RECOVERY OF α-LACTALBUMIN FROM WHEY PROTEIN ISOLATE AND OSTEOPONTIN FROM MILK BY ANION EXCHANGERS

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Abstract

A series of amines, DMEA, DMH, DMO, DMD, DMDo, and Do, were coupled to Sepharose, which was activated by epichlorohydrin first, to prepare amino anion exchangers DMEA-Seph, DMH-Seph, DMO-Seph, DMD-Seph, DMDo-Seph, and Do-Seph.

The batch binding of α-lactalbumin and β-lactoglobulin in 25 mM NaCl at around proteins' IEP to these exchangers and a commercial anion exchanger Q-Seph-ff were tested. Q-Seph-ff, DMEA-Seph, DMH-Seph, and DMO-Seph bound both of proteins at pH > IEP . Q-Seph-ff, DMEA-Seph, DMH-Seph did not bind either of the proteins at pH < IEP. DMO-Seph, DMD-Seph, DMDo-Seph, and Do-Seph bound both of the proteins, especially α-lactalbumin, by hydrophobic interaction at and below the proteins' IEP. The proteins bound by these exchangers except DMO-Seph could not eluted by HCl at low pH. HCl at pH 2.5 could be used to elute these proteins from DMO-Seph.

Recovery of α-lactalbumin from WPI in 25 mM NaCl at pH 3.8-5 by DMO-Seph, DMD-Seph, DMDo-Seph, and Do-Seph were tested. These exchangers were able to bind α-lactalbumin in preference to β-lactoglobulin at and below the proteins' IEPs. Thus DMD-Seph gave an α-lactalbumin yield and purity of 70 and 80% at pH 4.3, DMO-Seph 77 and 81% at pH 4.4. However DMD-Seph had difficulty in eluting all of the α-lactalbumin unless using ethanol.

The batch binding of α-lactalbumin and β-lactoglobulin in high concentration of NaCl at low pH (2.5) by DMO-Seph was tested. The exchanger showed strong hydrophobic affinity for α-lactalbumin but not b-lactoglobulin in 200-500 mM NaCl.

Recovery of native α-lactalbumin from WPI in 400 mM NaCl at pH 2.5 by DMO-Seph was tested. This gave an α-lactalbumin yield and purity of 79 and 73% and a capacity of DMO-Seph 0.73 g/g in 400 mM NaCl at pH 2.5, compared to 67%, 84% and 16 mg/g of DMO-Cell.

DMO-Cell was prepared. Cellulose was modified by propyl oxide and epichlorohydrin and then activated by ECH. The activated cellulose was then coupled with DMO.

The effects of cellulose particle size, cellulose type and substitution level of DMO-Cell on binding of whey proteins were investigated.

DMO-Cell, activated by 1,4-butanediol diglycidyl ether, with substitution level 0.55 meq/g was prepared. It showed a better binding capacity than DMO-Cell activated by epichlorohydrin.

Recovery of native α-lactalbumin from different WPI in 400 mM NaCl at pH 2.5 by DMO-cell was tested. DMO-Cell showed good selectivety for α-lactalbumin from all of three WPI. This gave an α-lactalbumin yield and purity of 13.5 mg/g from WPI (PT8253). From a low α-lactalbumin content WPI this gave an α-lactalbumin yield and purity of 70 and 91%.

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I hope to thank my supervisor J. S. Ayers for his guidance through the past year. I would also like to thank D. F. Elgar and J. Chan for their analysis of HPLC and advice, K. P. Coolbear for her advice and D. Poll for his instruction of equipment analysis.

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Chapter1. Recovery of α-Lactalbumin From Whey Protein Isolate by Anion Exchangers

1 Introduction

1.1 Production of Whey

Milk is a stable colloid consisting of large complex micelles (more than 10 million dalton), which are made up of several different casein components, calcium phosphate and other minor components, and whey. When milk is treated with rennet or its pH is adjusted to 4.6, the casein begins to coagulate. After centrifugation, the casein curd is separated. The supernatant, whey, is a yellow-green liquid. The production procedures of all kinds of whey are summarised in Figure 1.1. About seven litres of whey are separated with manufacturing of a kilogram of cheese. Depending on the production processes whey is divided in to three groups, rennet, acid, and industrial whey. Rennet and acid whey are obtained by separation of casein, effected predominantly by chymosin (rennet), an enzyme which induces coagulation of the caseins, and by lactic acid, respectively. Industrial whey is obtained when casein is precipitated with mineral acids (dilute hydrochloric acid at pH 4.4 or dilute sulphuric acid at pH 4.6) (Sieakiewicz T. & Riedel C., 1990).

The volume of whey continues to grow around the world and currently amounts to more than 190 billion litres/year (Zall R. R., 1984) (Morr C. V., 1984) (Van Hoogstraeten J. J., 1987). Whey had been considered to be a waste by-product by the dairy industry before modern process factories of dairy products were established. Most was used as a low cost animal feed by local farm or treated as waste because of expensive transportation costs.

1.2 Composition of Whey and Problems Caused by Whey

The whey dry matter content is 6-6.5% and is affected by season, region and the production process. It contains essentially 100% , 50 g/l, of the total milk carbohydrate (lactose), and about 20% , 5 g/l, of the total soluble proteins and vitamins in milk (Walstra P. & Jenness R., 1984). These components are responsible for the high putrescibility and biological oxygen demand of whey (Holder and Sewards 1976) When whey is used as a low cost animal feed it causes some problems too. The excess mineral proportions lead to increased urine production, while too high lactose proportions lead to diarrhoea, which in the case of fattened bulls and cows can result in reduced mass increase or reduced milk production (Sueakiewicz T. & Riedel C., 1990).

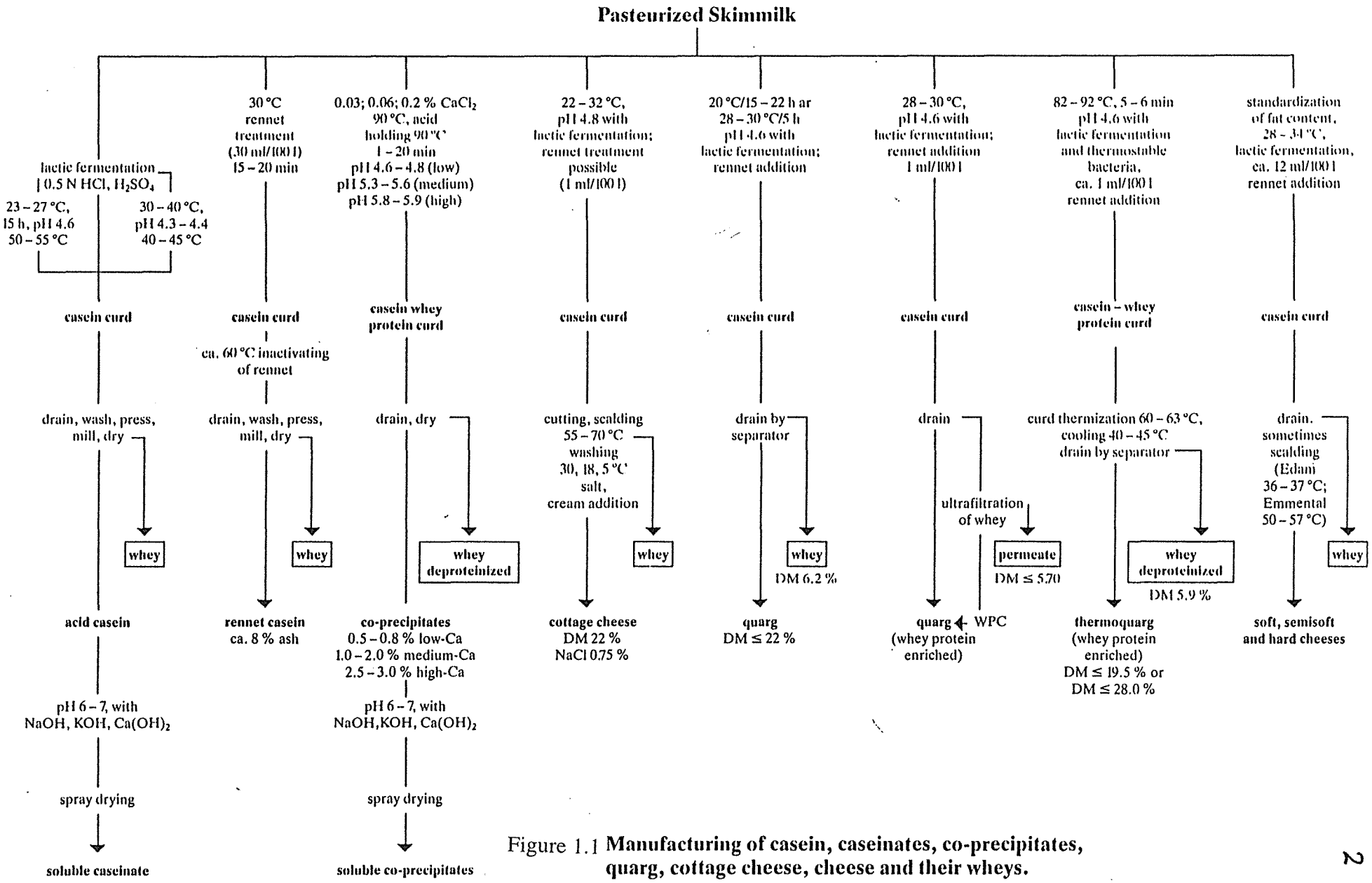


Figure 1.1 **Manufacturing of casein, caseinates, co-precipitates, quarg, cottage cheese, cheese and their wheys.**

On the other hand, whey proteins are judged to be the milk proteins with the highest value. Their biological value exceeds even that of whole egg protein since 17.4 g of egg protein is needed to satisfy the daily requirement of essential amino acids, whereas only 14.5 g of whey protein in the native condition will meet this need (Renner, et al, 1982). Thus the advent of strict environmental regulations worldwide and relatively high prices for whey protein powder has encouraged the dairy industry to recover these whey proteins.

1.3 WPC and WPI

A great amount of effort has been made to develop commercial recovery of whey protein concentration (WPC) and whey protein isolate (WPI).

WPC containing proteins 35-80% is produced by ultrafiltrating whey. Although WPC contains very high content of proteins, it is lack of functionality and flavour stability which is caused by the oxidation of lipid and by some volatile chemical. These disadvantages seriously affect the application of WPC in food.

To broaden the use of whey, non-fat whey protein isolate (WPI) is produced by a stirred-bed ion exchange adsorption process. The composition of WPI is displayed in Table 1.1. WPI contains much less fat, lactose and minerals than WPC. Its proteins consist of β-lactoglobin (β-lg), α-lactalbumin (α-la), bovine serum albumin (BSA), glycomacropeptide (GMP), immunoglobulins (Ig) and other minor proteins. The content of individual proteins in WPI varies depending on the production procedure.

1.4 Whey Proteins

The properties of whey proteins are summarised in Table 1.2. α-Lactalbumin is rich in lysine, leucine, threonine, tryptophan and cysteine. Its 8 cysteine residues form four intramolecular disulfide bonds, linking amino acid residues 6 and 120, 28 and 111, 61 and 77, and 73 and 91 (Swaisgood, 1982). It is nearly spherical and has a highly compact, globular structure. Its secondary structure is composed of 26% α-helix and 14% β-sheet. Its tertiary structure is very similar to lysozyme found in hen egg white. α-Lactalbumin binds calcium, zinc, manganese, copper and cobalt ion (Fox, 1989). These cations affect the association of α-lactalbumin and galactosyltransferase on the inner surface of the Golgi apparatus. Metal binding is also thought to stabilise the protein and protect it from thermal denaturation (Bernal, 1984) (Fox, 1989) (Owusu, 1992). Calcium ions in particular are important since its release at low pH leads to WPI conformational changes in the tertiary

structure of α-lactalbumin (Acharya et al, 1991) and was thought to affect the catalytic properties of the lactose synthase complex (Stuart et al, 1986). At pH above 4.2 calcium ion is strongly bound by α-lactalbumin in a cleft or pocket surrounded by three aspartic acid residues with abnormally low pK values, shown as asparagine residues 82, 87, and 88 in the amino acid sequence of α-lactalbumin. This tightly bound calcium ion stabilises its conformation in a complex structure. Release of the bound calcium renders α-lactalbumin more sensitive to heat-induced denaturation, but reduces its ability to renature upon cooling. It is denatured at 65.2 °C at pH 6.7 and 80 to 90% of the denaturation is reversed upon cooling. Because of this reversibility α-lactalbumin was considered to be the most heat stable of the whey proteins.

Three genetic variants of α-lactalbumin (A, B, and C) are known to exist. B is predominant variant in bovine milk. The A and B variants differ by a substitution of arginine for glutamine at position 10 in the B variant (Gordon et al, 1968).

Table 1.1 Chemical Composition (%) of Commercial WPI

Moisture	2.4-5.6
Protein	88.6-92.7
Nonprotein N compounds	0.29-0.34
Lactose	0.42-0.46
Total lipids	0.39-0.67
Phospholipids	0.11-0.31
Ash	1.37-2.15
Sodium	0.36-0.42
Potassium	0.10-0.16
Calcium	0.20-0.24
Magnesium	0.02-0.03
Phosphorus	0.05

*From Morr, C. V. and Foegeding, E. A., Composition and functionality of commercial whey and milk protein concentrates and isolates: a status report, *Food technol.*, **44**, (1990), 100

Table 1.2 Chemical and Physicochemical Properties of Whey Proteins

	β-Lg	α-La	BSA	Ig	GMP	Lf
Isoelectric point	5.2	4.2-4.5	4.7-4.9	5.5-8.3	2.8-4.5	8.0-9.0
Concentration.in whey, g/l	2-4	0.6-1.7	0.4	0.4-1.0	1.4	0.1
MW, kD	18	14	66	>14.6	6.0-8.0	76-86
Average hydrophobicity, Kcal/residue	1075	1020	995	NA	NA	NA
Total amino acid residue/mol	162	123	582	NA	64	708
Apolar residues/mol	54	44	163	NA	28	332
Cys. residues/mol	5	8	35	NA	0	35
Disulfide residues/mol	2	4	17	NA	0	NA
Sulphydryl residues/mol	1	0	1	NA	0	NA
Lys. residuals/mol	15	12	59	NA	3	55
Glu. residuals/mol	16	8	59	NA	8	39
Asp. residuals/mol	10	9	39	NA	2	36

1. From Eigel W. N., Butler J. E., Ernstrom C. A., Farrell H. M., Jr., Harwalker V. R., Jenness, R, and Whitney R. McL., *J. Dairy Sci.*, **67** (1984), 1599.
2. From Egan M. M. A., MSc thesis of Massey, 1994.

α-Lactalbumin is a glycoprotein with a N-linked oligosaccharide at asparagine 45 (Tilley et al, 1991). It consists of a major aglycoprotein (92%), a minor aglyprotein (5%) and two glycoproteins (total 3%) (Hopper & Mckenzie, 1973). The polysaccharide moiety of glycoproteins of α-lactalbumin is estimated to be 15% of the total molecular weight and is thought to contain 11-12 sugar residues per molecule (Barman, 1970). These two glycoproteins are soluble at low pH due to the carbohydrate moieties whereas two aglycoproteins are less soluble at low pH.

The biological function of α-lactalbumin is related to the synthesis of lactose in the mammary gland of lactating animals by regulating the activity of galactosyltransferase which is an enzyme responsible for the production of lactose. α-Lactalbumin associates with the galactosyltransferase on the luminal surface of the Golgi apparatus to form a lactose synthase complex (Mckenzie & White, 1991)

β-Lactoglobulin is the most abundant of the whey proteins, making up about 50% of the total whey proteins. It consists of 162 amino acid residues and a molecular weight of 18.3 Kd. Its five cysteine residues form two intramolecular disulfide bonds, linking residues 66 and 160, and residues 106 and 119 or 121 and leave one active SH group at residue 119 or 121. These sulfur-containing amino acid residues facilitate protein polymerisation by formation of covalent intermolecular disulfide bonds, which gives β-lactoglobulin the property of gelation, during high-temperature processing. β-Lactoglobulin is found as two genetic variants, A and B. The amino acid sequence differs at two locations: aspartic acid 64 and valine 118 are replaced with a glycine and alanine residues, respectively (Morr C. V. & Ha E. Y. W., 1993).

β-Lactoglobulin exists as a 36.7 Kd dimer in solution above its isoelectric point (IEP) of pH 5.2. Between pH 3.5 and 5.2 the dimer polymerises into a 147 Kd octomer (Swaisgood 1982 & 1985) (Ebeler et al., 1990). Below pH 3.5 and above pH 7.5, the dimer and octomer dissociate to the monomer. β-Lactoglobulin has about 15, 43 and 47% α-helix, β-sheet, and unordered structure, respectively. It is pH and temperature sensitive. It undergoes time-dependent and temperature-dependent denaturation above 66 °C, which is accompanied by an extensive conformational transition that exposes highly reactive SH and E-NH_2 groups.

Unfortunately, the biological function of β-lactoglobulin is not known.

Immunoglobulin (Ig) refers to a heterogeneous family of glycoproteins ranging in size from 15 to 1000 kd that share common antibody activity (Eigel et al, 1984). It consists of four classes: IgG1 and IgG2, IgA, IgM and IgE, with about 80% of Ig being IgG (150 kd). These proteins exhibit a higher denaturation temperature than α-lactalbumin and β-lactoglobulin, but in the presence of other whey proteins they are extremely thermolabile.

Immunoglobulin has the ability to interact with and agglutionate milκ-fat globules (MFG) in cold milk and also bind bacteria and fatty materials in desalted whey (de Wit J. N., 1989).

BSA has a 69 kd molecular weight and consists of 582 amino acid residues (Eigel et al, 1984). It is the second longest single polypeptide chain of all whey proteins. BSA is soluble up to 35% at 3 °C in distilled water but precipitates (denaturises) seriously between 40 and 45 °C. BSA denaturise at pH 4, which is attributed to the mutual repulsion of positively charged amino residues along the polypeptide chain. BSA is more compact in the

C-terminal region than in the N-terminal region, and the different domains are dissimilar in hydrophobicity, net charge, and ligand binding properties.

BSA is a well-known transport protein for insoluble fatty acids in the blood circulatory system (de Wit J. N., 1989) (Spector A. A.,1975).

There is a small amount of κ-casein glycomacropeptide (GMP) in acid whey. It mainly exists in rennet and cheese whey. GMP is one of two distinct regions of κ-casein mainly located at the surface of the milk micelles. It is hydrophilic and able to stabilise the micelles and prevent them from coagulating. When rennet cheese is made milk clotting enzymes split κ-casein at the junction between the para-κ-casein and the macropeptide regions at the bond between the phenylalanine residue 105 and the methionine residue 106. GMP diffuses into the whey.

1.5 Aim of Purification of Whey Protein

The principal protein constituent of human milk is α-lactalbumin, which represents approximately 30% of total protein in this milk. Moreover, human milk contains negligible quantities of β-lactoglobulin which has been considered a potential allergen for infants. Bovine and human α-lactalbumin also show excellent amino acid and structural homology, with 72% amino acid sequence homology (Heine et al, 1991). Furthermore, α-lactalbumin is considered to be of higher value than β-lactoglobulin as they give protein efficiency ratio (PER) values of 4.0 and 3.5 respectively (the PER value of casein and egg are 2.8 and 3.9 respectively). These factors provide support for use of bovine α-lactalbumin in enhanced formulas for human infants.

β-Lactoglobulin has excellent heat-set gelation properties and should find immediate application in manufactured meats, fish products, and a variety of formulated foods. It also shows excellent whippability and provides a superior and cost-effective replacement for egg protein in some food applications. β-Lactoglobulin shows high solubility at low pH (> 97%, pH 3), and is stable to UHT treatment under these conditions. These properties of β-lactoglobulin allow its use as the active agent in protein-fortified acidic beverages, such as fruit juices and sports drinks, and in varieties of these beverages with long shelf-life (Pearce, 1991) (Pearce et al, 1991).

Because of the different composition of human milk and bovine milk, WPC has been added to the bovine milk to adjust the ratio of whey protein to casein. However, this can not

adjust the ratio of α-lactalbumin to β-lactoglobulin at all. Long-feeding on this whey protein-rich milk may lead to brain and liver damage (Heine W. E. et al, 1991).

