preferred range for amylase stability. Elution samples were nearly colourless and of good purity (electrophoresis). The resins did not retain coloured foulants, after regeneration, despite using crude fermentation broths. The resins and methods described in the last three chapters appear to be especially well suited to amylase purification.

Catalase is also strongly adsorbed, although only resins of low ligand density were tested. Although these resins might be useful for purification of catalase from crude sources, the purpose of this study was to demonstrate the potential advantages of resins containing a low density of ionisable ligands for HIC of very hydrophobic proteins. The ionisable resins are clearly distinguished from a commercial HIC resin by the rapid elution of catalase at pH 4. The advantages of this type of elution are (i) target proteins can be recovered using aqueous buffers, (ii) rapid elution kinetics (low elution volume) and (iii) rapid removal of strongly bound protein foulants should improve regeneration of hydrophobic resins.

CHAPTER 8 CONCLUSIONS

Results summary

The matrix chosen as the focus of this study, Perloza bead cellulose, has been shown to have good properties for laboratory scale, low pressure chromatography. It has been found to compare favourably with Sepharose matrices. The results presented here, and previous reports (Peska et al., 1976; Stamberg et al., 1982; Stamberg, 1988; Gemeiner et al., 1989) have suggested it would also be useful for large scale chromatography.

The chemical reactivity of Perloza was demonstrated using the anhydrous CDI activation chemistry. The efficiency of this activation was greatly improved by the use of better solvent exchange techniques. Likewise, subsequent ligand attachment was improved by minimising water content of the reaction mixture. However, CDI methods were still not favoured for large scale use because of the solvent requirement, cost and the alkali sensitivity of the urethane linkage. Aqueous activation and ether linkages were preferred. Commonly used etherification reactions were not very effective in aqueous media. Carboxymethylation was improved by the inclusion of an organic solvent in the reaction mixture. However, ligand attachment to CM resins was not ideal because it required expensive carbodiimide chemistry. Low activation levels were obtained with standard (and preferred) bifunctional etherification reagents, regardless of the solvation used. The low activation levels were attributed to competition from hydrolysis and crosslinking side reactions. Therefore none of these conventional activation methods were considered ideal for large scale use.

The use of bifunctional reagents containing groups of unequal reactivity, would eliminate or reduce the side reactions. This quality was found with allyl bromide and allylglycidyl ether. Their reaction with Perloza resulted in covalent attachment of allyl groups by an ether linkage. The allyl groups were relatively stable and no indication of reaction with water or cellulose was found. This simplified the reaction chemistry and high activation levels were obtained (despite aqueous solvation) because side reactions were no longer limiting. It was demonstrated that the activation level could be easily controlled by the amount of reagent used.

Two distinct methods were demonstrated for ligand attachment to allyl matrices. The first of these was nucleophilic substitution with sulphite, amine or thiol ligands, after allyl groups were modified by bromination (addition of HOBr) of the double bond. Aqueous N-bromosuccinimide (NBS) was preferred because it was anticipated that a side reaction

found with bromine water addition would be eliminated. NBS is also easier and safer to use than bromine water. Nucleophilic substitution of brominated matrices occurred readily at alkaline pH (≥ 10). Reaction of thiols and sulphite at lower pH values was also effective if the mixture was heated at 60°C. Nucleophilic substitution allowed the preparation of a diverse range of resins for affinity, hydrophobic, ion exchange, IMAC and mixed mode chromatography.

The second method did not require an initial modification of the alkene group. Although the allyl group is comparatively unreactive, it will undergo specific free radical addition reactions other than bromination. Several thiols and the bisulphite ion were found to react readily with allyl Perloza without any (deliberate) catalysis. This was consistent with the facile addition of these molecules reported for simple alkenes, despite the use of a macromolecular alkene substrate and aqueous solvation. Advantages of addition were simplicity and specificity (e.g. glutathione adds readily through its thiol group, without competition from the amino group). Thiol attachment was simplified because there was no requirement to remove oxygen from reaction mixtures, in contrast to conventional methods (Simons and Vander Jagt, 1977). One less reaction step is required compared to the substitution chemistry. Resins for affinity and ion exchange chromatography were prepared by free radical addition and a wider range of resins might be produced if catalytic methods (e.g. irradiation) were employed. Addition of a mercaptoacid ligand followed by carboxyl group titration was used to analyse allyl activation level. The high activation levels obtained in aqueous media and efficient ligand attachment recommended these allyl chemistries for large scale use especially.