A possible way to modify bovine milk is to add α-lactalbumin to match the ratio of α-lactalbumin to casein in human milk. To do so α-lactalbumin must be purified from whey, WPC, or WPI.

1.6 Purification of Whey Proteins

Although whey proteins have wide ranginging functional attributes for nutritional, biological, and food purposes their utilisation as individual proteins has not been widespread. One of the reasons is that viable industrial technologies are lacking for the isolation of individual whey protein species. Such technologies are an essential prerequisite if the unique properties of each protein constituent are to be exploited.

Because purified individual proteins exhibit unique and better functionality than in their native protein mixtures as mentioned previously there is great interest in developing easier methods to prepare pure whey proteins on large scale. α-Lactalbumin and β-lactoglobulin have such close molecular weights and isoelectric points that it is very difficult to separate them from each other. On the other hand, they are the most abundant protein species in whey so that major efforts to purify individual proteins have been focused on the separation of these two proteins from each other.

Current methods to purify α-lactalbumin or β-lactoglobulin involve:

A. Precipitation
B. Ion Exchange Adsorption.
C. Immobilised Metal Ion Affinity Chromatography

Almost all of the published sodium hydroxideature, using methods A and B, only prepared β-lactoglobulin, rather than α-lactalbumin which would be the more interesting and valuable product. A satisfactory method for the large scale purification of α-lactalbumin has not yet been found.

A. Precipitation

The technology of precipitation has been used to purify β-lactoglobulin and α-lactalbumin from whey. It is based on the different solubilities of α-lactalbumin and β-lactoglobulin in inorganic salt solutions. Temperature and pH affect the procedures.

Large scale thermal separation of β-lactoglobulin and α-lactalbumin from fresh defatted Cheddar cheese whey was described by Pearce (Okumura et al, 1990). The UF retentate of whey at pH 4.2 was heated at 65 °C for 30 minutes. α-Lactalbumin precipitated in very fine form and was separated by centrifugation. The α-lactalbumin was contaminated with Ig, BSA, and some unidentified highly aggregated materials. The supernatant liquid contained the remaining whey proteins and was enriched in β-lactoglobulin. Although the condition was claimed to be very mild, the process may cause denaturation of whey proteins. Heating caused denaturation of 60% of α-lactalbumin recovered by a pilot plant using this method.

Other precipitation processes were used to precipitate β-lactoglobulin. Ammonium sulphate (200-264 g/l) at pH 3.5 was the most usual salt solution used (Armstrong J. McD, et al, 1967) (Aschaffengurg R. & Drewry J., 1957). After the salts were removed by dialysis 1.3-2.1 g of β-lactoglobulin was recovered from 1 litre of whey. The yield of β-lactoglobulin could be increased to 3.5-4.25 g from per litre of whey by using TCA (34.2 g/l) (Fox et al, 1967). Acetone (25-33%) was also used to precipitate β-lactoglobulin from whey at low pH (Mehrens et al, 1983). The most successful separation of native β-lactoglobulin was possibly achieved by Mailliart and Rubadeau (1988). β-Lactoglobulin with high purity (84%) was recovered from whey by removing all other whey proteins at pH 2.0, using 7% NaCl.

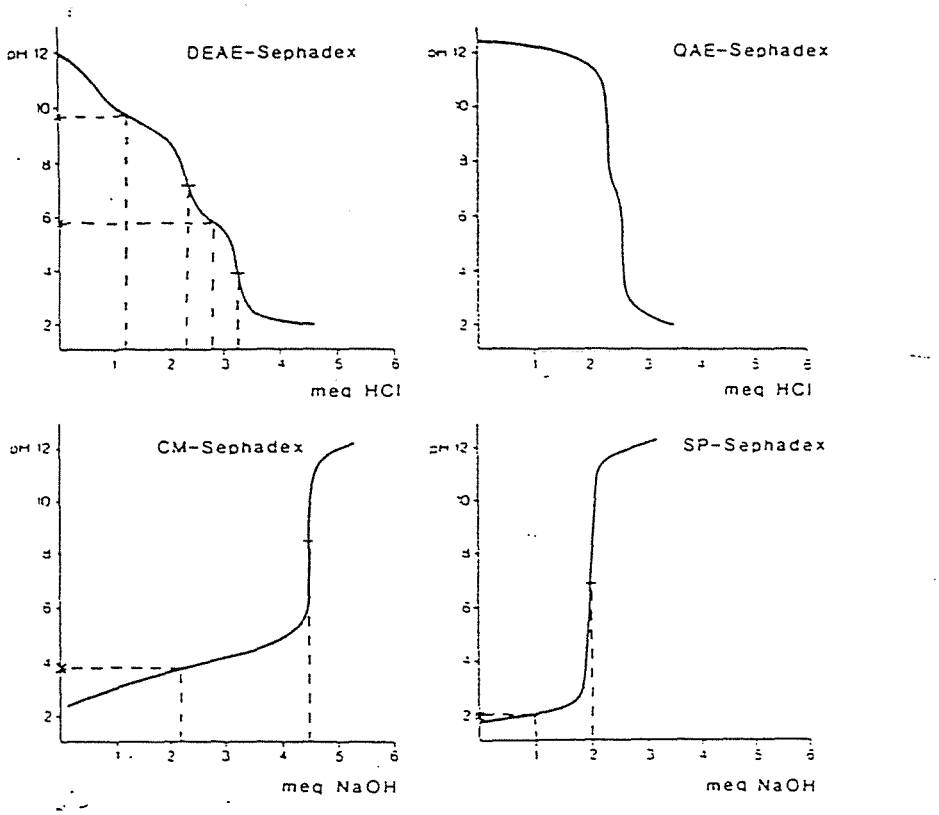
Although some of the fractions obtained by precipitation processes were rich in α-lactalbumin, its isolation with high purity from such fractions could not be achieved readily by salting-out procedures. Even the crystallised forms of α-lactalbumin and β-lactoglobulin were not completely homogeneous according to the more critical methods of detecting impurities; hence various chromatographic techniques have been developed as final purification steps.

Table 1.3 Functional groups used in ion exchangers(Ryden, 1989).

Name	Designation	pK	Structure
Anion exchangers			
Diethyl aminoethyl	DEAE	9.0 to 9.5	$-OCH_2CH_2NH^+(C_2H_5)_2$
Trimethyl hydroxypropyl	QA		$-OCH_2CH(OH)N^+(CH_3)_3$
Quaternary aminoethyl, diethyl-(2-hydroxypropyl) -aminoethyl	QAE		$-OCH_2CH_2N^+(C_2H_5)_2CH_2CH(OH)CH_3$
Quaternary aminomethyl	Q		$-OCH_2N^+(CH_3)_3$
Triethyl aminomethyl	TEAE	9.5[a]	$-OCH_2N^+(C_2H_5)_3$
Triethylaminopropyl	TEAP		$-OCH_2CH_2CH_2N^+(C_2H_5)_3$
Polyethyleneimine	PEI		polymerized $CH_3—CH=NH$
Cation exchangers			
Methacrylate		6.5	polymerized $CH_2=C(CH_3)COOH$
Carboxymethyl	CM	3.5 4	$-OCH_2COOH$
Orthophosphate	P	3 and 6	$-OPO_3H_2$
Sulfonate	S	2	$-OCH_2SO_3H$
Sulfoethyl	SE	2	$-OCH_2CH_2SO_3H$
Sulfopropyl	SP	2-2.5	$-OCH_2CH_2CH_2SO_3H$

[a] The pK value apparently does not refer to quaternary groups

Figure 1.2 Titration curves for ion exchangers used in protein chromatography (Ryden, 1989).



B Ion Exchange

Ion exchange adsorption is one of the common chromatographic techniques to purify individual proteins. Some functional groups are coupled directly or indirectly to an insoluble matrix. These functional groups are balanced by counter-ions which can be exchanged by other ions with same property of charges. Depending on the property of the counterion exchanged, ion exchangers can be divided into anion exchangers in which anions are exchanged and cation exchangers in which cations are exchanged. Depending on the relationship of charges and pH, ion exchangers can be divided into strong ion exchangers which hold their charges over the whole range of pH and weak ion exchangers which lose whole or part of their charges when pH is higher than a certain value for anion exchangers and lower than a certain value for cation exchangers. The classical ion exchangers were diethyaminoethyl (DEAE) and carboxymethyl (CM) derivatives (Sober H. A. & Perterson E. A., 1954 & 1956) (Sober H. A., et al 1956). They are weak ion exchangers. More derivatives have been introduced to make strong ion exchangers which allow the adsorption process for proteins to occur over a wider pH range. The most common of these are the quaternary ammonium derivatives for the anion exchangers and the sulfonate derivatives for the cation exchanger. These and other derivatives are listed in Table 1. 3. The titration curves of the four most common derivatives are shown in Figure 1.2.

Proteins bind to ion exchangers by electrostatic force between the proteins' surface charge (mainly) and the dense clusters of charged groups on the exchangers. The interaction of proteins and ion exchangers depends on the type of ion exchangers, pH, ionic strength, net charge and distribution of charges on the surface of the protein (Karlsson E., et al). A protein molecule carries strong net positive charge at a pH much lower than its isoelectric point and is able to bind to cation exchangers. It carries strong net negative charge at pH much higher than its isoelectric point and is able to bind to anion exchangers. It is probably able to bind to cation exchangers at a pH a little higher than its isoelectric point or bind to anion exchangers at a pH a little lower than its electric point because of uneven distribution of charges on proteins.

Both cation and anion exchangers have been used to recover whey proteins from whey on a commercial scale. Their utilisation in purifying individual whey proteins has been reported too. Strong and weak anion exchangers such as DEAE (Armstrong et al, 1967) (Mckenzie et al, 1971), QMA (Skudder, 1995), QAE (Imafidon et al, 1992) were used to bind β-lactoglobulin rather than other whey proteins in low concentration of salt at pH 6-8.

C. Immobilised Metal Ion Affinity Chromatography (IMAC)

Immobilised metal ion affinity chromatography (IMAC) recovered 80% of the α-lactalbumin in whey with 90% purity (Blomkalns et al, 1997), using stationary phases that chelate selected metal ions (copper ion for α-lactalbumin) that have affinities for specific amino acid residues (histidine, cysteine and Tryptophan) in proteins. β-Diketoamine as stationary phase chelates Cu^{2+} much more strongly than iminodiacetate and thus it had much higher capacity, 86 mg/g, than the latter (less 10 mg/g). The advantage of this separation technique over precipitation and ion exchange adsorption is to bind α-lactalbumin rather than other whey proteins and thus purer α-lactalbumin was obtained. Unfortunately, the fact that copper ion chelated on the resins could be released to solution so readily that the α-lactalbumin obtained was blue (Chilcott, 1996), because of the contaminant copper ion, and that imidazole was used to elute α-lactalbumin from the resin, precluded the commercial application of this technique for the purification of α-lactalbumin for food use.

The chelation reactions are summarised in Figure 1.3

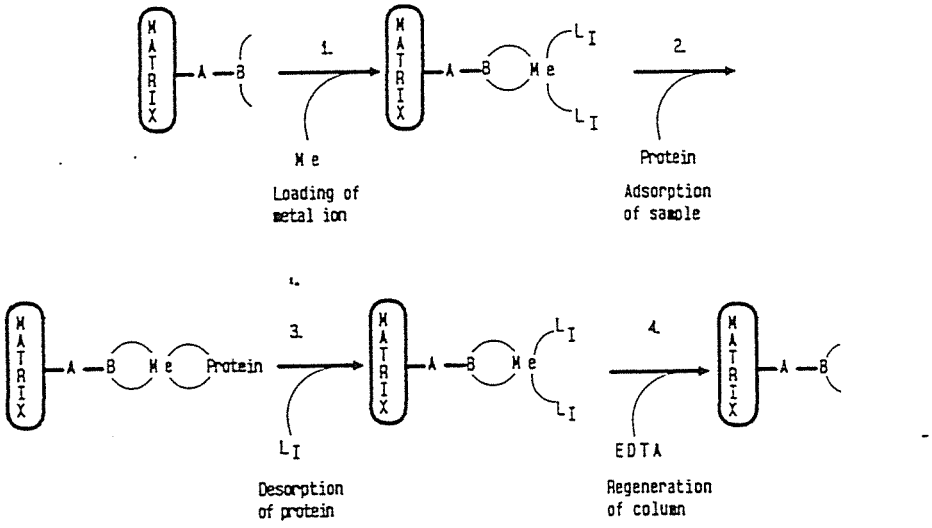


Figure 1.3. Principle of immobilized metal ion affinity chromatography. A = linkage (spacer) group, B = chelating group, Me = metal ion, L_I = solvent or buffer molecule.

1.7 Hydrophobic Interaction Chromatography (HIC)

Hydrophobic molecules in an aqueous solvent will self-associate. This association is due to hydrophobic interaction. The driving force of hydrophobic interaction is the high surface tension among polar water molecules which forces new hydrogen bonds to be formed when old bonds are broken when apolar solute is added into water. According to this, hydrophobic interaction chromatography (HIC) has been developed as a technique to purify proteins in the last decades. Its principle is based on the hydrophobic interaction between hydrophobic functional groups, for example alkyl or aryl groups coupled to matrix and the apolar branch chains of amino acid residues in proteins in media favouring hydrophobic interaction, eg., an aqueous solution with a high salt concentration.

The first gels of practical use for HIC were of a mixed hydrophobic-ionic character (Yon, 1972) (Er-el et al, 1972) (Hofstee, 1973). Neutral adsorbent (alkyl and aryl ethers) were later prepared by Porath et al (1973). and Hjerten et al (1974), the latter leading to the introduction of the commercial products Octyl- and Phenyl-Sepharose.

Some typical HIC-gels are A to E:

A [M]-O-C(NH_2^+)-$NHCH_2(CH_2)_6CH_3$

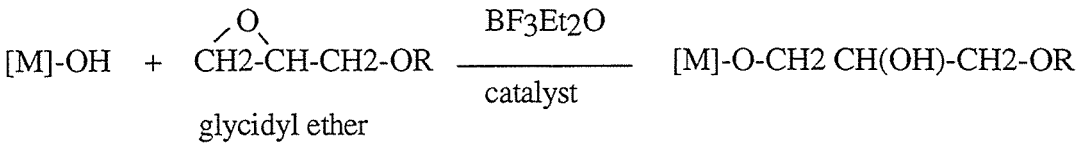
B [M]-O-CH_2-CH(OH)CH_2-O-$CH_2(CH_2)_6CH_3$

C [M]-O-CH_2-CH(OH)CH_2-O-C_6H_6

D [M]-O-Si(OH)-$(CH_2)_3$-O-CH_2CH(OH)$CH_2(CH_2)_6CH_3$
[M]-O

E [M]-O-CH_2CH(OH)CH_2-O-$(CH_2)_4$-O-CH_2CH(OH)CH_2-S-$(CH_2)_6CH_3$

A, B, and C are commercially available. All of A to E (except C) are octyl-gel. A was prepared by activating agarose by cyanogen bromide and then coupling with an alkylamine (Axex et al, 1967). It is not a pure HIC gel but an anion exchanger with positive charge. The others are pure HIC gel. The synthesis of B and C were base on the glycidyl ether (with an epoxide, oxirane, functional group) coupling procedure (also used for the production of Octyl- and phenyl-Sepharose) (Hjerten,1974). Because this coupling method is the most widely used, it is shown in the following formula:



D was introduced recently. It was prepared by activating agarose by a r-glycidoxypropyltrimethoxy silane in water then coupling hydrophobic ligand (Hjerten et al, 1986). E was prepared by activating agarose with a bis-epoxide, 1,4-butanediol diglycidyl ether and then coupling with an alkyl mercaptan (Maisano et al, 1985).

Because HIC gels have lower substitution levels of hydrophobic ligands (about 40 u meq/ml gel bed for commercially available Octyl- and Phenyl-Sepharose) than reversed phase chromatogrphy (RPC), its ligands are considered individual rather than continuous as in the case of RPC. Therefore, HIC is a more general technique so that the denaturation of protein molecules does not usually occur when it is applied for the separation of proteins.

Four factors of great importance for HIC are: the type and concentration of salt used; the additives which change the polarity of the solvent; temperature; and pH.

A. High salt concentration promotes hydrophobic interaction. The effectiveness of the ions is in the following order: (Hjerten, et al, 1977).

Anion: $SO_4^{2-} > Cl^- > Br^- > NO_3^- > ClO_4^- > I^- > SCN^-$

Cation: $Mg^{2+} > Li^+ > Na^+ > K^+ > NH_4^+$

B. Apolar additives, such as ethanol, decrease the hydrophobic interactions between proteins and ligands.
C. Hydrophobic interactions are promoted by temperature increase.
D. Most hydrophobic interactions are promoted by decreasing pH.

Protein adsorption capacities of HIC matrixs are high, in the same range as that for ion exchangers, so they are suitable for large scale use. Unfortunately the cost of the matrixs is high which means they can only be used for the laboratory purification or for high value products.

A number of publications have investigated the hydrophobicity of α-lactalbumin and β-lactoglobulin. α-Lactalbumin was discovered to interact strongly with the apolar regions of phospholipid vesicles (Hanssens et al, 1983,1981) and to bind to alkyl-agaroses with

hydrocarbon chains of 4-8 carbon atoms (Jost, 1974). α-Lactalbumin binding on octyl agarose could not be eluted with 1 M NaCl. Although the proteins were possibly eluted by using detergent, the detergent were not often accepted by food industry so that there are problems with trying to purify the proteins using HIC.

1.8 Preparation of Quaternary Amino Anion Exchangers

Anion exchanger derivatives are usually prepared by attaching the charged organic molecules directly to the matrix via an ether linkage which is stable to acid and alkali (Peterson & Sober, 1974), eg.

$$[C]\text{-}OH + ClCH_2CH_2NEt_2 \xrightarrow{NaOH} [C]\text{-}O\text{-}CH_2CH_2NEt_2$$

Cellulose — Diethylaminoethyl-Cell (DEAE-Cell)

DEAE-Cell is a weak base exchanger. Porath treated DEAE-Cell with ethyl bromide to attempt obtaining strong quaternary ammonium anion exchanger. However little quaternization was actually achieved (Peterson, 1970).

$$[C]\text{-}O\text{-}CH_2CH_2NEt_2 + EtBr \longrightarrow [C]\text{-}O\text{-}CH_2CH_2NEt_3^{+}Br^{-}$$

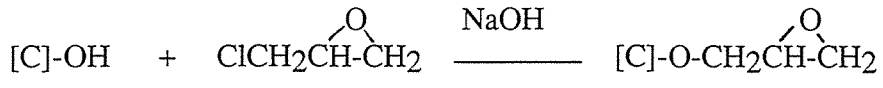
Strong base anion exchanger

To obtain quaternary amino anion exchangers with satisfactory substitution levels some quaternary ammonium salts were synthesed first and then coupled with cellulose matrix. Two of these salts were 2,3-epoxypropyltrimethylammonium chloride and 3-chloro-2-hydroxypropyl-trimethylammonium chloride (Hellwig, 1992) (Egan, !994) (Japanese Patent 54042385). In this way a substitution level of 1.0 meq/g could be attained in a single processing cellulose.

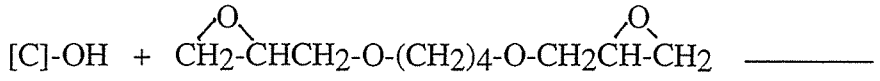
All of these methods put on secondary and tertiary amines first and then quaternized or then put on quaternary amino groups directly via an alkylating agent bearing the quaternary groups.

A third method involved activating a cellulose matrix first with functional reagents and then coupling amines. Epichlorohydrin (ECH) (Ayers, 1980) and 1,4-butanediol diglycidyl ether (Bisoxiranes) (Sundberg & Porath, 1973) were used to add active epoxy groups to the matrix. These groups were then coupled with various amines to form weak or strong base anion exchangers. Because the immobile ligands contain only ether bonds they are stable in acidic and basic solution. So in this work we select epichlorohydrin (ECH) and 1,4-butanediol diglycidyl ether as activating reagents. The reactions are displayed below:

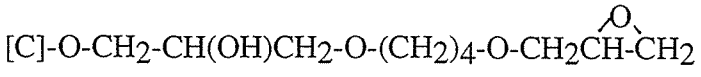
Activation:



Matrix Activated matrix



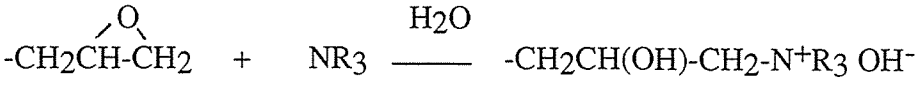
Matrix



Activated matrix

Coupling:

Activated matrix



Activated matrix Quaternary anion exchanger

Conversion:

$$-CH_2CH(OH)-CH_2-NHR_2 \xrightarrow{HCl} -CH_2CH(OH)-CH_2-N^+HR_2\ Cl^-$$

Weak basic anion exchanger

$$-CH_2CH(OH)-CH_2-N^+R_3OH^- \xrightarrow{HCl} -CH_2\ CH(OH)CH_2N^+R_3\ Cl^-$$

Stronger basic anion exchanger

By this method anion exchangers are able to be synthesised from common chemicals rather than special alkylating reagents.

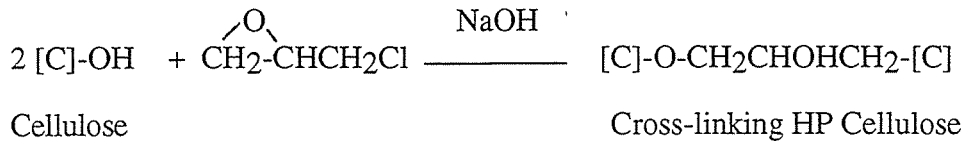
The cellulose ion exchangers for proteins were introduced in 1954 by Sober (1954) (1956) and Perterson (1956). These exchangers have been limitation because of their fibrous structure. They generally have low flow rates, become easily clogged by suspended particles and produce fines easily when handled. Chemically crosslinked regenerated cellulose was used as matrix by Grant (1968). This cellulose was robust, with low attrition properties and high flow rates but relatively few binding sites for proteins. Some efforts were done to increase its binding sites.

Propylene oxide and epichlorohydrin were used to swell and cross-link regenerated cellulose by Ayers. J. (1980) and Lilly M. J (1988). The modified cellulose was semi-rigid and suitable for large scale use. The reaction formulas are described as follows:

$$[C]\text{-}OH + CH_2\text{-}CHCH_3 \xrightarrow{NaOH} [C]\text{-}O\text{-}CH_2CH(OH)CH_3$$

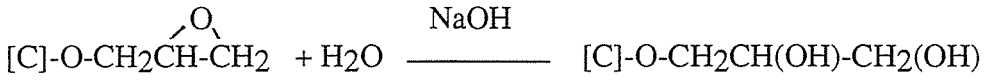
Cellulose

Hydroxypropyl group
HP Cellulose

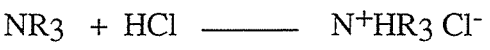


The swollen cellulose was then activated by epichlorohydrin. Finally, the activated cellulose was coupled with amines to make anion exchangers as shown in the activation and coupling reaction on the previous pages.

When the modified cellulose was activated in the way in the presence of sodium hydroxide, epoxy groups are hydrolysed by the sodium hydroxide hydrolysed (shown below). To reduce this hydrolysis a low activation temperature and excess epichlorohydrin were used.



When activated cellulose was coupled with amine, the high pH of the aqueous solution of amine also hydrolysed epoxy groups on the cellulose. To reduce this hydrolysis hydrochloric acid was added to lower the pH of slurry. The amount of hydrochloriic acid added was sufficient to neutralise about 10% of the amine present. This significantly reduced the pH without changing the concentration of the amine and thereby favoured the coupling reaction over the hydrolysis so that it was quantitative.



1.9. Aim of the thesis

α-Lactalglobumin was able to bind to hydrophobic ligand (Jost et al, 1974). This work hopes to take the advantage of ion exchangers, eq. desorbility of proteins by acid or alkaline, and of HIC, e. q., high selectivity. A series of quaternary anion exchangers with long alkyl chains will be investigated if they have higher selectivities for α-lactalbumin than β-lactoglobulin in isolating former from whey proteins and if they are able to provide native α-lactalbumin on the food industrial scale.

2 Materials and Methods

2.1 Materials

Deionised water was used unless otherwise stated. Deionised water was made with MILLI-Q REAGENT SYSTEM WATER.Other chemicals were obtained from the following supplier:

Sepharose 4B-200 (40-150 um)	Sigma Chemical Company
VDC cellulose (40-100 um)	Phoenix Chemicals Ltd
VDC cellulose (70-150 um)	Phoenix Chemicals Ltd
VDC cellulose (150-210 um)	Phoenix Chemicals Ltd
VDC cellulose (250-300 um)	Phoenix Chemicals Ltd
VDC cellulose (300-350 um)	Phoenix Chemicals Ltd
NaOH (AR)	Rhone-Poulenc Ltd
NaCl (AR)	Ajax Chemicals
Epichlorohydrin (ECH)	Dow
HCl (AR)	B.D.H.
N,N-Dimethyloctylamine	Aldrich
N,N-Dimethylhexylamine	Aldrich
1-Dodecylamine	Ega
Sodium acetate	Ajax Chemicals
Whey protein isolate:	
WPI (MP0162) and WPI (PT8253)	New Zealand Dairy Research Institute
Cheese whey retentate	Kiwi Dairy company (NZ)
α-Lactalbumin (α-la)	New Zealand Dairy Research Institute
β-Lactoglobulin (β-lg)	New Zealand Dairy Research Institute
Tris	Sigma
1,4-butanediol diglycidyl ether (70%)	Sigma

2.1.2 Equipment

8452A Diode Array Spectrophotometer	Hewlett Packard
VIT90 Video Titrator	Radiometer Copenhegen
Smart System & mono-Q column	Pharmacia
LCC-500 (FPLC)	Pharmacia
Econo System	Bio-Rad

2.2 Experimental Methods

2.2.1 Preparation of Quaternary Amino Anion Exchangers with a Sepharose Matrix.

To prepare quaternary amino anion exchangers on an agarose matrix, Sepharose 4B was first activated and then coupled with tertiary amines. The detailed procedures are described as follows.

2.2.1.1 Activation of Sepharose 4B

Sepharose 4B-200 was washed extensively with water and then drained of excess water under vacuum filtration. Twenty grams of this moist Sepharose was then transferred into a 100 ml jar. Water (14.4 ml) and NaOH (3.6 ml of 30%) were added to make a thick slurry. The jar was cooled to below 5 °C in an ice bath before 2 ml of epichlorohydrin was added into it. The jar was then sealed and tied to a wheel which was then rotated so that the jar was turned end-over-end to mix the contents. This was continued overnight at room temperature. After that, the slurry was transferred to a sintered glass filter and washed thoroughly with water to remove excess epichlorohydrin and other soluble products. The Sepharose was then drained of excess water. By this process a Sepharose matrix, with an activation degree of 1.2-1.3 meq/g (dry matrix), was obtained.

2.2.1.2 Coupling

The activated Sepharose was transferred to a 100 ml jar. Ethanol (20 ml), N,N-dimethyloctylamine (2.0 ml), and 1 M HCl (2 ml) were first mixed and then added into the jar. The jar was then sealed and tied to a wheel which was then rotated so that the jar was turned end-over-end to mix the contents. This was continued overnight at room temperature. This was followed by heating at 70 °C for three hours in a water bath with only occasional shaking of the jar by hand. The jar was then cooled to room temperature and the mixture was transferred to a sintered glass filter. The Sepharose was washed with 100 ml of solution containing 50 ml of ethanol and 50 ml of 1 M HCl, followed by 50 ml of 1M HCl to remove the access amine. The Sepharose was then washed thoroughly with water until the pH of the washing was neutral to pH 6-8 indicator paper. The exchanger was then drained of excess water before it was stored in a sealed jar in a fridge. The subsititution level of the exchanger (DMO-Seph) was about 1.1 meq/g (dry exchanger).

To obtain other quaternary amino anion exchangers from Sepharose, N,N-dimethylethanolamine (DMEA), N,N-dimethylhexylamine (DMH), N,N-dimethyloctylamine (DMO) and N,N-dimethyldecylamine (DMDo) were coupled to further batches of activated Sepharose (prepared as in Section 2.2.1.1) by the same coupling process. To maintain the mole ratio of amine to epoxy groups on the activated Sepharose, different volumes of the amines were used. Water (20 ml) and 50% ethanol (20 ml) were used respectively to make thick slurries for DMEA-Seph and DMH-Seph. The chemicals used are summarised in Table 2.1.

2.2.1.3 Reprocessing of DMO-Sepharose

To obtain DMO-Seph with substitution levels above 1.1 meq/g DMO-Seph with substitution level 1.1 meq/g was used as the starting matrix. It was activated with epichlorohydrin and coupled with DMO as described for Sepharose 4B in Sections 2.2.1.1 and 2.2.1.2.

2.2.2 Preparation of Secondary Amino Anion Exchanger with a Sepharose Matrix

The primary amine dodecylamine (Do) was attached to Sepharose 4B using the same process as that for DMO-Seph. The chemicals used are shown in Table 2.1.

2.2.3 Synthesis of a Series of Dimethyloctylamino Anion Exchangers with a Cellulose Matrix

To make quaternary amino anion exchangers with a cellulose matrix a three stage reaction sequence was required. First, the cross-linked hydroxylpropyl (HP) cellulose matrix was prepared. The HP cellulose matrix was then activated. Finally the activated HP cellulose was coupled with tertiary amines. The detail processes was as follows.

2.2.3.1 Modification of Cellulose with Epichlorohydrin and Propylene Oxide

Cellulose was cross-linked with epichlorohydrin and propylene oxide in the presence of NaOH. The reactions were described in the Introduction. The procedure for the preparation and some factors were discussed by J. S. Ayers (1980). Thus, to synthesise a modified cellulose HP8-50 matrix using 8% cross-linking agent and 50% propylene oxide (volume/weight of cellulose), NaOH (30 ml of 30%) below 5 °C and a solution consisting

of 1.6 ml of epichlorohydrin and 10 ml of propylene oxide were simultaneously added to 20 g of cellulose powder in a stainless steel bomb below 5 °C. All reagents were mixed thoroughly with a stainless steel spatular. The bomb was then sealed and stirring was commenced immediately to allow the liquids to wet all of the powder before it swelled, particularly that at the bottom of the bomb. Stirring was continued 2-3 minutes. The bomb was heated in a water bath at 45 °C for 1.5 hours. The bomb was then cooled to room temperature in a cold water bath for half hour.

To obtain the modified celluloses using 4 and 6% cross-linking agent, ie., HP4-50 and HP6-50 cellulose, 0.8 and 1.2 ml of epichlorohydrin respectively were used for 20 g of cellulose. All other processes and chemicals were exactly same as those for HP8-50 cellulose.

Table 2.1 Synthesis of Sepharose Anion Exchangers

Exchanger	DMEA-Seph	DMH-Seph	DMO-Seph	DMD-Seph	Do-Seph	DMDo-Seph
Chemical						
Activation						
ECH (ml)	3.0	3.0	3.0	3.0	3.0	3.0
30% NaOH (ml)	3.6	3.6	3.6	3.6	3.6	3.6
water(ml)	14.4	14.4	14.4	14.4	14.4	14.4
Sepharose (g)	20	20	20	20	20	20
Coupling						
amine (g)	0.89	1.29	1.54	1.85	1.85	2.13
water (ml)	20.0	0.00	0.00	0.00	0.00	0.00
ethanol (ml)	0.00	20.0	20.0	20.0	20.0	20.0
1M HCl (ml)	1.0	1.0	1.0	1.0	1.0	1.0
Time						
(hours)						
Activation	16	16	16	16	16	16
Coupling						
at 21 °C	16	16	16	16	16	16
at 70 °C	3	3	3	3	3	3

2.2.3.2 Activation of HP cellulose matrix

The cross-linked cellulose from 2.2.3.1 contained about 8 g of excess sodium hydroxide. The excess NaOH could be used for the preparation of activated matrix of ion exchangers with high substitution level. The method of activation was as given by Lilly, M. J (1988). Thus, water (240 ml), NaOH (10 ml of 30%) and the unwashed modified HP 8-50 cellulose containing about 8 g of excess NaOH were mixed and cooled down to below 5 °C in a 500 ml jar. Epichlorohydrin (28 ml) was added into the jar. The jar was sealed and rotated on a wheel slowly at 4 °C overnight. Further reaction was allowed at room temperature for 4 hours. The activated cellulose was transferred to a sintered glass filter, washed with excess water and finally drained. The activated HP8-50 cellulose matrix had an activation level of about 1.2 meq/g (dry resin).

The activations of cellulose HP4-50 and HP6-50 were similar to that for HP8-50 cellulose. When the slurries of HP4-50 and HP6-50 celluloses were made more water than that for HP8-50 cellulose was used since cellulose with low cross-linkage had a greater swollen volume. To maintain the concentration of NaOH constant and the ratio of epichlorohydrin to NaOH constant more NaOH and epichlorohydrin had to be added to the cellulose activation. All other processes were the same as those for HP8-50. The chemicals are shown in Table 3.10.

To obtain activated HP8-50 celluloses with low activation levels the amount of NaOH remaining in the HP 8-50 cellulose was greater than that required for further reaction. Thus, HP8-50 cellulose with excess NaOH was transferred to a filter, washed with excess water until the washing was neutral to pH 6-8 indicator paper. The resin was then drained of excess water. It was then divided into four even parts. Each part was transferred into a reaction jar. Sufficient water was added into it to make a thick slurry. NaOH (30%) was added to the slurry to make up the required concentration of NaOH. Epichlorohydrin (25% excess) was added after the slurry was cooled to below 4 °C in an ice bath. All other processes were the same as those for the activated HP8-50 cellulose with substitution level 1.2 meq/g as described above in this section.

2.2.3.3 Preparation of Dimethyloctyl amino Anion Exchangers from Activated HP Cellulose

Tertiary amines can be coupled to the activated HP cellulose. Thus the moist activated HP8-50 cellulose (150 g) with activation level 1.2 meq/g was added to a 500 ml jar containing a

mixture of ethanol (120 ml), N,N-dimethyloctylamine (33 ml) and HCl (3 ml of 5 M). The jar was sealed and placed on a roller. The reaction was maintained at room temperature over night, followed by 2-3 hours at 70 °C in a water bath. After cooling to room temperature, the exchanger was transferred to a sintered glass filter. It was then washed with 400 ml of solution containing 200 ml of ethanol and 200 ml of 1 M HCl, followed by 400 ml of 1 M HCl, followed by water until the washing was neutral to pH 6-8 indicator paper. The exchanger was then drained of excess water before it was stored in a sealed jar in a fridge. The subsititution level of the exchanger was 0.94 meq/g.

Those exchangers with low substitution levels were made of HP8-50 celluloses with low activation levels. The chemicals used are summarised in Table 3.10.

2.2.3.4 Reprocessing of DMO-Cell

To obtain DMO-Cell with substitution levels above 1.1 meq/g DMO-Cell with substitution level 0.94 meq/g was used as starting matrix. It was activated with epichlorohydrin again and coupled with DMO as described for the preparation of DMO-Cell with substitution level 0.94 meq/g from HP8-50 cellulose.

2.2.4 Preparation of DMO-Cell using 1,4-Butanediol Diglycidyl Ether

To obtain this exchanger cellulose was first modified with epichlorohydrin and propylene oxide in presence of NaOH to prepare HP8-50 cellulose. HP8-50 cellulose was then activated by 1,4-butanediol diglycidyl ether. The activated cellulose was finally coupled with N,N-dimethyloctylamine. The details for the latter two stages are as follows.

2.2.4.1 Activation of HP8-50:

The procedure followed the instruction of Maisano, F. with minor modifications. Thus thoroughly washed and suction-dried HP8-50 cellulose (10 g) was mixed with 10 ml of 1.8 M NaOH in a glass jar. The jar was cooled down to below 5 °C in an ice bath before 60 mg of sodium borohydride was added as well as 20 ml of 70% 1,4-butanediol diglycidyl ether. The jar was then sealed and turned end-over-end slowly to mix the contents at room temperature overnight. The slurry was transferred to a sintered glass filter and the activated cellulose was then washed thoroughly with water, drained and transferred back to a glass jar.

2.2.4.2 Coupling

The drained activated cellulose was then added to a mixture of 8 ml of ethanol, 2 ml of N,N-dimethyloctylamine and 1 ml of 1 M HCl. The jar was sealed and turned end-over-end slowly to mix the contents. The reaction was continued at room temperature overnight, followed by 2-3 hours at 70 °C. After cooling to room temperature, the exchanger was transferred to a sintered glass filter where it was then washed with 50 ml of solution containing 25 ml of ethanol and 25 ml of 1M HCl. This was followed by 30 ml of 1M HCl and then water until the washing was neutral to of pH 6-8 indicator paper. The exchanger was then drained of excess water before it was stored in a sealed jar in a fridge. The subsititution level of the resin was 0.61 meq/g.

2.2.5 Determination of Dry Matter Content of Ion Exchangers

A sample of drained Sepharose or cellulose exchanger (4 or 2 g respectively) was weighed accurately in a accurately weighed 5 ml-beaker. The beaker was then placed in an oven at 60 °C for 2 hours. The temperature was then increased to 105 °C for another two hours. The beaker was then cooled to room temperature in a desiccator. It was then weighed. The process of heating at 105 °C was continued until the beaker weight was constant. The dry matter content of the exchanger was calculated as follows:

$$\text{Dry Matter Content (\%)} = 100 * (W_2 - W_1) / M$$

W_2: Weight of beaker with dried resin (g)
W_1: Weight of beaker (g)
M: Weight of wet resin (g)

2.2.6 Analysis of Activation Level.

The determination of epoxy activation level was described by Koh. T. S. (1985). Sodium sulphite (0.25 g) and sodium metabisulphite (0.25 g) in water (10 ml) were added to a sample of the moist activated cellulose (3g) or Sepharose (5 g). These were mixed overnight at room temperature. The sample was washed with water, followed by 1M HCl (ca. 50 ml) to transfer the Na^+ form of the resin into the H^+ form, followed by water until the washing was neutral. It was then dispersed in 0.5 M NaCl (ca.10 ml) and titrated with 0.1 M NaOH to pH 8. The sample was transferred to a weighed sintered glass filter and

washed thoroughly with water before being dried at 60 °C overnight, followed by at 105 °C for about 2 hours. The filter with dried sample was weighed again. This process was carried on until the weight of the filter was constant. The activation level was calculated from the following equation.