A range of ion exchange resins were produced from allyl Perloza, using simple methods, and high charged group densities were obtained. With one exception the physical properties of these resins were excellent, with only a small loss of performance relative to unmodified Perloza. Flow rates superior to Sepharose Fast Flow resins were obtained. Chromatographic (bovine serum albumin elution) properties of the strong ion exchange resins were similar to those of Sepharose Fast Flow. Protein capacities were also high, although the BSA capacity of anion exchange resins was lower than that of Q Sepharose Fast Flow. These results indicated that the products of allyl Perloza were useful for protein chromatography.

CDI and allyl Perloza were used for preparation of mixed mode (hydrophobic and ionic) resins (of high ligand density) for chymosin purification. Some resins were also produced from CDI and ECH Sepharose. Chymosin was adsorbed to these resins at both high and low ionic strengths. Most forms of chromatography have specific ionic

strength requirements (e.g. low ionic strength for ion exchange chromatography and addition of ammonium sulphate for hydrophobic interaction chromatography). Therefore an intermediate step to alter ionic strength is typically required before crude fermentation broths can be processed. Therefore, the adsorption found at high and low ionic strength was a preferred characteristic, because an intermediate step was not required.

Despite the high ligand density (and hydrophobicity) which imparted strong adsorption properties, near homogeneous (by SDS PAGE) chymosin was recovered, in one step, by a small pH change. High capacities were also found. Fouling of resins by crude broths was significant for amine but not carboxylate containing resins and the latter resins also had higher capacity. Mercaptoalkyl carboxylate derivatives of allyl Perloza were preferred for large scale use.

The features of high ligand density, adsorption at high and low ionic strengths and elution by a small pH change were retained for a new form of hydrophobic chromatography. This relied on weakly ionisable ligand groups which titrated in a pH range near neutrality. At adsorption pH these resins were neutral and hydrophobic, while at elution pH they had mixed mode characteristics. The use of a variety of ligands (and functional groups) allowed production of resins with differing hydrophobicity and titration range. The latter property meant a range of titration "windows" were available and a rational selection of ligand/resin could be made to suit the preferred pH requirements of a target protein.

Salt promoted adsorption of various proteins to ionisable (pyridyl) resins of low ligand density, in comparison with Phenyl Sepharose, suggested the uncharged forms functioned as HIC adsorbents. At moderate ligand density, lysozyme and chymotrypsinogen were not completely eluted and at high ligand density they were adsorbed at high and low ionic strength. The ionisable character was then exploited, by a pH change, to obtain rapid elution. High ligand density (pyridyl) resins were also used for chymosin adsorption independent of salt concentration, at pH 5.5-6.5. Elution was again obtained by a pH change (to 2).

Chromatography of two other crude enzymes was investigated. The more hydrophobic of these (α -amylase) was also adsorbed at low and high ionic strength to the mixed mode (carboxylate) and hydrophobic ionisable resins used for chymosin. Rapid elution was again obtained by a small pH change. Eluted α -amylase was near colourless and homogeneous (by SDS PAGE). Subtilisin is less hydrophobic and was only retarded at high ionic strength on the carboxylate resins. It was however adsorbed by the more

hydrophobic of these resins at an intermediate ionic strength, similar to that of the crude broth. Elution from these resins was also more difficult than was found for α -amylase and chymosin. Stronger adsorption was found with the uncharged form of hydrophobic ionisable (especially pyridyl) resins. Nevertheless, rapid elution was found following a pH change to the partially ionised form. Although purification was less evident (by SDS PAGE), eluted subtilisin was largely colourless and separated from the bulk of material absorbing at 280 nm.

Catalase, an enzyme which required 50% ethylene glycol for elution from a conventional (Phenyl Sepharose) resin, was chromatographed on low ligand density pyridyl resins. Similar adsorption properties were found using a salt gradient at pH 7.5 (<10% activity eluted). However, catalase was recovered (rapidly) by a pH change to 4. No elution was found at this pH using Phenyl Sepharose. Regeneration of the pyridyl resins was apparently superior to that of Phenyl Sepharose.

Future prospects

Further applications of allyl chemistry could exploit the high ligand density potential of this method. High ligand density has been considered unfavourable in many applications because non-specific interactions become more significant and therefore resolution is reduced. However for some large scale applications, it may be preferred to increase capacity at the expense of resolution. High ligand densities have already been obtained for IMAC and glutathione resins and these might be useful for large scale, high capacity chromatography, especially for purification of fusion proteins. Allyl chemistry appears to be particularly suited to use with Perloza, because low activation levels are obtained with the conventional etherification reagents.