Activation Level (meq/g) = N * V / W

N: Concentration of NaOH (moles/litre)
V: Volume of NaOH used (ml)
W: Weight of Dry Resin (g)

2.2.7 Determination of Substitution Level of Anion Exchangers

The analysis of an amino anion exchanger was as described by Lilly, M. J. (1988). A sample (about 5 g wet weight) of an anion exchanger was washed with 1 M NaOH to transfer it into the hydroxide form, followed by water until the washing was neutral to pH 6-8 indicator paper. The exchanger was then transferred to a titration vessel and covered with 10 ml of 0.5 M NaCl. The hydroxide ion was disassociated from the resin by chloride ion and titrated by 0.1 M HCl to pH 4. The exchanger was transferred by washing to a dry sintered glass filter of known weight and washed thoroughly with water. It was dried at 60 °C overnight and further dried at 105 °C for 2-3 hours to constant weight. The substitution level was calculated as follows:

Substitution Level (meq/g) = N * V / W

N: Concentration of HCl (moles/litre)
V: Volume of HCl used (ml)
W: Weight of Dry Exchanger (g)

2.2.8 Determination of Swollen Volume

A moist exchanger (3 g) was placed in a 10 ml-measuring-cylinder, topped up with water and allowed to settle for 48 hours. The cylinder was tapped occasionally until a constant volume was recorded. The swollen volume of the resin was calculated as follows:

Swollen Volume (ml/g) = Volume of Resin / Dry Weight of Resin

2.2.9 Determination of Capacity of Exchangers for Individual Proteins in 25 mM NaCl

To set up a batch adsorption capacity test in 25 mM NaCl, α-lactalbumin or β-lactoglobulin (200 mg) was dissolved in 19.6 ml of water by stirring gently. NaCl (0.4 ml of 5 M) was added to the protein solution to make up an approximate 1% protein stock solution in 100 mM NaCl. The pH of the solution was adjusted with 1 M or 0.2 M HCl. The wet anion exchanger (0.25 g), water (7.5 ml) and the protein stock solution (2.5 ml) were added to a 25 ml bottle. This made up 10 ml of 0.25% protein solution in 25 mM NaCl for the batch capacity test. The bottle was sealed and mixed end-over-end on a slowly rotating wheel. After mixing for 1 hour for Sepharose and 2 hours for cellulose, the pH of the solution was recorded. The exchanger was allowed to settle for 15 minutes. The supernatant was filtered through a dry Pierce filter (This filtration was not necessary for the celluloses because of their high densities and large particle size). A sample of the filtrate (1 ml) was diluted with 4 ml of water and its UV absorbence (A_1) measured at 280 nm. Another 2.5 ml of the 1% protein stock solution was mixed with 7.5 ml of water and 1 ml of sample of this diluted similarly with 4 ml of water. Its UV absorbence at 280 nm (A_2) was measured. The capacity of the exchanger was calculated as follows:

$$\text{Capacity} = (A_2 - A_1) / (2 * C * M * D)$$

Capacity:	gram of bound protein / gram of dry exchanger
C:	Equal to 20.1 for α-lactalbumin and 9.6 for β-lactoglobulin
M:	Weight of wet exchanger (g)
D:	Dry matter content of exchanger (%/100)

2.2.10 Determination of Capacities of Exchangers for Individual Proteins in High Ionic Strength

To measure the capacities of the exchangers for α-lactalbumin or β-lactoglobulin at high NaCl concentration the 0.25% protein solution was prepared by either of two methods.

Method 1.
α-Lactalbumin or β-lactoglobulin (200 mg) was dissolved in 19.6 ml of water by stirring gently. NaCl (0.4 ml of 5 M) was added to prepare a clear 1% stock solution in 25 mM NaCl. The pH of the solution was adjusted to the required value with 5, 1 and 0.1 M HCl.

NaCl (4 ml of 1 M) was mixed with 3.5 ml of water first to make up 7.5 ml of salt solution. This solution was mixed with 2.5 ml of 1% protein stock solution at 25 mM NaCl. By this method it was convenient to prepare 0.25% protein solution in 400 mM NaCl over a pH range. However, the mixture pH of the exchanger and the protein solution must be measured after adsorption.

Method 2.
α-Lactalbumin or β-lactoglobulin (200 mg) was dissolved in 40 ml of water by stirring gently to prepare a 0.5% protein stock solution without salt.

NaCl (6.4 ml of 5 M) was diluted with 33.6 ml of water to make an 800 mM NaCl solution. The salt solution was mixed with an equal volume of 0.5% protein stock solution by stirring gently. The pH of the mixture was adjusted to the required value with 5, 1 and 0.1 M HCl. This method was especially suitable in summer when the proteins were readily salted out. By this method it was convenient to prepare a 0.25% protein solutions in 400 mM NaCl at the same pH.

Preparation of protein solutions in other ionic strengths at other pHs used either of these two methods.

Determination of the capacities of exchangers for the proteins in high ionic strength was same as that used in 25 mM NaCl.

2.2.11 Batch Recovery of α-Lactalbumin

All WPI used in this research was batch MP0162 from New Zealand Dairy Institute unless otherwise stated.

A WPI stock solution (1 or 4%) in 25 mM NaCl was prepared according to the method for 0.25% α-lactalbumin stock solution in 25 mM NaCl described in the previous section. Thus, a WPI powder (1 g for 1% solution or 4 g for 4% solution) was dissolved in 99 (for 1% solution) or 96 ml (for 4% solution) of water by stirring gently, followed by adding 0.5 ml of 5 M NaCl. The solution pH was adjusted with 5 and 1 M HCl to the required value.

WPI solutions in other low salt concentrations at other pHs were prepared by a similar method. Vigorous stirring was avoided during the whole process to prevent the proteins from denaturation, especially at pH close their isoelectric points.

A WPI stock solution (1 or 4%) in 400 mM NaCl was prepared according to the second method in Section 2.2.10 described for 0.25% α-lactalbumin stock solution in 400 mM NaCl. Thus, WPI powder (1 g for 1% solution or 4 g for 4% solution) was dissolved in 60 ml of water by stirring gently. The solution pH was adjusted with 5 and 1M HCl to the required value, for example, pH 2.4. NaCl (40 ml of 1 M) was then added into the protein solution carefully with stirring gently. The solution pH was adjusted to 2.5 with 1 M HCl again. WPI solutions at other salt concentrations at other pHs were prepared by a similar method. Vigorous stirring was avoided during the whole process to prevent the proteins from denaturation, especially under the condition of high salt, low pH and high temperature.

When a recovery test was carried out in 25 mM NaCl a sample of the exchanger was not equilibrated with 25 mM NaCl. When a recovery test was carried out in 400-500 mM NaCl a sample of the exchanger of known weight (0.5-5.0 g) was equilibrated with same salt concentration and pH as that used for the 1 or 4% WPI solution and then drained by vacuum filtration. After equilibration the weight of the exchanger sample decreased. The sample was then mixed with an 1 or 4% WPI solution (10 ml) in a 25 ml bottle on a slowly rotating wheel for 1 hour for Sepharose or 2 hours for cellulose. The product was then filtered through a 10 ml Pierce filter. The solution remaining in exchanger was expelled out as much as possible with air pressure. The exchanger in the filter was washed twice with the same volume of salt solution and pH as that used for the test. All of the washings were combined and made up to 25 ml with water. This was further diluted 100 fold (or 300 fold for 4% WPI stock solution) to analyse by HPLC. The proteins bound on the exchanger were eluted by suitable solvents, eg., dilute HCl at pH 1.5 for the exchangers made from dimethyloctylamine or a solvent (ethanol/HCl at pH 1.5 : 1/1) for the exchangers prepared from DMD, Do and DMDo. The eluate was diluted with water to 25 ml (or 100 ml for 4% WPI stock solution) for analysis by HPLC.

2.2.12 Column Recovery of α-Lactalbumin

An ion exchanger (1 or 2 g or ml) was suspended in 5 ml of equilibration buffer to make a thick slurry. It was stirred to remove air bubbles. The slurry was then transferred to a 2 ml Pierce column (or FPLC column for FPLC analysis). The exchanger was allowed to settle under gravity with the flow of additional equilibrium buffer through the column to make a 1 or 2 g (or ml) column. The column was equilibrated with 5 bed volume (BV) of equilibration buffer at a flow rate 0.2 ml/minute.

A WPI solution in NaCl was loaded onto the column at a flow rate 0.1 ml/minute by a peristaltic pump. Fractions of the breakthrough were collected and analysed by HPLC. After the WPI solution finished loading the column was washed with small amount of equilibration buffer. The proteins was then eluted by the method described in batch process. The eluate was analysed by HPLC.

2.2.13 Protein Analysis by HPLC

All HPLC analysis was carried out at New Zealand Dairy Research Institute, using a 1 ml RPC Resource column and a program developed by this institute.

2.2.14 Purification of Crude α-Lactalbumin by Q Resin

The crude α-lactalbumin from New Zealand Dairy Research Institute consisted of approximately 60% α-lactalbumin, 10% β-lactoglobulin and the rest contaminants. Before it was used in the research it was purified by ion exchanger chromatography on a QA anion exchanger.

A commercial QA anion exchanger (1 litre) kept in 20% iso-propanol was mixed with 1.0 litre of 20 mM Tris at pH 7.5 (equilibration buffer) to make a thin slurry. Air bubbles were removed by stirring. The slurry was then poured into a 1.0 litre column and the resin was allowed to settle by gravity under flow. The inlet of the column was connected to a Bio-Econo System. Its outlet was connected to a fraction collector through a 280 nm monitor. The column was equilibrated with 2 litres of equilibration buffer at a flow rate of 5 ml/minute.

The crude α-lactalbumin (10 g) was dissolved in 1 litre of equilibration buffer. The solution pH was adjusted to 7.5 with 5 and 1 M HCl. After centrifugation at 4300 rpm for 10 minutes the supernatant was separated and passed through a filter with pore size of 30 um, being degassed at the same time.

The filtrate was loaded onto the QA anion exchanger column at a flow rate 5 ml/minute. After loading, the column was washed with 1 litre of equilibration buffer to remove the unbound proteins. α-Lactalbumin was eluted by passing 1.5 litre of 20 mM Tris in 200 mM NaCl at pH 7.5 through the column at the same flow rate. The other proteins remaining on the resin were eluted by 20 mM Tris in 500 mM NaCl at pH 7.5.

The eluate of α-lactalbumin was dialysed against water three times to remove the salt and Tris at 4 °C. It was then freeze-dried.

2.2.15 Batch Recovery of WPI from UF Retentate of Cheese Whey by GibcoCel Q2H

UF retentate of cheese whey (100 g, 15.5% solids) was diluted with 150 g of water. The pH of the diluted retentate was adjusted to 8.0 with 5 and 1.0 M HCl. Q2H exchanger (88 g) was added to it. The mixture was stirred with a magnetic stirrer gently for 40 minutes. The mixture was then filtered. The exchanger in the filter was washed with water (2 * 100 ml) and drained. The breakthrough and the washings were combined and analysed by HPLC.

The exchanger was then transferred back to a 500 ml beaker. Water (100 ml) was added to the beaker and the pH was adjusted to 3.2 with dilute HCl. The mixture was stirred gently with a magnetic stirrer for 20 minutes to desorb the WPI. It was then filtered. The exchanger was transferred back to a 500 ml beaker. NaCl (100 ml of 0.1 M) was added to the beaker and the mixture was stirred gently with a magnetic stirrer for 20 minutes while maintaining the pH at 2.5 with dilute HCl. The mixture was then filtered. The filtrates were combined and analysed by HPLC.

The exchanger was cleaned by being soaked in 100 ml of 500 mM NaCl at 4 °C overnight. The exchanger was then filtered The breakthrough was collected and analysed by HPLC.

3 RESULTS AND DISCUSSION

3.1 Use of Agarose Matrix.

The beaded derivatives of agarose have many of the properties of the ideal matrix and have been used successfully in numerous recovery procedures. They have a very loose structure which allows ready penetration by macromolecules with molecular weight in the order of several millions. The diffusion equilibrium of substances of low diffusion constant should be attained most readily by fractionation in a gel of fine particles with uniform spherical shape. Abundant hydroxyl group in the polysaccharide backbones can be activated or functionalised to allow the covalent attachment of a variety of ligands. These derivatives are stable, rigid and have a moderately high capacity for substitution. Agarose's hydrophilic nature and nearly complete absence of charged groups allow it to hardly cause any denaturation or adsorption of sensitive biochemical substances. Based on these features agarose was chosen for this work.

3.1.1 Synthesis of Alkyl Quaternary Amino Sepharoses

To investigate the effect of hydrophobicity of adsorbent on the adsorption of individual proteins a series of amines with various lengths of straight aliphatic chains were coupled to Sepharose 4B. The amines coupled were those shown in Table 3.1. The hydrophobicity of these amines increases in the order, DMEA < DMH < DMO < DMD < Do < DMDo. The method used was that of M. J. Lilly (1988) to achieve substitution level of about 1 milli-equivalent of charged amino groups per gram of dry product (meq/g of dry resins). The procedure involved:

(1) Activation of the matrix with epichlorohydrin (ECH) in the presence of 3% sodium hydroxide.
(2) Coupling of the amines to the activated matrix.
(3) The final products were converted from hydroxide to chloride form ready for their use in adsorption experiments. The conversion was necessary to maintain the constant pH when proteins replaced counter-ions on the resins during the protein adsorption tests.

Table 3.1 Amines and Their Agarose Derivatives

Abbreviation.	Amines	Agarose Derivatives
DMEA	N,N-Dimethylethanolamine	DMEA-Seph
DMH	N,N-Dimethylhexylamine	DMH-Seph
DMO	N,N-Dimethyloctylamine	DMO-Seph
DMD	N,N-Dimethyldecylamine	DMD-Seph
DMDo	N,N-Dimethyldodecylamine	DMDo-Seph
Do	Dodecylamine	Do-Seph

The structure of the agarose derivatives are as follows:

DMEA-Seph: [S]-O-$CH_2CH(OH)CH_2N^+(CH_3)_2CH_2CH_2OHCl^-$

DMH-Seph: [S]-O-$CH_2CH(OH)CH_2N^+(CH_3)_2(CH_2)_5CH_3Cl^-$

DMO-Seph: [S]-O-$CH_2CH(OH)CH_2N^+(CH_3)_2(CH_2)_7CH_3Cl^-$

DMD-Seph: [S]-O-$CH_2CH(OH)CH_2N^+(CH_3)_2(CH_2)_9CH_3Cl^-$

DMDo-Seph: [S]-O-$CH_2CH(OH)CH_2N^+(CH_3)_2(CH_2)_{11}CH_3Cl^-$

Do-Seph: [S]-O-$CH_2CH(OH)CH_2N^+H_2(CH_2)_{11}CH_3Cl^-$

All of the activation and coupling reaction formulas were described in the Introduction section.

All of these products except Do-Seph were quaternary amino exchangers. The titration curves of all of the exchangers except Do-Seph were characteristic of strong base anion exchangers as described in the Introduction. Do-Seph was a weak base secondary amine.

Although the substitution levels of the exchangers shown in Table 3.2 were different, ie., the exchangers made from the longer alkyl chains had lower substitution levels, these figures did not represent the true amount of epoxy group reacting with amines because of the different size of the alkyl ammonium groups. When the levels of epoxide groups converted were calculated by the following equation it was found that almost same amount of epoxy group reacted with DMEA, DMH and DMO despite of the different lengths of these alkyl chains (shown in Table 3.2). These results indicate that steric hindrance did not affect the coupling of these amines. Too long alkyl chains of amines (above 10 carbons)

caused difficulty in coupling and consequently caused the decreases in substitution level and conversion efficiency.

Epoxide substitution level (meq/g) = $N * V / (M * D - M * D * S * MW*10^{-3})$

N: Concentration of hydrochloric acid (mole/l)
V: Volume of hydrochloric acid (ml)
M: Mass of wet exchanger (g)
D: Dry matter content of wet exchanger
S: Substitution level (meq/g)
MW: molecular weight of amine

Table 3.2 Substitution Levels of Sepharose Anion Exchangers

Exchanger	DMEA -Seph	DMH- Seph	DMO- Seph	DMD- Seph	Do- Seph	DMDo -Seph
Substitution level (meq/g)	1.2	1.1	1.1	0.92	0.95	0.83
E.* substitution level (meq/g)	1.3	1.3	1.3	1.1	1.1	1.0
Conversion (%)	100	100	100	85	85	77

E.*: Epoxide

Only quaternary amino ion exchangers would be synthesised further in this work. They retain their positive charge over the full pH range 1-14, whereas tertiary amino Sepharose, Do-Seph, carries positive charge between pH 1-5 and progressively loses its charge above pH 5 and consequently converts to free secondary amine form.

3.1.2 Adsorption Properties of Amino Sepharoses in Low Ionic Strength

3.1.2.1 Effect of pH and Size of the Alkyl Ammonium Groups

Before the anion exchangers (amino Sepharoses) were used to separate proteins, it was essential to investigate their adsorption capacities for the individual proteins to be purified under a range of conditions of pH and ionic strength. High capacity and selectivity at neutral pH and low ionic strength (below 100 mM) is usually the preference of industry. However, the solubility of β-lactoglobulin was low at low salt concentration between pH 4 and 5.5 (ie., near its isoelectric point). β-Lactoglobulin (1%) without salt was cloudy in this pH range, possibly due to the presence of small amount of denatured β-lactoblobulin.

Adding a small amount of salt was advantageous to increase its solubility but adverse to the capacity of ion exchangers. Earlier work of Ayers, J. S. and Elgar, D. F. (1991) suggested that some ionic strength (25-50 mM sodium chloride) be used when attempting to separate β-lactoglobulin and α-lactalbumin from whey protein isolate (WPI) by appropriate choice of pH. As long as the proteins were completely soluble in solution the lowest ionic strength (25 mM sodium chloride) was used in this step of this work.

A commercially available quaternary amino Sepharose, Q-Sepharose-fast-flow (Q-Seph-ff) was included in the series of anion exchanger to compare adsorption properties.

α-Lactalbumin and β-lactoglobulin (20 ml of 0.25%) were applied to 0.2 g of Q-Seph-ff and DMEA-Seph while 0.5 g of DMH-Seph, DMO-Seph, DMD-Seph, DMDo-Seph and Do-Seph were used. Figures 3.1-3.4 display the binding capacities of each of the anion exchangers for α-lactalbumin and β-lactoglobulin by electrostatic and/or hydrophobic interaction. (Unless stated otherwise solid lines are used for α-lactalbumin, broken lines for β-lactoglobulin in the figures).

At a pH above the isoelectric point the proteins had net negative charge and bound on all of these anion exchangers by electrostatic interaction. The closer the pH came to the isoelectric point, the smaller the net negative charge the proteins had. Thus, the capacities of the exchangers decreased dramatically with the decrease of pH from 6 to 4.5, especially for β-lactoglobulin (Figures 3.1 and 3.2). The capacities of these exchangers for β-lactoglobulin were always higher than those for α-lactalbumin at pH above isoelectric point. This indicates that it is difficult to purify α-lactalbumin in this pH range by binding α-lactalbumin in preference to β-lactoglobulin from a mixture containing the two proteins.

It was worth noting the binding of the proteins to some of the exchangers at pH below isoelectric points of the proteins where the proteins carried net positive charge. DMEA-Seph and DMH-Seph hardly bound any protein below pH 5. However, with the increase in the hydrobicity of the amines the anion exchangers started to bind both proteins below their isoelectric points, especially α-lactalbumin. The anion exchangers with long alkyl chains not only showed their preference for α-lactalbumin but also showed higher capacities for α-lactalbumin than those with short alkyl chain. At pH 4.5 a gram of DMH-Seph bound only 0.08 g of α-lactalbumin. DMO-Seph bound about 0.3 g while DMD-Seph bound 0.75 g of α-lactalbumin. After the size of the alkyl group had increase to 10, this trend levelled off. DMD-Seph, Do-Seph and DMDo-Seph had very similar capacities at pH 4.

DMO-Seph bound slightly more α-lactalbumin than β-lactoglobulin at pH below 4.4. When the aliphatic chain was longer than octyl the exchangers bound greatly more α-lactalbumin than β-lactoglobulin (Figures 3.3 and 3.4). The capacities of DMD-Seph, DMDo-Seph and Do-Seph for α-lactalbumin were about 2-3 times higher than those for β-lactoglobulin at pH 3.5-4.

When pH dropped to 3.8 β-lactoglobulin desorbed from DMO-Seph. In FPLC experiment it was found that all α-lactalbumin was eluted at pH 2.5. However the proteins still bound on DMD-Seph, DMDo-Seph even at pH 2.3. To elute them 50% ethanol was used.

This discovery that the capacities of the anion exchangers with hydrophobic groups attached, DMO-Seph, DMD-Seph, DMDo-Seph and Do-Seph, for α-lactalbumin were bigger than those for β-lactoglobulin at pH levels below the isoelectric points supplied a possible opportunity to purify α-lactalbumin by selecting an anion exchanger to bind α-lactalbumin in preference to β-lactoglobulin below their isoelectric points.

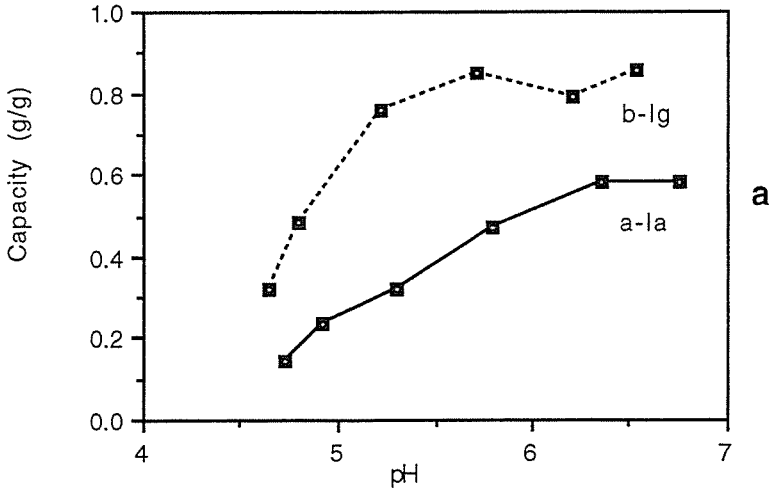
The fact that proteins still bound on anion exchangers with long alkyl chains at pH levels below their isoelectric points indicates that the binding was not possibly caused by electrostatic interaction but another attractive interaction, eg., hydrophobic interaction, between proteins and these anion exchangers. This attractive interaction was strong enough to overcome the repulsive force of static charge at pH 3.5-4.3 for DMO-Seph (Figure 3.2b) and at pH < 4.5 for DMD-Seph, DMDo-Seph and Do-Seph (Figure 3.3 and 3.4).

The result that alkyl chains longer than octyl was necessary to obtain sufficient hydrophobicity to bind α-lactalbumin was similar to that of Jost (et al, 1974). He prepared two series of alkylamino-agarose and aryl-agarose:

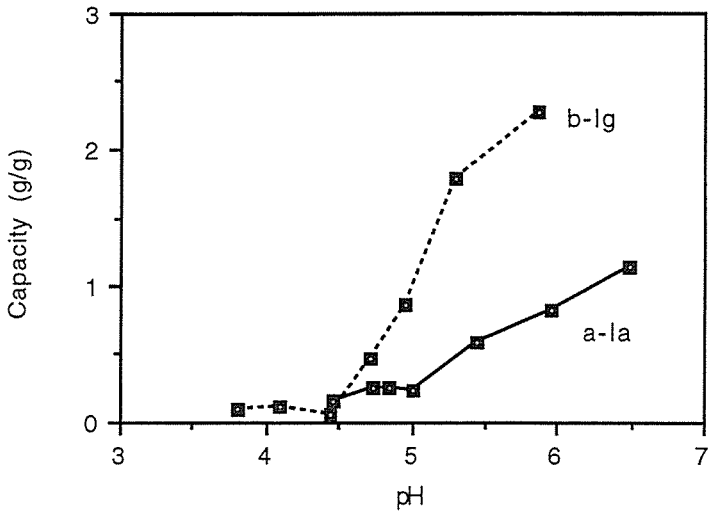
1. agarose-NH-$(CH_2)_{3\text{-}7}CH_3$
2. agarose-NH-NH-CO-$(CH_2)_nCH_3$
3. agarose-NH $CH_2CH_2C_6H_4OH$
4. agarose-$NHNHCOCH_2C_6H_4OH$

The last two alkylamino-agaroses (3 and 4) with hydrophilic hydroxyl group at the end of alkyl chains were not able to bind any α-lactalbumin, whereas α-lactalbumin bound tightly to the alkylamino-agaroses (1) by electrostatic and hydrophobic interaction, whereas it did

Figure 3.1 Effect of pH on binding of a-lactalbumin and b-lactoglobulin in 25 mM NaCl to (a) Q-Seph-ff and (b) DMEA-Seph

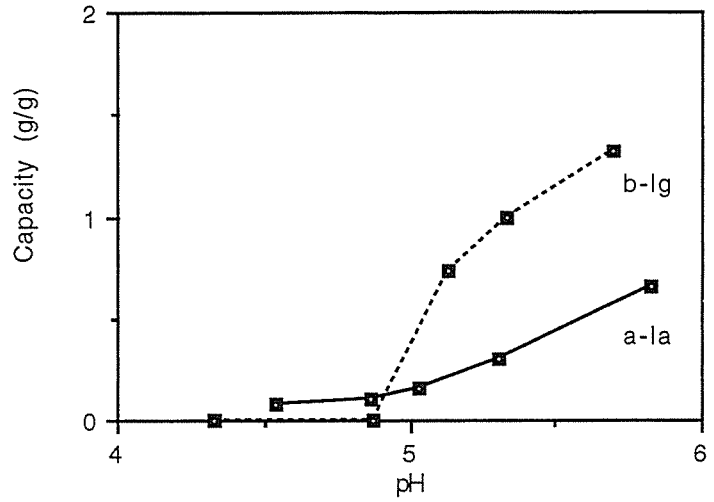


a

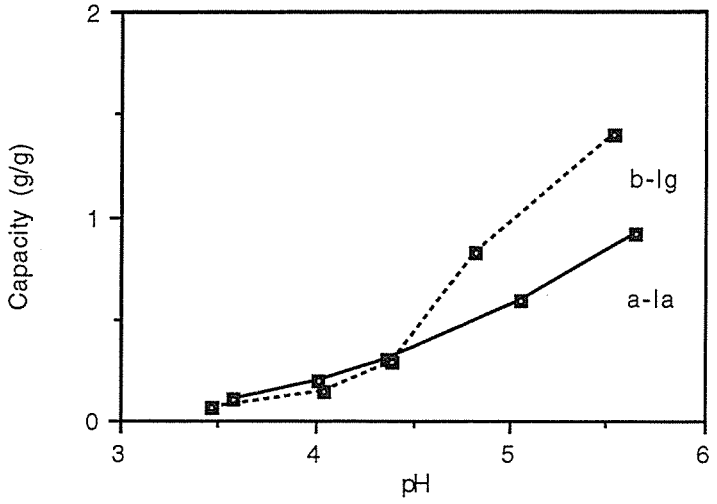


b

Figure 3.2 Effect of pH on binding of a-lactalbumin and b-lactoglobulin in 25 mM NaCl to (a)DMH-Seph and (b) DMO-Seph

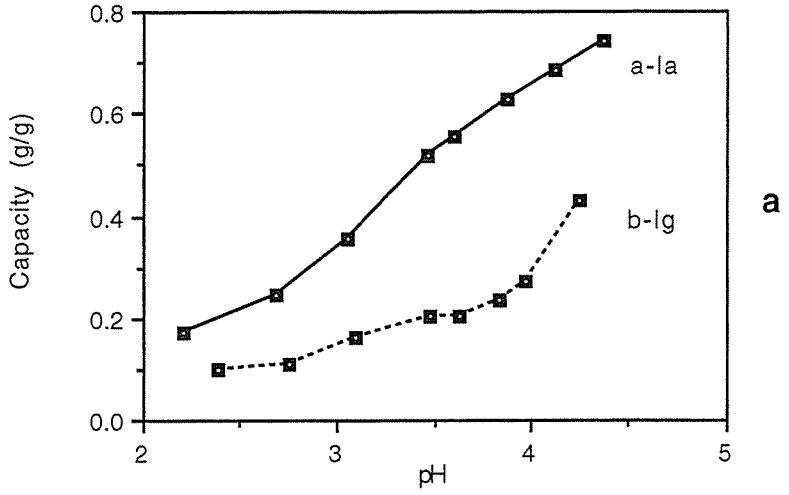


a

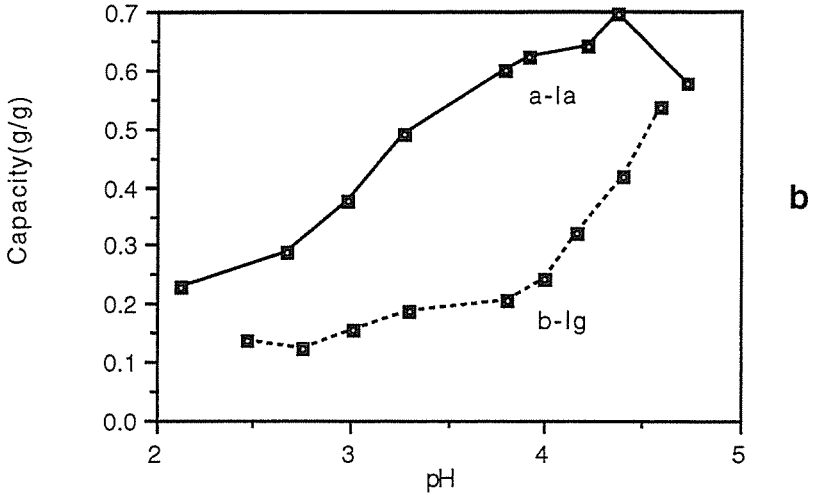


b

Figure 3.3 Effect of pH on binding of a-lactalbumin and b-lactoglobulin in 25 mM NaCl to (a) DMD-Seph and (b) DMDo-Seph

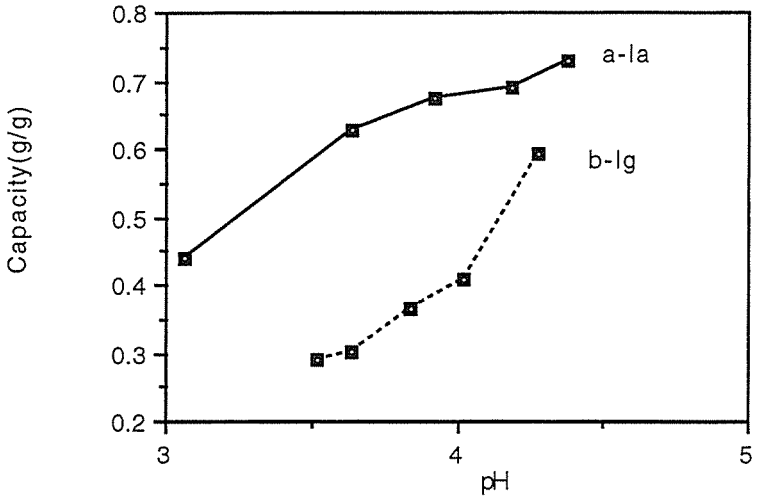


a



b

Figure 3.4 Effect of pH on binding of a-lactalbumin and b-lactoglobulin in 25 mM NaCl to Do-Seph



not bound to alkylamino-agaroses (2) by hydrophobic interaction until n was 8. To explain the hydrophobic affinity of α-lactalbumin with the alkyl chain on the matrix, Jost proposed that the immobilised alkyl group formed a detergent-like structure with a polar charged polysaccharide backbone from which the hydrophobic alkyl or aryl chains stick out to avoid the hydrophilic matrix. In this model, the function of hydrophobic chain consists in penetrating the surface of a protein molecule, causing a partial unfolding of the polypeptide chain. A conformational change of α-lactalbumin, induced by acidification at and below pH 5, was the result of this behaviour.

The lengths of the hydrophobic hydrocarbon chains did not affect the dispersion of the exchanger particles in water. All of the anion exchangers in Table 3.1 were readily dispersed into small individual particles in water and even in higher polarity media, 500 mM sodium chloride at any pH. However, after the anion exchangers had been used and then stored for two months at 4 °C it was found that those with long hydrocarbon chains (more than eight carbon atoms) did not disperse well again in water, even with vigorous stirring.

3.1.2.2 Effect of Matrix

Although Q-Seph-ff had higher substitution level than DMEA-Seph at 1.2 meq/g, its protein capacity was significantly lower (Figure 3.1). This was a result of Q-Seph-ff having a higher dry matter content (16%), compared with about 6% of the derivatives of Sepharose 4B,. This result showed the crucial effect of macroporous structure of the matrices on the binding of proteins to the anion exchangers, even for the binding of small proteins such as α-lactalbumin and β-lactoglobulin.

3.1.3 Effect of Ratio of Exchanger to Proteins

The capacities of 0.25 g of DMO-Seph (1.4 meq/g) for α-lactalbumin and β-lactoglobulin were tested using 10 ml of various concentrations of the proteins in 25 mM sodium chloride at pH 4.4. The results are displayed in Figure 3.5. The capacities for α-lactalbumin and β-lactoglobulin were nearly proportional to the residual concentrations of the proteins. This suggests that the adsorption was weak (Lowe C. R. & Dean. P. D. G., 1974) because the curvature was a function of the ratio of spaces available to the concentration of the protein molecules to fill them. This suggestion is consistent with

Figure 3.5 Adsorption isotherm of a-lactalbumin and b-lactoglobulin in (B1) 25 mM NaCl at pH 4.4, (B2) 500 mM NaCl at pH 2.5 to DMO-Seph

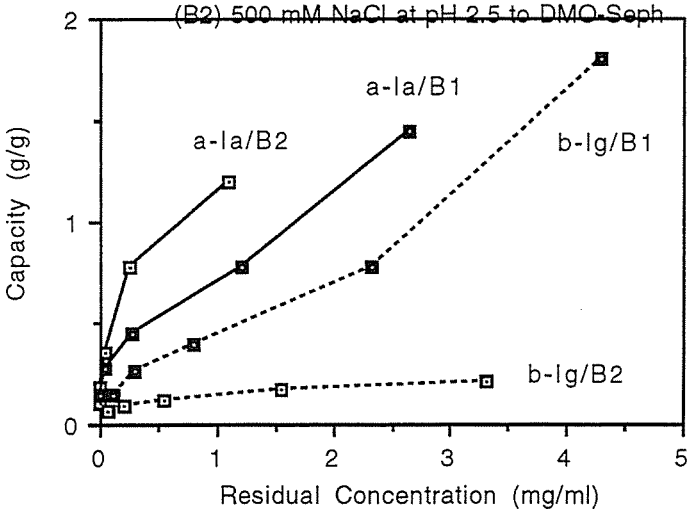
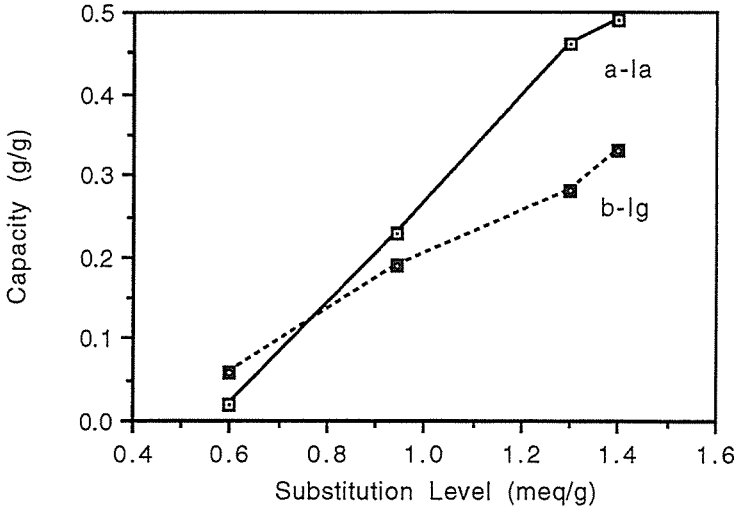


Figure 3.6 Effect of substitution level of DMO-Seph on binding of a-lactalbumin and b-lactoglobulin in 25 mM NaCl at pH 4.3



hydrophobic interaction rather than electrostatic interaction between the proteins and the exchangers.

The effect of concentration of the proteins on the capacity of the resin was subject to the limitation that sub-saturating amounts of complementary α-lactalbumin and β-lactoglobulin were used to the exchangers. β-Lactoglobulin even in low concentration, 1 mg/ml, occupied more pores of resins or had bigger steric hindrance than α-lactalbumin and thus lead to low capacity and adsorption. To bind more proteins, more pores or more exchanger must be used.

3.1.4 Effect of Substitution Level

A series of DMO-Seph were prepared with a range of substitution levels from 0.6 to 1.4 meq/g. This was achieved by varying the concentration of sodium hydroxide in the activation step and by reprocessing DMO-Seph. The capacities of DMO-Seph (0.25 g) with different substitution levels were determined with 10 ml of 0.25% protein solution. The results are displaced in Figure 3.6. A high substitution level of DMO-Seph was advantageous for α-lactalbumin and β-lactoglobulin to bind. The influence of substitution level on capacity was almost linear in 25 mM sodium chloride at pH 4.3 in the range of 0.6 to 1.4 meq/g. It was interesting that DMO-Seph at high substitution levels, 1.3 and 1.4 meq/g, made by reprocessing treatment was advantageous to the binding of the proteins. This reprocessing treatment probably did not increase the cross-linking level of Sepharose seriously because the reprocessing treatment only slightly increased the dry matter content of Sepharose, by about 20%. The linear relationship between capacity and substitution level indicates that because of low capacities of the resin in 25 mM sodium chloride at pH 4.3, the binding of proteins to DMO-Seph were hardly affected by steric hindrance. This result was different to that in high concentration of salt at pH 2.5 where substitution levels greater than 0.94 meq/g did not lead to significantly more binding of the proteins (Figure 3.13).

3.1.5 Recovery of α-Lactalbumin from WPI in Low Salt Concentration by DMO-Seph and DMD-Seph

3.1.5.1 By DMO-Seph and DMD-Seph

As shown in Figures 3.1-3.4, only those resins with alkyl chains more than 8 carbon atoms long had significant hydrophobic affinity with the proteins at low salt concentration, ie., 25 mM sodium chloride. DMDo-Seph and Do-Seph did not show much bigger capacities than DMD-Seph. Furthermore, too long an alkyl chain or too strong an interaction can possibly cause denaturation of proteins and has the added difficulty of being hard to elute the proteins from the exchangers. Thus, only DMO-Seph, DMD-Seph and DMDo-Seph were investigated further. All of them had higher capacities for α-lactalbumin than for β-lactoglobulin at and below their isoelectric point. Especially DMD-Seph, its capacity for α-lactalbumin was 2-3 times higher than that for β-lactoglobulin at pH 3.5-4.1

Table 3.3 Recovery of α-Lactalbumin from WPI (Alacen895) Using DMO-Seph.

Mass of wet DMO-Seph (g)	0.50	0.50	1.04	1.07
pH	4.46	4.94	4.44	4.86
Load (mg)				
α-la	30.3	30.3	30.3	30.3
β-lg	109	109	109	109
BSA	2.5	2.5	2.5	2.5
Filtrate (mg)				
α-la	23.3	22	17	13.6
β-lg	97	63.8	90.7	39
BSA	2.2	2.4	2.0	1.2
Eluate (mg)				
α-la	6.4	6.7	11.7	13.6
β-lg	7.3	28	10.1	46
BSA	0	1.0	0	1.4
Purity of α-la (%)	47	19	54	22
Yield of α-la (%)	21	22	39	45
Capacity for α-la (g/g)	0.22	0.24	0.20	0.22

*Capacity (gram of protein/ gram of dry anion exchanger)

(Figure 3.3). This result provided the possibility of binding α-lactalbumin in preference to β-lactoglobulin from a mixture of the two. However, as seen in Figure.3.3, DMD-Seph still retained capacity for α-lactalbumin even at pH 2.2. This disadvantage hampered its application as it was difficult to see how to elute the α-lactalbumin at low pH levels. In contrast, α-lactalbumin did not bind on DMO-Seph at pH 2.5. (This was found in the FPLC experiments.)

To investigate the possibility of recovering α-lactalbumin from a mixture of α-lactalbumin and β-lactoglobulin a solution of whey protein isolate (WPI) was used. WPI is obtained from whey by ion exchange and contains about 20% α-lactalbumin and rest is mainly β-lactoglobulin. Samples (0.5 and 1 g) of DMO-Seph were mixed with 10 or 20 ml of 1% WPI solution in 25 mM sodium chloride at pH 3.8 to 5.0 for 1 hour, then filtered, and washed with the starting buffer. The proteins bound on the resins were eluted with a 2:1:1 mixture of ethanol, 10 mM hydrochloric acid and 100 mM sodium chloride or 0.1 M acetic acid in 0.025 M sodium chloride. The breakthroughs (filtrates) and the eluates were collected and analysed by HPLC. (After elution, all breakthroughs and eluates were diluted with 20 mM sodium phosphate at pH 8.0 before being analysed by HPLC). The analytical results are displayed in Table 3.3.

The results in Table 3.3 show that a change from pH 4.4 to pH 4.9 caused the change of the predominant interaction between DMO-Seph and α-lactalbumin and β-lactoglobulin from weak hydrophobic affinity to strong electrostatic attraction. This change caused a greater increase in capacity for β-lactoglobulin than that for α-lactalbumin. This had a disastrous effect on the selectivity for α-lactalbumin and thus purity of the recovered α-lactalbumin dropped from 39 to 21%, whereas, the yield of α-lactalbumin hardly changed. This result is consistent with that in Figure 3.2b where two curves crossed at pH 4.5 and the increase in capacity for β-lactoglobulin had been much more than that for α-lactalbumin above pH of 4.5.