Lower ligand densities can still be obtained with efficiency and accuracy using allyl chemistry. Mercaptoacetic and mercaptopropionic acid derivatives of allyl matrices should provide cheap, chemically stable "spacer arm" resins, for attachment of amine ligands for affinity chromatography. These would be alternatives to aminocaproic acid resins, and results here (subtilisin chromatography at high ionic strength) indicate that the mercaptoacid spacer arm would be less hydrophobic. If a hydrophobic spacer arm is required, this can still be obtained by using a longer mercaptoacid (e.g. mercaptohexanoic acid). Allyl matrices are relatively stable in aqueous media, facilitating storage and shipment. Another application of allyl chemistry would be to test a wider range of chromatographic matrices.

Specific ligand addition reactions may be useful for directed attachment of thiol containing ligands such as peptides (Englebretsen, 1992). This attachment would be simplified by lack of competition from amine groups and water for reaction with the allyl matrix. The scope of addition possibilities will be determined by the effectiveness of irradiation catalysis of general thiol ligand additions.

Other applications of the mixed mode resins described are also likely to be for large scale protein purification. Hydrophobic ionisable resins may be useful for analytical (FPLC and HPLC) as well as preparative chromatography. This would require adjustments of matrix (to higher performance) and ligand density. At lower ligand density a more traditional chromatographic separation on the basis of retention time rather than the strong adsorption methods used here. Therefore decreasing salt gradients could be used for elution. A pH step or gradient could be used concurrently or after a salt gradient to provide further resolution. Catalase results suggested that low ligand density resins would be useful for HIC of very hydrophobic proteins (simple recovery) and/or HIC applications where severe hydrophobic fouling occurs.

High ligand density mixed mode and hydrophobic ionisable resins may be applied to adsorption of a wider range of proteins, particularly for industrial separations. Another possible application is non-covalent enzyme immobilisation (to the neutral resin form). If the pH is maintained in an appropriate range, the enzyme should be strongly retained. Hydrophobic resins have been used before (Butler, 1975; Nemat-Gorgani and Karimian, 1983; Hutchinson and Collier, 1986) for non-covalent immobilisation. Ionisable resins have the advantages that the enzyme may be recovered and the resin regenerated (foulants removed) by pH adjustment.

The activation chemistries and hydrophobic ligands described may also be used for modification of polymers other than chromatographic resins. Possibilities include membranes for protein adsorption (Heath and Belfort, 1992) and soluble polymers for two phase liquid extraction (Jensen et al., 1993).

APPENDIX 1 : Molar prices for activation reagents and ligands

Activation reagents

allyl bromide	\$3.50
allylglycidylether	\$6.40
bromine	\$3.50
N-bromosuccinimide	\$8.50
butadiene diepoxide	\$310
butanediol diglycidylether	\$157 (95% grade)
carbonyldiimidazole	\$200
chloroacetic acid	\$1.10
cyanogen bromide	\$47
cyclohexylmorpholinoethylcarbodiimide	\$1200
divinylsulphone	\$154
epichlorohydrin	\$1.00
ethoxycarbonylethoxydihydroquinoline	\$190
ethyldimethylaminopropylcarbodiimide	\$1000
ethyleneglycoldiglycidyl ether	\$238
fluoromethylpyridinium paratoluene sulphonate	\$3290
tosyl chloride	\$6.00
tresyl chloride	\$4250

Ion exchange and spacer arm ligands

aminocaproic acid	\$8.00
cysteamine HCl	\$70
diethylamine	\$0.90
mercaptoacetic acid	\$2.20
mercaptopropionic acid	\$5.40
mercaptosuccinic acid	\$30
sodium (meta)bisulphite	\$0.67*
sodium sulphite	\$1.10
trimethylamine	\$1.10

* price per mole of NaHSO₃ obtained when dissolved in water

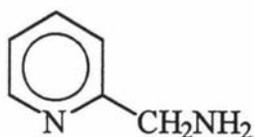
Mixed mode and hydrophobic ionisable ligands

2-(aminomethyl)benzimidazole	\$588
2-(aminomethyl)pyridine	\$75
3-(aminomethyl)pyridine	\$24
4-(aminomethyl)pyridine	\$57
1-(3-aminopropyl)imidazole	\$40
4-(3-aminopropyl)morpholine	\$16
3-chloro-4-hydroxyphenylacetic acid	\$115
3,5-dichlorosalicylic acid	\$86
histamine	\$530
histidine	\$47
4-hydroxybenzoic acid	\$5.70
4-hydroxy-3-nitrobenzoic acid	\$71
4-hydroxyphenylacetic acid	\$90
hydroxythiophenol	\$355
2-mercaptobenzimidazole	\$6.10
2-mercapto-1-methylimidazole	\$74
2-mercaptopyridine	\$110
4-mercaptopyridine	\$1020
3-nitrotyrosine	\$620
thiolacetic acid#	\$9.90
tyramine HCl	\$270
4-vinylpyridine#	\$10

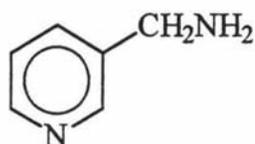
raw materials for mercaptoethylpyridine synthesis

Prices (US\$) were obtained from Janssen or Aldrich catalogues (cheapest value quoted).