Almost double the amount of α-lactalbumin was recovered by increasing the DMO-Seph used from 0.5 to 1.0 g. Therefore, to achieve a high yield of α-lactalbumin a high ratio of DMO-Seph to α-lactalbumin was essential. Before further investigation of this it was noted that there were some problems with the WPI.

The WPI (Alacen895) used in the case of Table 3.3 was an industrial product and had been stored for 2-3 years. When it was dissolved in 25 mM sodium chloride, its 1% solution

was slightly cloudy at pH 6.6 and seriously cloudy at pH 4.4. Another WPI sample (MP0162) was prepared by New Zealand Dairy Research Institute recently. Its 1% solution in 25 mM sodium chloride was clear at pH 4.4-6.6. As shown in Figure 3.7 it is suspected that there was small amount of dimer or octomer of β-lactoglobulin, whose retention time was 20 minutes, in WPI (Alacen895). The amount of this polymer was discovered to change with pH. There was much more at pH 4.9 than at pH 4.4. This dimer or octomer bound so strongly on DMO-Seph, especially at pH 4.9 that little of it was found in the breakthrough (filtrate), but it made up about 25% of β-lactoglobulin present in the eluate and thus destroyed DMO-Seph's capacity and selectivity for α-lactalbumin significantly.

The structural change of β-lactoglobulin with pH was reported by Swaisgood, H. E. (1982 and 1985). β-Lactoglobulin exists as a 36.7 Kd dimer in solution above its isoelectric point (pH 5.2). Below pH 3.5 and above pH 7.5 the dimer dissociates into a slightly expanded monomer, and between pH 3.5 and 5.2 the dimer polymerises to a 147 K doctomer. On the other hand, the polymerisation of β-lactoglobulin is also affected by temperature and storage. The polymerisation by formation of covalent intermolecular disulfide bonds during high-temperature processing (> 70 °C) is irreversible. Partial denaturation and progressive polymerisation of this protein during storage does not involve formation of intermolecular disulfide cross-links and is reversible (Rechtor et al, 1991). This polymerisation was facilitated by its five cysteine residues as two intermolecular disulfide bonds linking residues 66 and 160, and residues 106 and 119 or 121, and one unreacted SH group at residue 119 or 121 (Godovac-Zimmermann & Braunitzer, 1987). The difficulty in dissolving at pH 4.2 was probably caused by the formation of dimer or octomer too. According to this and the result of the adsorption experiments, fresh WPI recovered by ion exchanger should be selected for the recovery of α-lactalbumin. Therefore, the fresh WPI (MP0162) prepared by New Zealand Dairy Research Institute, in which the dimer or octomer was not found, was used for the rest of the investigation.

Because a higher yield of α-lactalbumin could not be obtained at pH 4.9 than at pH 4.4-4.5, the latter was chosen in the recovery of α-lactalbumin from WPI (MP0162). Since Table 3.3 also showed that a higher yield of α-lactalbumin could be obtained by increasing the amount of DMO-Seph used, this was investigated further. To obtain an optimal ratio of exchanger to WPI, samples of DMO-Seph from 0.25 to 0.75 g were mixed with 10 ml of 1% WPI (MP0162) in 25 mM sodium chloride at pH 4.5 to recover the α-lactalbumin. The results are displayed in Table 3.4.

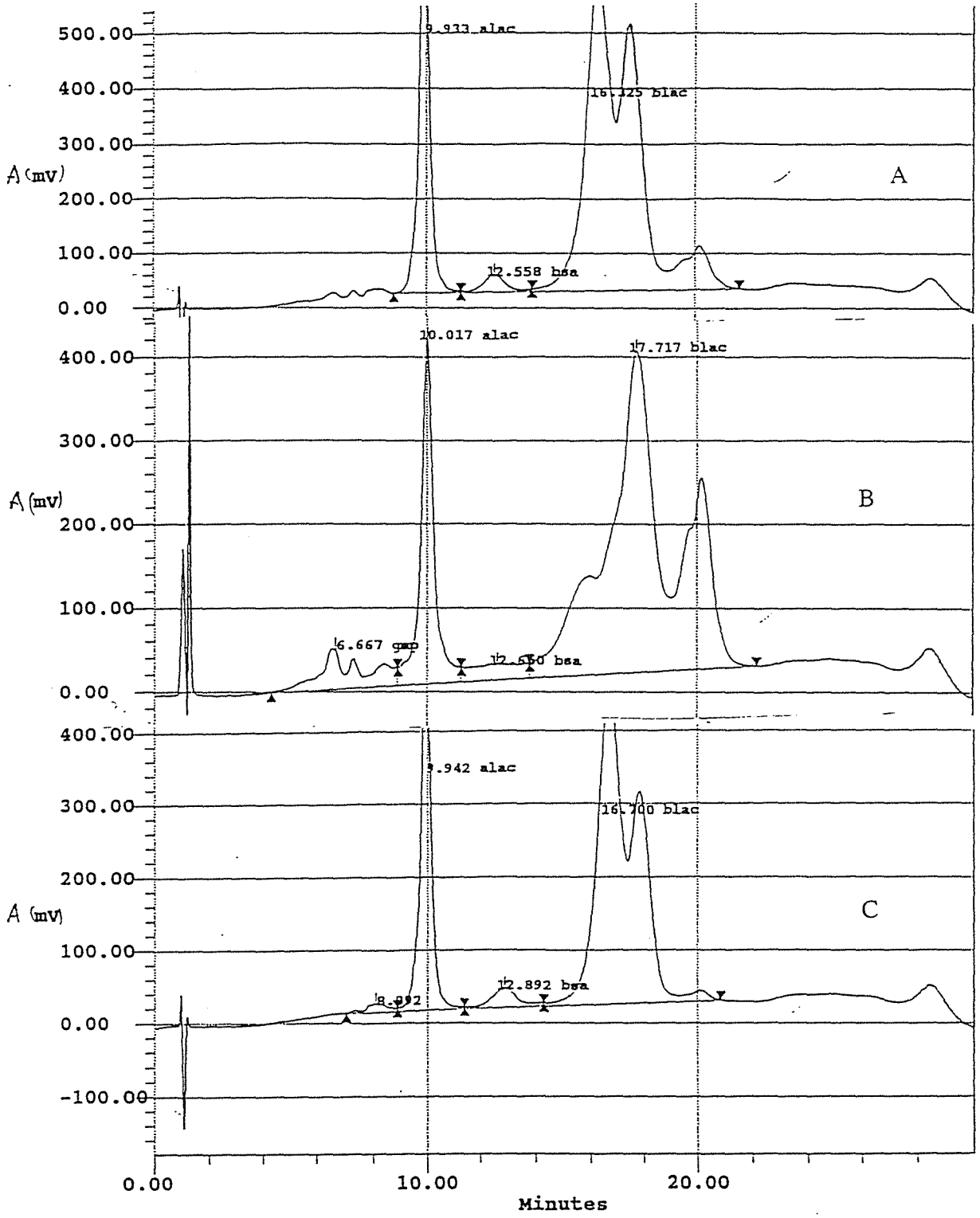


Figure 3.7 Distribution of β-lactoglobulin dimer at pH 4.9
A Load, B Eluate, C Breakthrough

Using 0.38 g or less of wet DMO-Seph per 10 ml of 1% WPI did not recover α-lactalbumin efficiently. Less than 40% was adsorbed. However, using 0.51 - 0.75 g of the exchanger, the yield increased to 70-80%. It was impossible to recover all of the α-lactalbumin in the WPI even with a higher ratio of exchanger to WPI. The maximum yield of α-lactalbumin was 80%. Furthermore, using excess exchanger decreased the purity of the α-lactalbumin recovered because when the ratio of exchanger to WPI was too great the exchanger bound additional β-lactoglobulin but not α-lactalbumin. For example, 0.62 and 0.75 g of the exchanger bound 0.4 and 1 mg more β-lactoglobulin than when 0.5 g of resin was used. Therefore, the optimal ratio was about 20 ml of 1% WPI containing 29 mg of α-lactalbumin to 1 g of the wet DMO-Seph. At this ratio DMO-Seph recovered 77% α-lactalbumin in the purity of 81% from 1% WPI. This is a significant achievement from such a simple method.

One possible reason why the yield of α-lactalbumin could not be increased beyond 80% is that about 10% of α-lactalbumin is glycosylated and less hydrophobic. This is less able to bind to the DMO-Seph.

In the adsorption experiment with the individual proteins, in which the starting concentration of both proteins were the same, the capacity of DMO-Seph for α-lactalbumin was similar to that for β-lactoglobulin at pH 4.4 (Figure 3.2b). In the experiment with WPI, although the concentration of β-lactoglobulin was 3.6 times higher than that of α-lactalbumin, DMO-Seph bound much more α-lactalbumin than β-lactoglobulin. This is the first time an ion exchanger has been identified which binds α-lactalbumin preferentially to β-lactoglobulin.

The capacity of DMO-Seph for α-lactalbumin from 1% WPI dropped to 0.08 g/g at pH 4.2 (Table 3.5) and purer α-lactalbumin was not obtained at this pH than at pH 4.4. Below its isoelectric point, electrostatic repulsion dramatically decreased the binding for α-lactalbumin. GMP was negative at pH 4.2 and the small amount presented in WPI was bound on the anion exchanger DMO-Seph. Thus, although there was little β-lactoglobulin in the eluate, GMP became the main contaminant although only small amount of GMP was present in the WPI. Thus, the optimal pH for purifying α-lactalbumin should be 4.4-4.5.

A disadvantage of DMO-Seph was that its selectivity was so sensitive to pH that it was hard to achieve good reproducibility.

Table 3.4 Recovery of α-lactalbumin from WPI (MP0162) by DMO-Seph

Mass of DMO-Seph (g/g)	0.25	0.38	0.51	0.62	0.75
pH	4.5	4.5	4.5	4.5	4.5
Load (mg)					
α-la	14.6	14.6	14.6	14.6	14.6
B-lg	59.7	59.7	59.7	59.7	59.7
GMP	1.5	1.5	1.5	1.5	1.5
BSA	0	0	0	0	0
Filtrate (mg)					
α-la	6.5	3.5	/	2.2	4
β-lg	56	53	/	51	50
GMP	0.7	0.8	/	0.5	0.5
BSA	0	0	/	0	0
Eluate (mg)					
α-la	4.4	5.4	11.2	11.7	10.4
β-lg	0.4	0.4	1.6	2.0	2.6
GMP	0.5	0.4	1.1	1.0	1.1
BSA	0	0	0	0	0
Purity of α-la (%)	83	87	81	80	74
Yield of α-la(%)	30	37	77	80	71
α-la (load)/resin (mg/g)	58	38	29	24	19
Capacity for α-la (g/g)	0.29	0.24	0.37	0.31	0.23

* WPI (10 ml) was 1% MP0162 in 25 mM NaCl.at pH 4.5.

3.1.5.2 Effect of Hydrophobicity of Alkyl Ammonium Group on Selectivity.

As illustrated in the last section (Figures 3.1-3.3), the hydrophobicity of the alkyl group attached to the ammonium group on the resins had significant effect on its properties. DMD-Seph and DMDo-Seph were expected to have better selectivity than DMO-Seph at and below the isoelectric point of α-lactalbumin.

The experiments with DMO-Seph and WPI (MP0162) reported in the previous section were repeated using DMO-Seph, DMD-Seph and DMDo-Seph. Because DMD-Seph and DMDo-Seph present the best selectivity at pH 3.5-4.2 (Figure 3.3), pH 3.8 and 4.2 were selected for this work. The results are displayed in Table 3.5. DMD-Seph and DMDo-Seph indeed had better recovery efficiencies and selectivities and were suitable to be used over a wider range of pH than DMO-Seph. More and purer α-lactalbumin was obtained. The main contaminant was GMP but not β-lactoglobulin. GMP is still negatively charged at pH 4 and binds to anion exchangers by electrostatic attraction. Even though there were only traces of GMP (1%) present in the WPI it was not surprising to find some in the eluate. No BSA was bound by these anion exchangers.

The difficulty with DMD-Seph and DMDo-Seph was that α-lactalbumin must be eluted by solvents with low polarity, for example, ethanol. Elution with ethanol, however, is not suitable for large scale production and has the risk of irreversible denaturation of proteins.

When recovery of α-lactalbumin from the WPI was carried out with DMD-Seph, greater recovery of α-lactalbumin could not be obtained by increasing the pH, even up to pH 5.2. Table 3.6 shows that the amount of α-lactalbumin recovered was relatively constant (5-6 mg) across the pH range. However, an increase in pH caused more binding of β-lactoglobulin and thus decreased the purity of the recovered α-lactalbumin from 95 to 46%.

Although complete recovery of the α-lactalbumin could not be achieved the yield was increased from about 40 to 70% by increasing the amount of the exchanger used from 0.25 to 0.50 g. A further increase in the amount of exchanger used to 0.75 g did not result in a significant improvement in yield and gave only a small further increase in yield to 76%.

The optimum conditions for use of the DMD-Seph in 25 mM sodium chloride were at pH 4.3 with a load of 10 ml of 1% WPI for 0.5 g of exchanger. This gave an α-lactalbumin yield and purity of 70 and 80% respectively. Shifting the pH to 3.8 could improve the purity further because of a reduction in the amount of GMP bound at lower pH. The

isoelectric point of the aglycofraction of GMP is around 4 and ceases to bind below this pH.

Table 3.5 Recovery of α-Lactalbumin from WPI (MP 0162) by DMO-Seph DMD-Seph and DMDo-Seph

Exchangers	DMO-Seph	DMO-Seph	DMD-Seph	DMD-Seph	DMDo-Seph	DMDo-Seph
Mass of resin (g)	0.25	0.25	0.25	0.25	0.25	0.25
pH	4.2	3.9	4.2	3.8	4.2	3.8
Sub. level (meq/g)	1.1	1.1	0.92	0.92	0.83	0.83
Load (mg)						
α-la	14.8	14.8	14.8	14.8	14.8	14.8
β-lg	71	71	71	71	71	71
BSA	2.5	2.5	2.5	2.5	2.5	2.5
GMP	0.9	0.9	0.9	0.9	0.9	0.9
Filtrate (mg)						
α-la	13.7	14.1	6.4	6.8	5.2	5.0
β-lg	68.5	70	68.3	69	68	69
BSA	2.3	2.4	2.3	2.4	2.2	2.4
GMP	0.5	1.0	0.6	0.9	0.5	0.7
Eluate (mg)						
α-la	1.2	0.7	7.4	5.2	6	6.8
β-lg	0.3	0.1	0.3	0.1	0.2	0.0
BSA	0	0	0	0	0	0
GMP	0.5	0.3	0.7	0.5	0.5	0.3
Purity of α-la (%)	60	64	88	90	90	96
Yield of α-la (%)	8	5	50	35	41	46
Capacity for α-la (g/g)	0.08	0.05	0.47	0.33	0.34	0.38

*WPI (10 ml of 1%) in 25 mM sodium chloride was applied to every sample.

3.1.5.3 Column Recovery of α-Lactalbumin from WPI

As a separation method column chromatography is more common and efficient than batch. So this method was investigated.

3.1.5.3.1 Recovery of α-Lactalbumin from WPI by DMD-Seph Column

A WPI solution (100 ml of 1% in 25 mM sodium chloride at pH 4.26) was passed through a column containing 2.5 g of wet DMD-Seph, at a flow rate of 0.28 ml/min. Fractions were collected and analysed by HPLC. The results are displayed in Figure 3.8. Within the first 30 ml of WPI loaded, no α-lactalbumin was found in the breakthrough stream, whereas β-lactoglobulin increased to 90% of that in WPI loaded. Beyond this point α-lactalbumin increased slowly. At 55 ml the resin still bound 80% of α-lactalbumin and all GMP. Beyond 55 ml the adsorption for α-lactalbumin decreased rapidly and α-lactalbumin in the breakthrough reached the same level as that in the WPI loaded at about 90 ml. Because the concentration of β-lactoglobulin in the late breakthrough did not exceed the level in the load WPI significantly (α-lactalbumin = 1.5 mg/ml and β-lactoglobulin = 6.0 mg/ml), and its decrease beyond 80 ml was only small, it is hard to conclude that β-lactoglobulin adsorbed in the early stage was subsequently displayed from the exchanger by α-lactalbumin when the passage of WPI through the column was continued.

This experiment was repeated except that the breakthrough was collected only as two fractions, ie., $Breakthrough_1$ until 55 ml and $Breakthrough_2$ until 100 ml of the WPI solution had been loaded. After a sample had been taken from $breakthrough_1$, $breakthrough_1$ was combinded with $Breakthrough_2$ to make $Breakthrough_3$. The proteins bound by the column were eluted by very dilute hydrochloric acid at pH 1.5. The residual proteins in the column were eluted with a mixture of ethanol and dilute hydrochloric acid at pH 1.5 (1/1, V/V). The two eluates were combined. The eluate and the breakthroughs were analysed by HPLC. The results are displayed in Table 3.7. Only 1.7 of 80 mg of α-lactalbumin contained in the first 55 ml of WPI solution was not bound by DMD-Seph. Beyond that point the exchanger gradually reached saturation. It bound only about 28 of 64 mg in the rest of the WPI solution. The eluate by dilute hydrochloric acid at pH 1.5 was clear. The second eluate using ethanolic hydrochloric acid was slightly cloudy. Although high concentration (50%) of ethanol was used to elute the proteins from DMD-Seph, the total amount of α-lactalbumin in the two breakthroughs and eluate was nearly equal to that in the load, so to with β-lactoglobulin and BSA. Therefore, this suggested that DMD-Seph

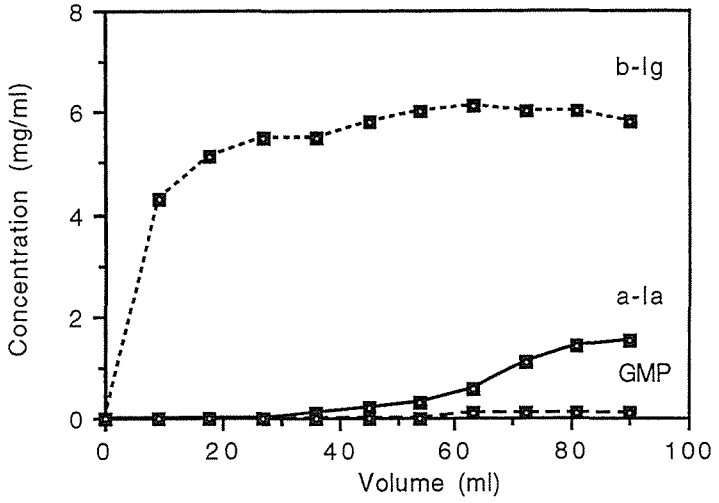
is safe as a hydrophobic adsorbent and that the two-step washing used here hardly cause any denaturation of the proteins.

Table 3.6 Effect of pH on capacity of DMD-Seph for WPI

Mass of DMD-Seph (g)	0.25	0.25	0.25	0.25	0.50	0.50	0.75	0.75
pH	5.2	4.8	4.4	3.8	4.7	4.3	4.7	4.3
Loads (mg)								
α-la	14.6	14.6	14.6	14.6	14.6	14.6	14.6	14.6
β-lg	59.7	59.7	59.7	59.7	59.7	59.7	59.7	59.7
GMP	1.5	1.5	1.5	1.5	1.5	1.5	1.5	1.5
BSA	0	0	0	0	0	0	0	0
Filtrates (mg)								
α-la	8.5	8.0	8.0	8.0	3.3	2.9	1.0	1.0
β-lg	45	52	56	57	49	45	45	47
GMP	0	0	0	0	0	0	0	0
BSA	0	0	0	0	0	0	0	0
Eluates (mg)								
α-la	5.2	6.3	5.8	5.6	10.0	10.1	12.8	11.1
β-lg	5.2	1.3	0.9	0	3.0	1.7	5.0	2.0
GMP	0.8	0.8	0.6	0.3	0.9	0.8	1.0	0.7
BSA	0	0	0	0	0	0	0	0
Purity of α-la (%)	46	75	79	95	72	80	68	80
Yield of α-la (%)	36	43	40	38	69	69	88	76
Cap. for α-la (g/g)	0.32	0.39	0.36	0.35	0.32	0.32	0.27	0.23
α-la (in load)/resin (mg/g)	58	58	58	58	29	29	19	19

1. WPI (10 ml) was 1% MP0162 in 25 mM NaCl
2. Elution by the mixture of ethanol, 0.1 M NaCl and 0.01 M HCl (2 :1 :1)
3. All filtrates and eluates was diluted with the same volume 20 mM sodium phosphate at pH 8.0 and kept in thefreezer until being analysed
4. Cap.= Capacity

Figure 3.8 Adsorption of protein from 1% WPI in 25 mM NaCl at pH 4.3 by DMD-Seph column



Compared with the results presented in Table 3.6 for the batch method, the yield of α-lactalbumin increased from 40 to 73% by using column processing at a same ratio of α-lactalbumin to resin. The purity of the recovered α-lactalbumin decreased slightly. This possibly was that column processing not only increase binding efficiency for α-lactalbumin but also for β-lactoglobulin.

Table 3.7 Column Recovery of α-Lactalbumin from WPI (MP0162) by DMD-Seph

Protein (mg)	Load	BT_1	BT_3	Eluate
α-la	145	1.7	44	106
β-lg	744	366	717	24
BSA	28	4.1	27	0
GMP	10.7	0	0	7.5
Purity of recovered α-la (%)				77
Yield of recovered α-la (%)				73

*BT_1: breakthrough$_1$
*BT_3: breakthrough$_3$ (the whole breakthrough)

3.1.5.3.2 Separation by Using DMO-Seph Column by FPLC.

In order to better explore several gradient elutions for the separation of α-lactalbumin and β-lactoglobulin from WPI (MP 0162), a 1 ml column was packed with DMO-Seph and coupled to a Pharmacia FPLC instrument. The results are shown in Figure 3.9. Individual whey protein was able to be separated by DMO-Seph on FPLC.

Method 1. Salt gradient in 50 mM sodium acetate at pH 5.0.

A 1.0 ml sample of 1% WPI in 50 mM sodium acetate at pH 5.0 was loaded onto a 1.0 ml DMO-Seph (1.1 meq/g) column at a flow rate of 0.1 ml/min. A linear salt gradient from 0 to 200 mM sodium chloride in 50 mM sodium acetate at pH 5.0 was run for 80 minute, followed by elution with a mixture of 1:1 (V/V) of hydrochloric acid at pH 2.5 and ethanol. Multiple peaks were observed. Peak 1, 2, 3 were confirmed to be β-lactoglobulin, peak 4 to be α-lactalbumin. It was seen that α-lactalbumin still bound to the column even the gradient finished. Stronger eluent (dilute hydrochloric acid) had to be used to elute. The separation of α-lactalbumin from β-lactoglobulin had baseline resolution.

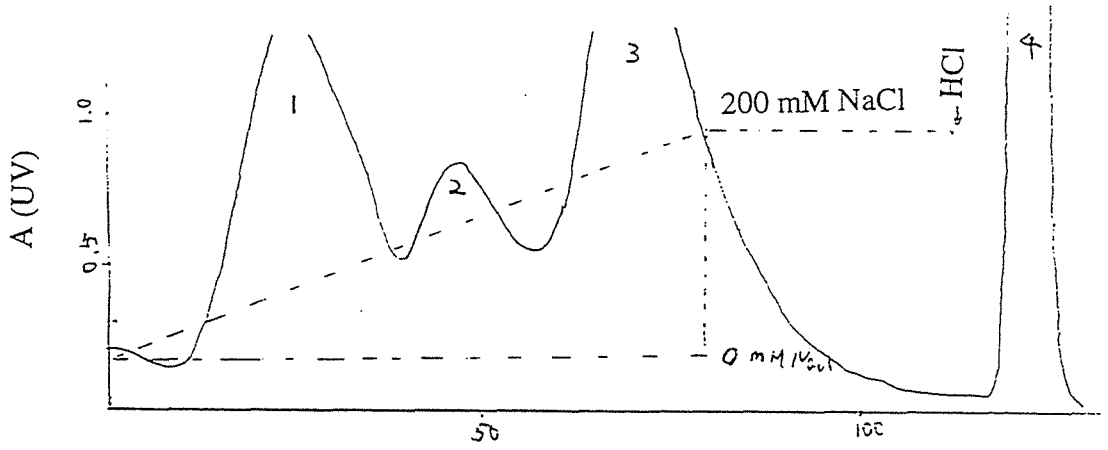
Method 2. pH gradient in 50 mM sodium acetate

A 1.0 ml sampleof 1% WPI in 50 mM sodium acetate at pH 5.0 was loaded onto a 1.0 ml DMO-Seph (Sub. 1.1 meq/g) column at a flow rate of 0.1 ml/min. A pH gradient from 5 to 2 was run for 80 min. Two peaks, representing β-lactoglobulin (peak 1) and α-lactalbumin (peak 2) with close to baseline resolution were obtained. The resolution decreased when a longer column was used.

Method 3

A 1.0 ml sample of 1% WPI in 300 mM sodium chloride at pH 2.0 was loaded onto a 1.0 ml DMO-Seph (Sub. 1.1 meq/g) column at a flow rate of 0.1 ml/min.. The column was then eluted by dilute hydrochloric acid at pH 2.5 without any salt. Two peaks with good baseline resolution were obtained. The first one was β-lactoglobulin which were passed straight through the column. The second peak was α-lactalbumin which was eluted when the salt strength was reduced from 300 to 0 mM at pH 2.5. Later work (Figure 3.10) showed that the capacity of DMO-Seph for α-lactalbumin under acid conditions was high at high salt concentration but decreased to zero when the salt was absent.

Figure 3.9 Separation of WPI by FPLC
1, by a linear salt gradient
2, by a pH gradient
3, in hight salt concentration



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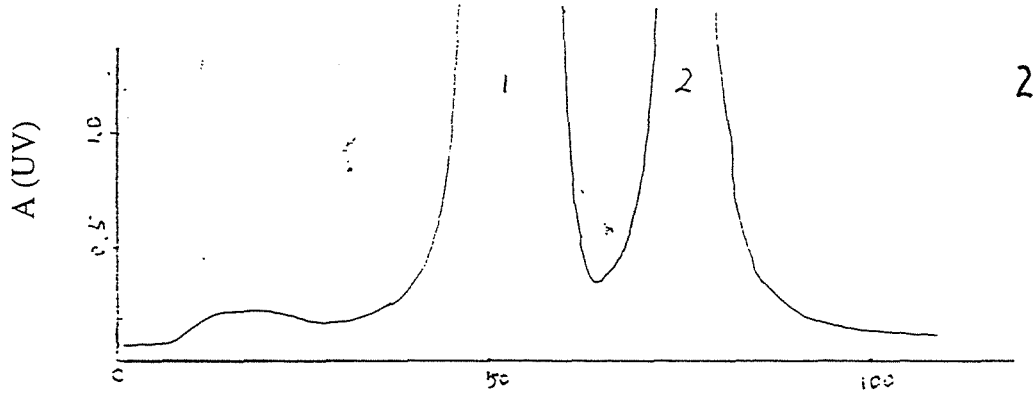
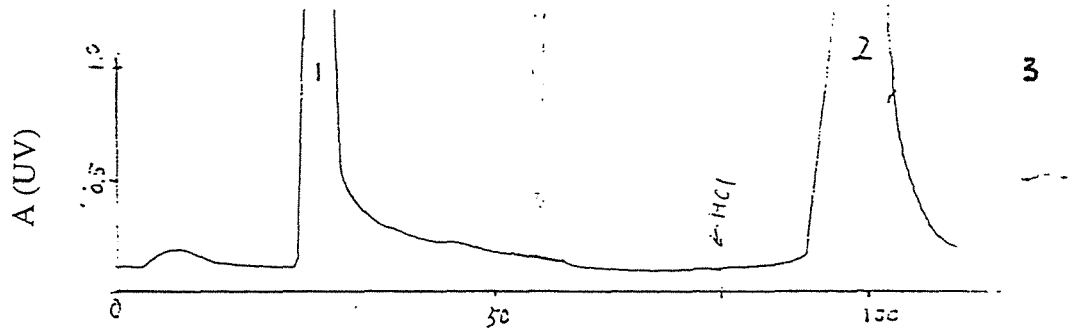


2.



3

3.1.6. Adsorption Properties of Amino Sepharose in High Ionic Strength

As discussed in the previous section, DMO-Seph and DMD-Seph had relatively good recovery, adsorption and elution properties for α-lactalbumin. However, some factors hindered their application on a large scale. Firstly, the fact that WPI had the lowest solubility at pH 4-5, where DMO-Seph and DMD-Seph worked well, meant that the recovery process had to be done at low protein concentration. It would have low efficiency and need a large adsorption vessel. Adding salt was able to increase solubility of α-lactalbumin, but decreased the capacity of the resins. Secondly, the pH range suitable for DMO-Seph was very narrow, eg., pH 4.4 to 4.5. Small deviations in pH could possibly cause an absolutely different result. This would lead to difficulty in operation. Thirdly, adsorption at pH 4.4 was suitable for only acid whey but not for rennet whey because large amount of GMP in rennet whey will bind to the anion exchanger at this pH. Finally their capacities were still unsatisfactory.

In previous FPLC experiments, it was not possible to accelerate the elution of α-lactalbumin by increasing the ionic strength to 500 mM even at pH 2 which was one of the common methods for elution of protein from anion exchangers. In contrast to that, less α-lactalbumin was eluted. In another experiment, it was easy for α-lactalbumin to be separated from β-lactoglobulin by increasing ionic strength and decreasing pH. WPI in 25 mM sodium chloride at pH 5.0 was loaded. Then, β-lactoglobulin was eluted by 300 mM sodium chloride at pH 2.0, followed by the elution of α-lactalbumin by very dilute hydrochloric acid at pH 2.5 without salt. The two peaks of elution separated very well and were quite sharp. These two results suggest that high concentration of salt at low pH enhances the binding of α-lactalbumin to DMO-Seph.

3.1.6.1 Effect of Ionic Strength and pH on the Adsorption of Proteins to DMO-Seph

The adsorption capacities of DMO-Seph for α-lactalbumin and β-lactoglobulin at a range of ionic strengths was determined by mixing 0.25 g of DMO-Seph with 10 ml of each protein solution (0.25%) for 1 hour in a batch method. The results are displayed in Figures 3.10 - 3.12. Ionic strength had a completely different influence on the binding of these proteins at low pH compared with pH 5.0 (Figure 3.10). At pH 5.0 increasing the sodium chloride concentration from 25 to 200 mM caused the capacity for β-lactoglobulin and α-lactalbumin to drop from 1.3 to 0.7 g/g and 0.7 to 0.4 g/g, respectively. The decrease for β-lactoglobulin was more than that for α-lactalbumin. Further increasing the salt concentration to 500 mM had little further influence on the binding capacities. They

remained constant at 0.6-0.7 and about 0.4 g/g respectively. The capacity for β-lactoglobulin was greater than that for α-lactalbumin over the full range of ionic strength, 0-500 mM.

The exchanger predominantly played a role of ion exchanger in low ionic strength (sodium chloride concentration below 200 mM). Adding chloride ion replaced the proteins by competitive adsorption and bound to the cation group on the exchanger. Higher ionic strength enhanced the hydrophobic binding of the proteins to the alkyl chain of the exchanger, which overcame the loss of weaken electrostatic interaction. However, at pH 1.7 with the increasing in the sodium chloride concentration from 200 to 500 mM the capacity for α-lactalbumin and β-lactoglobulin increased from 0.1 to 0.95 g/g nearly linearly and 0.06 to 0.38 g/g, respectively. At this pH without salt the capacities of the anion exchangers for α-lactalbumin and β-lactoglobulin are zero because of the electrostatic repulsion between the positive charged groups on the exchangers and of proteins. The adsorption here was clearly caused by hydrophobic interaction and increased with rising ionic strength. Even at low ionic strength (below 100 mM sodium chloride) there was still some proteins binding. The ionic strength has to be practically zero in order to eliminate completely the adsorption of the proteins.

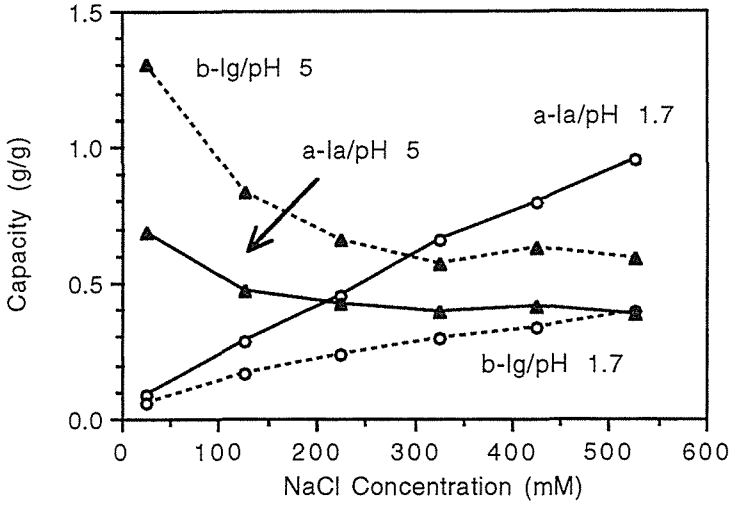
The effect of ionic strength on the adsorption of proteins in hydrophobic interaction chromatography (HIC) was explained by Benedek et al (1984). Salt of the starting mobile phase increased the surface tension of the mobile phase of HIC, and correspondingly, the solvent-stationary phase interfacial tension. The free energy of adsorption of the protein to the stationary phase was negative. Decreasing the salt concentration thus decreased the interfacial tension and permitted the elution of the protein.

The dramatic desorption of β-lactoglobulin by salt at pH 5.0 was characteristic of the property of ion exchange adsorption. In contrast, slow increase in binding for proteins, especially for α-lactalbumin, by salt at pH 1.7 was characteristic of HIC where the value of partition coefficient of proteins changes only slowly with altering condition.

A conformation change for α-lactalbumin at pH < 4 has been mentioned in the previous section. At pH < 4 the calcium ion bound by α-lactalbumin is probably replaced by hydrogen ion and thus its structure loosened. More hydrophobic side chains of amino acids of the protein in the core extend into the aqueous phase than at pH above isoelectric point.

These hydrophobic patches were bound by hydrophobic ligands of the resin. The affinity was weak without salts and was enhanced by salts.

Figure 3.10 Effect of NaCl concentration on binding of a-lactalbumin and b-lactoglobulin at pH 1.7 and 5.0 to DMO-Seph



We also concluded that β-lactoglobulin was more sensitive to ion exchange adsorption than α-lactalbumin at pH above isoelectric point, whereas α-lactalbumin was more sensitive to hydrophobic interaction than β-lactoglobulin at pH below isoelectric point. This is clearly related to their structures. There is no binding site for metal ion, especially calcium ion, in β-lactoglobulin. This feature means that the structure of β-lactoglobulin is more stable than that of α-lactalbumin at pH < 4 as it is not dependant on calcium binding for its stability.

In our work high salt concentration also weakened the repulsion between the exchanger and the proteins at pH below isoelectric point so that it was advantageous to the adsorption of the proteins by hydrophobic adsorption.

Figure 3.10 and the above discussion show the importance of pH for determining whether DMO-Seph binds α-lactalbumin and β-lactoglobulin by ion exchange or hydrophobic interaction. It was therefore decided to explore further the effect of pH by measuring the adsorption capacities over a range of pH levels at different ionic strengths.

DMO-Sephs (0.25 g) were mixed with 10 ml of 0.25% solutions of α-lactalbumin and β-lactoglobulin in 200 and 500 mM sodium chloride at pH 1-8 for 1 hour. The results are displayed in Figures 3.11 (pH 6-8) and 3.12 (pH 1-6). The capacity for β-lactoglobulin was higher than that for α-lactalbumin in 200 and 500 mM sodium chloride at pH > 5 (Figure 3.11). This was similar to the result in 25 mM sodium chloride at and pH > 4.5 in Figure 3.1 and 3.2. It was mentioned that α-lactalbumin and β-lactoglobulin did not bind at pH 7 in high salt to conventional anion exchangers. So in 500 mM sodium chloride adsorption must be obtained by hydrophobic interaction. But in this pH range the capacity for β-lactoglobulin was higher than that for α-lactalbumin.

There was no significant effect of pH on binding of β-lactoglobulin in 200 and 500 mM sodium chloride between pH 2 and 5 (Figure 3.12). However, more protein was bound at pH 1.5. This suggests that too low a pH like 1.5 should be avoided in trying to bind α-lactalbumin in preference to β-lactoglobulin.

Ionic strength above 200 mM sodium chloride had little effect on the binding of β-lactoglobulin in the pH range. In contrast, it had a crucial influence on the binding of α-lactalbumin (Figure 3.12). These results confirm that α-lactalbumin can be bound more efficiently to hydrophobic ligands than β-lactoglobulin although β-lactoglobulin has higher hydrophobicity than α-lactalbumin, 1199 and 1087 cal, respectively (Chaplin 1986).

The dependence of the hydrophobicity of α-lactalbumin on pH was reported by Mulqueen et al (1982). α-Lactalbumin did not show its strong hydrophobicity until it lost a bound Ca^{2+} ion at and below pH 4.

The high value of capacity for α-lactalbumin at pH 3.5 did not represent the real amount of this protein bound by DMO-Seph since α-lactalbumin had lowest solubility in high content of salt between pH 2.8 and 4. The solution of the protein in high salt at this pH range became very cloudy and precipitated soon after it had been prepared and mixed with the exchanger. In contrast, the solution of α-lactalbumin (0.8%) or WPI (4%) in 400 mM sodium chloride were stable below pH 2.8. The solution of β-lactoglobulin (3%) in the same salt concentration was stable at least between pH 1.5 and 5.

Proteins usually have the lowest solubility at their isoelectric points. However, this work found that the least amount of α-lactalbumin dissolved between 2.8 and 4.2, eg., below its isoelectric point in 200 mM sodium chloride. This possibly is the result of the conformational change for α-lactalbumin only occurring below pH 4 which prompts the

precipitation of the protein in high salt concentration (salting-out). The reason why its solubility was high at pH below 2.8 was unknown.

The capacity for α-lactalbumin was more than 0.9 g/g in 500 mM sodium chloride below pH 2.8, about 3.5 times more than that for β-lactoglobulin. This should provide a more advantageous opportunity to purify α-lactalbumin under these conditions than with 25 mM sodium chloride at pH 4.4. It was not known if the high capacity was also relevant to the rapid formation of dimer or trimer of α-lactalbumin below pH 4.2 (Lyster, 1972).

Although there are some reports that α-lactalbumin was readily soluble in 1 M sodium chloride at pH 2-8 (Slack. A. W et al, 1985), we found that 0.25% α-lactalbumin in 500 mM sodium chloride at pH 2.5 was soluble for no more than 5-6 hours in winter and it was not soluble in summer. When its concentration increased to 0.5%, it precipitated in the same buffer immediately even in winter. Instead, even up to 0.8% α-lactalbumin or 5% WPI solution in 400 mM sodium chloride at pH 2.8 was quite stable. Therefore 400 mM sodium chloride was chosen for most of the further recovery experiments, especially for those done in summer.