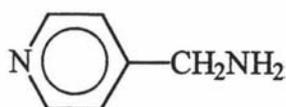
APPENDIX 2 : Structures of hydrophobic ionisable ligands



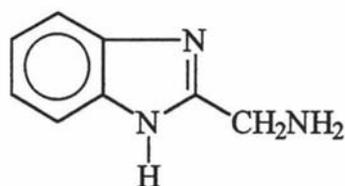
2-(aminomethyl)pyridine



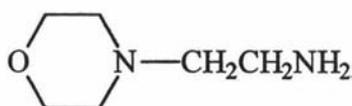
3-(aminomethyl)pyridine



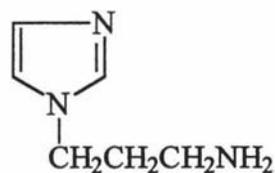
4-(aminomethyl)pyridine



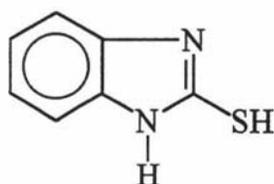
2-(aminomethyl)benzimidazole



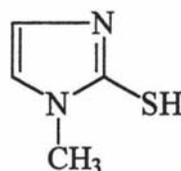
4-(3-aminopropyl)morpholine



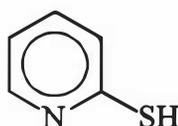
1-(3-aminopropyl)imidazole



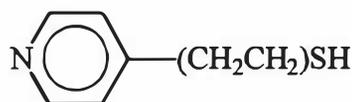
2-mercaptobenzimidazole



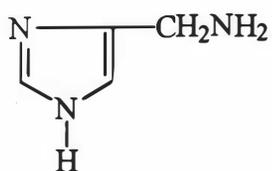
2-mercapto-1-methylimidazole



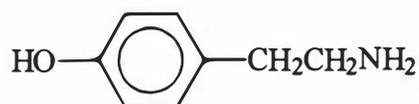
2-mercaptopyridine



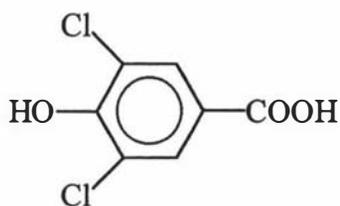
4-mercapto(ethyl)pyridine



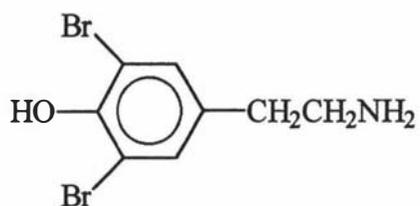
histamine



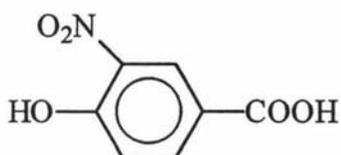
tyramine



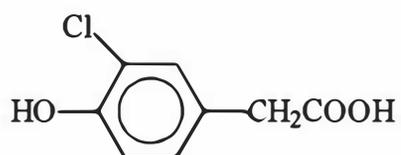
3,5-dichlorosalicylic acid



3,5-dibromotyramine



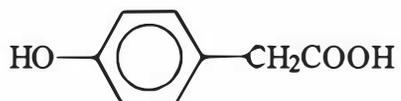
4-hydroxy-3-nitrobenzoic acid



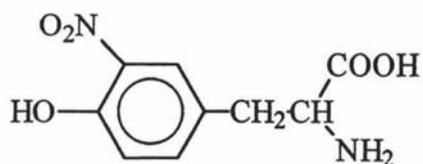
3-chloro-4-hydroxyphenylacetic acid



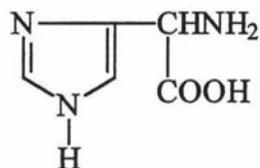
4-hydroxybenzoic acid



4-hydroxyphenylacetic acid



3-nitrotyrosine



histidine

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