Figure 3.11 Binding of a-lactalbumin and b-lactoglobulin in (B1) 200 mM NaCl and (B2) 500 mM NaCl to DMO-Seph (1.3 meq/g)

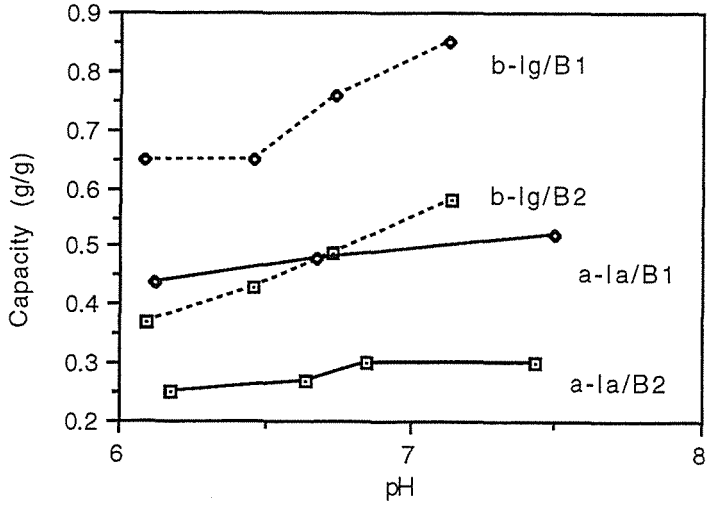
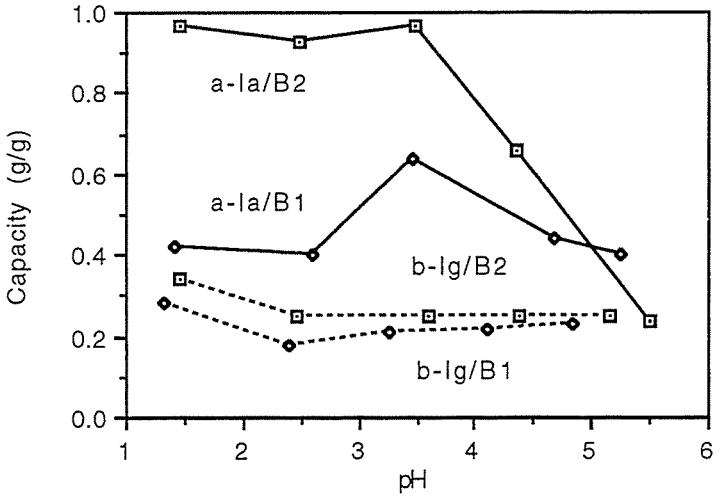


Figure 3.12 Effect of pH on binding of a-lactalbumin and b-lactoglobulin in (B1) 200 and (B2) 500 mM NaCl to DMO-Seph



3.1.6.2 Adsorption Isotherm in 500 mM sodium chloride at pH 2.5

The capacity of 0.25 g of DMO-Seph (1.4 meq/g) was tested with 10 ml of different concentration of α-lactalbumin and β-lactoglobulin in 500 mM sodium chloride at pH 2.5. The results are displayed in Figure 3.5 along with the results for adsorption in 25 mM sodium chloride at pH 4.4 discussed earlier in Section 3.1.3. The binding of α-lactalbumin in high salt concentration at low pH to DMO-Seph was similar to that at pH 4.4 in 25 mM sodium chloride. But the binding of β-lactalbumin reached saturation at low concentration and levelled off. Thus α-lactalbumin and β-lactoglobulin behaved very differently under these conditions of low pH and high ionic strength with DMO-Seph compared to the smaller difference seen between the two proteins at pH 4.4 and low ionic strength. This difference was investigated further.

3.1.6.3 Application of DMO-Seph for Recovery of α-Lactalbumin from WPI in High Salt Concentration.

The recovery of α-lactalbumin from WPI was investigated using batch and column processes.

A. By Batch Process

Table 3.8 shows the effect of high salt concentration at pH 2.7 on the recovery of α-lactalbumin from 10 ml of 1% WPI (MP0162) by 0.25 g of DMO-Seph (1.4 meq/g). With an increase in salt concentration from 200 to 500 mM the α-lactalbumin bound by 0.25 g of moist exchanger increased from 4.1 to 8.2 mg. This result was consistent with that shown in Figure 3.10. Unfortunately, the exchanger bound more of the other proteins, ie, β-lactoglobulin and bovine serum albumin at the same time. So while the yield of α-lactalbumin increased from 37 to 75% the purities remained practically constant at 62-65%.

It was interesting that the binding of bovine serum albumin (BSA) was fairly complete in 500 mM salt even though its concentration was very low in the WPI. This is the result of BSA being very hydrophobic.

The isotherm in Figure 3.5 showed high capacity of DMO-Seph for α-lactalbumin was able to be achieved from high concentration of α-lactalbumin solution. The recovery of α-lactalbumin from 5 ml of 4% WPI solution in 400 mM sodium chloride at pH 2.5 by DMO-Seph (1.3 meq/g) was tried. The results are displayed in Table 3.9. There was only a small increase of the yield of α-lactalbumin when going from 0.5 to 0.7 g of DMO-Seph. Purity of the α-lactalbumin remained the same.

Compared with the results when 1% WPI was used (Table 3.8), the purity and yield of α-lactalbumin increased from 62 and 66% to 73 and 79% respectively when 4% WPI solution was used (Table 3.9) at the same ratio of WPI to resin (0.4 g/1.0 g). Therefore, concentrated WPI was advantageous for increasing the recovery of α-lactalbumin from WPI by DMO-Seph.

Table 3.8 Effect of Ionic Strength on Recovery α-lactalbumin

mass of resin (g)		0.25		0.25		0.25		0.25	
Salt (mM NaCl)		200		300		400		500	
Solution	Load	Fil.	Elu.	Fil.	Elu.	Fil.	Elu.	Fil.	Elu.
Protein (mg)									
α-la	11	6.2	4.1	5	5.8	2.8	7.3	1.9	8.2
β-lg	56	48	0.6	50	1.9	40	2.4	40	3
BSA	2.6	1.5	1.5		1.3	0	2	0.1	2.1
GMP	0.7	0.7	0.1	0.7	0	0.7	0	0.7	0
Yield of α-la (%)			37		55		66		75
Purity of α-la (%)			65		64		62		62
Cap for α-la (g/g)			0.27		0.38		0.48		0.54

* Fil. = Filtrate, Elu. = Eluate, Cap =capacity

Table 3.9 Effect of Ratio of DMO-Seph to α-Lactalbumin

Mass of DMO-Seph (g)		0.5		0.7	
Protein (mg)	Load	Filtrate	Eluate	Filtrate	Eluate
α-la	30.5	3.8	24	1.5	26
β-lg	138	129	3.6	123	5.2
BSA	4.8	0	5.2	0	4.2
GMP	2.0	1.8	0	1.8	0
Yield of α-la (%)			79		85
Purity of α-la (%)			73		73
Capacity for α-la (g/g)			0.73		0.57

B. By Column Process

To test the binding of WPI to DMO-Seph in a column process. WPI solutions (100 ml of 1%) in 500 mM sodium chloride at pH 2.5 were passed through columns containing 1.0 g DMO-Seph (0.94 and 1.3 meq/g) at a flow rate 0.1 ml/min. The fractions were analysed by HPLC. The results are displayed in Figure 3.13. α-Lactalbumin broke through the column after 40 ml and 48 ml respectly had been loaded. There was thus a small (20%) increase in capacity with the greater substitution level of the exchanger. Like the case in Figure 3.8, DMO-Seph continued to bind some α-lactalbumin beyond the breakthrough point until 80 and 90 ml was collected. The exchanger bound less β-lactoglobulin in 500 mM sodium chloride at pH 2.5 than in 25 mM sodium chloride at pH 4.3 (Figure 3.8). Only about 15% of the protein was bound at 5 ml, compared with about 50% in Figure 3.8. This confirms that DMO-Seph can separate of α-lactalbumin from WPI solution in high salt concentration at low pH more readily than from WPI solution in low salt concentration at higher pH.

When this experiment was repeated using 4% solution of WPI in 400 mM sodium chloride at pH 2.5 α-lactalbumin broke through the column of DMO-Seph (0.94 meq/g) at 10 ml (Figure 3.14). Therefore, column process was not very efficient at separating α-lactalbumin from WPI solution.

Figure 3.13 Adsorption of a-lactalbumin from 1% WPI in 500 mM NaCl at pH 2.5 by DMO-Seph (0.94 and 1.3 meq/g) column

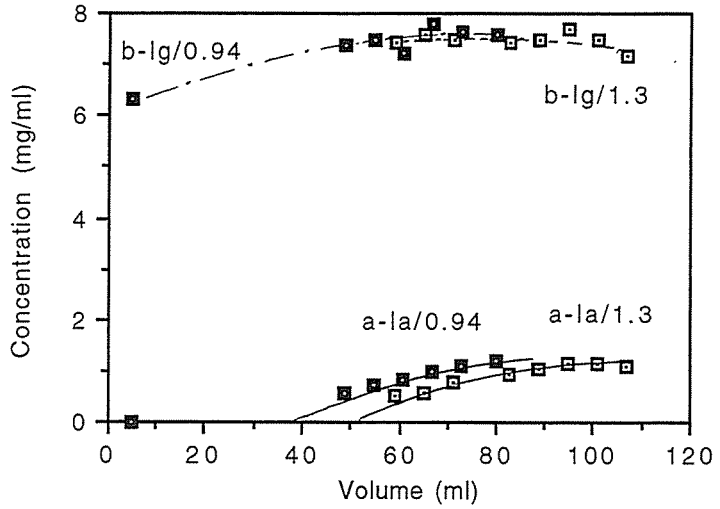
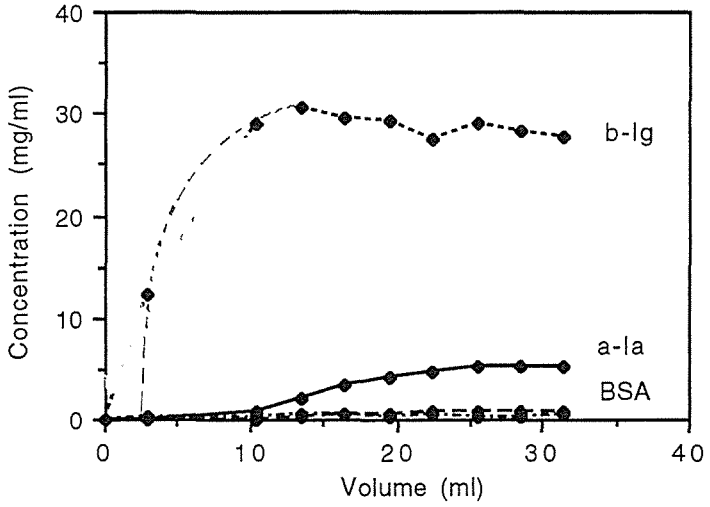


Figure 3.14 Adsorption of a-lactalbumin from 4% WPI in 400 mM NaCl at pH 2.5 by DMO-Seph (0.94 meq/g) column



3.2 Use of Cellulose Matrix

3.2.1 Background

As discussed in the previous section DMO-Seph and DMD-Seph showed satisfactory performance in recovery of α-lactalbumin from WPI. Both of them were suitable for preparation of α-lactalbumin in the laboratory but are not suitable for industry for the following reason.

a. Sepharose is too soft to bear pressure in an industrial process and high pressures are required to obtained a suitable flow rate.
b. Sepharose is very expensive .
c. Sepharose has very fine particles (40-200 um) which further limits the flow rate. Although Sepharose big beads are available they are still expensive.

One of the alternatives to Sepharose is cellulose. Cellulose has been used as a main matrix material for ion exchangers since 1950s because of its relatively low cost and its tough, rigid nature.

The disadvantage of cellulose is its high density or the consequent low porosity which hinders the penetration of protein molecules. This penetration of protein molecules available in matrices with macroporous structures is crucial to the capacity of ion exchangers. For this reason cellulose has not been used as a matrix for affinity chromatography or hydrophobic interaction chromatography. Much work has been done to improve the porosity of cellulose. One of these efforts (Ayers, seen in the Instruction) was to add hydroxypropyl groups to cellulose to swell it and at the same time crosslink it to prevent dissolution. This was achieved by adding propylene oxide (PO) and epichlorohydrin (ECH) in the presence of base. The modified cellulose had a much larger swollen volume than the starting material. A cross-linking level of 6-8% (ECH/cellulose: v/w) was optimum in terms of rigidity and swollen volume.

Table 3.10 Preparation of DMO-Cell with Different Substitution Levels

Mass of wet HP8-50 Cellulose (g)	31	31	31	31	31
Activation					
H_2O (ml)	18	22	24	25	26
30% NaOH (ml)	12	8	6	5	4
ECH (ml)	9.25	6.20	4.60	3.85	3.10
(Conc. of NaOH in slurry) (%)	6.3	4.2	3.2	2.6	2.1
Coupling					
DMO (ml)	10	10	10	10	10
CH_3CH_2OH (ml)	40	40	40	40	40
5M HCl (ml)	0.67	0.67	0.67	0.67	0.67
Substitution level (meq/g)	1.14	1.06	0.94	0.86	0.72

Conc. =concentration

3.2.2 Synthesis of Dimethyloctylamino-Cellulose (DMO-Cell) with Different Substitution Levels and Cross-linking Levels.

As summarised in Table 3.10, five DMO-Cell products with substitution levels from 0.72 to 1.14 meq/g were synthesised by using cellulose HP8-50 as initial material and activation by epichlorohydrin in 2.1-6.3% sodium hydroxide, followed by coupling with N,N-dimethyloctylamine (The preparation of HP8-50 was described in the Experimental section). Small increases in sodium hydroxide concentration used for the activation at the low end of the range (2.2-3.2%) resulted in significant increases in substitution levels (0.72-0.92 meq/g) for the DMO-Cell. Over this range the dry matter content of the products did not change significantly. However when higher sodium hydroxide concentrations were used (4.2-6.3%) there was little increase in substitution (1.06-1.14 meq/g) but a significant rise in the dry matter content from 13.8 to 15.7%. This suggests that extra cross-linking reaction occurred over this sodium hydroxide concentration range. Because higher dry matter content is adverse when calculating the capacity (g/g) of the resin, these concentrations of sodium hydroxide were avoided in further experiments.

Higher substitution levels, eg., 1.6 meq/g, can be achieved by reprocessing DMO-cell (1.0 meq/g) but unavoidable extra cross-linkage occurs in the activation procedure causing much higher dry matter content and lower porosity.

3.2.3 Effect of Cross-Linkage Level

As mentioned in Egan's work (1994), the cross-linking level of modified cellulose is one of the major factors affecting to the properties of cellulose derivatives. Thus, DMO-Cell with 4-8% cross-linking levels were prepared by hydroxypropylation and cross-linking, activation and coupling. Although it had a similar dry matter content as Sepharose, DMO-Cell with 4% cross-linking level was much softer than Sepharose derivatives so that it collapsed when washed with 1 M hydrochloric acid and was difficult to filter. Reprocessing treatment (including activation and coupling) of this product was not able to achieve satisfactory rigidity for the DMO-Cell.

Low cross-linkage was advantageous for supplying a loose structure and optimum binding for proteins. DMO-cell (35-36 mg of dry matter) with different cross-linking levels (4-8%) were used to bind protein from 10 ml of 0.25% α-lactalbumin and β-lactoglobulin solutions in 400 mM sodium chloride at pH 2.5 by batch process. The results are displayed on Table 3.11. The product with a cross-linking level of 4% had an especially high capacity for α-la, ie., 0.46 g/g. However its low rigidity precludes its use in industry. Therefore to obtain rigid enough exchangers only those cellulose derivatives with 8% cross-linking level were used in the further experiments unless mentioned otherwise.

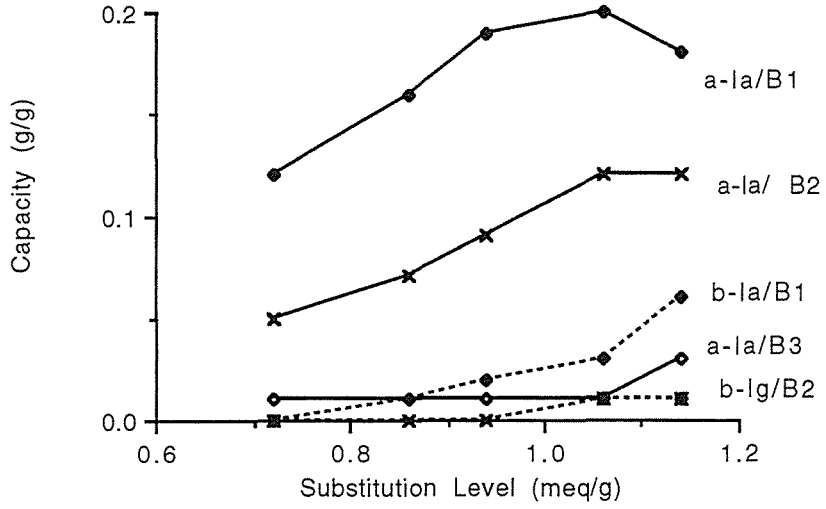
Table 3.11 Effect of Cross-linking of DMO-Cell

Cross-linking level of resin	4%	6%	8%
Capacity for α-la (g/g)	0.46	0.26	0.20
Capacity for β-lg (g/g)	0.13	0.07	0.03

3.2.4 Effect of Substitution Level and Ionic Strength

Because proteins bind on the hydrophobic ligands of the resins, the ligand concentration is a crucial parameter in the preparation of effective adsorbent. Maisano et al (1985) reported that pure HIC resins with straight chain alkyl groups with 6-12 carbons need a ligand concentration of no more than 0.3 meq/g (dry resin) to achieve the maximum capacity for serum proteins. This work obtained a different result. The five of DMO-Cells (36 mg of dry matter) with substitution levels from 0.72 to 1.14 meq/g were tested with 10 ml of 0.25% of α-lactalbumin and β-lactoglobulin in 200-400 mM sodium chloride at pH 2.5. The results are displayed in Figure 3.15.

Figure 3.15 Binding of a-lactalbumin and b-lactoglobulin in (B1) 400, (B2) 300 and (B3) 200 mM NaCl at pH 2.5 to DMO-Cell



The capacity of the DMO-Cell for α-lactalbumin in 400 mM sodium chloride at pH 2.5 increased rapidly until the substitution level was 0.94 meq/g. Further increasing the substitution level to 0.94 meq/g did not cause significant increase of the capacity. DMO-Cell with substitution level 1.14 meq/g bound less α-lactalbumin than that with substitution level 0.96 meq/g. At lower salt concentration a higher substitution level was needed to achieve maximum capacity, ie., 1.06 meq/g for 300 mM sodium chloride and > 1.14 meq/g for 200 mM sodium chloride. On the other hand, the capacity for β-lactoglobulin in 400 mM sodium chloride at pH 2.5 increased from 0 to 0.05 g/g with the increasing of the substitution level of DMO-Cell from 0.72 to 1.14 meq/g. This suggests that a substitution level 0.9-1.0 meq/g is the best for adsorption of α-lactalbumin in high salt concentration at low pH.

It was seen from Maisano's work that long alkyl chains needed less substitution to obtain the same capacity as the short chains. This result and those in Figure 3.16 indicated that like other HIC resins, DMO-Cell bound protein molecules by multi-point attachment and more adsorption groups were necessary to bind a protein molecule under the weak hydrophobic affinity. The reason that DMO-Cell needed a higher substitution level than octyl-Silica of Maisano was possibly that the cellulose has a lower porosity and the alkyl chains are not as accessible to the proteins as they are in silica.

When the substitution level of DMO-Cell was increased to 1.6 meq/g by further reprocessing treatment, its capacities for α-lactalbumin and β-lactoglobulin in 400 mM sodium chloride at pH 2.5 plummeted to 0.08 and 0 g/g respectively. Even another reprocessed DMO-Cell product (cross-linking level of 4%) did not have good capacities for α-lactalbumin and β-lactoglobulin, 0.21 and 0.09 g/g, respectively. The further reprocessing treatment to DMO-Cell is thus adverse to the binding of the proteins and unnecessary. This possibly is the result of increasing of the cross-linking level of cellulose by twice activation which caused the rapid increase of dry matter content of cellulose, ie., from 6 to 17% for DMO-Cell (4%) and from 15 to 26% for DMO-Seph (8%).

3.2.5 Beaded DMO-Cell

Beaded-cellulose, when used to prepare DMO-Cell, did not provide better protein capacity in 200 and 500 mM sodium chloride at pH 2.5 than ground-cellulose (Figure 3.16). Its capacity for α-lactalbumin was less than the latter's. Its capacity for β-lactoglobulin was similar with that of ground cellulose. Therefore, ground cellulose were used in further experiments.

3.2.6 DMO-Cell Activation by 1,4-Butanediol Diglycidyl Ether

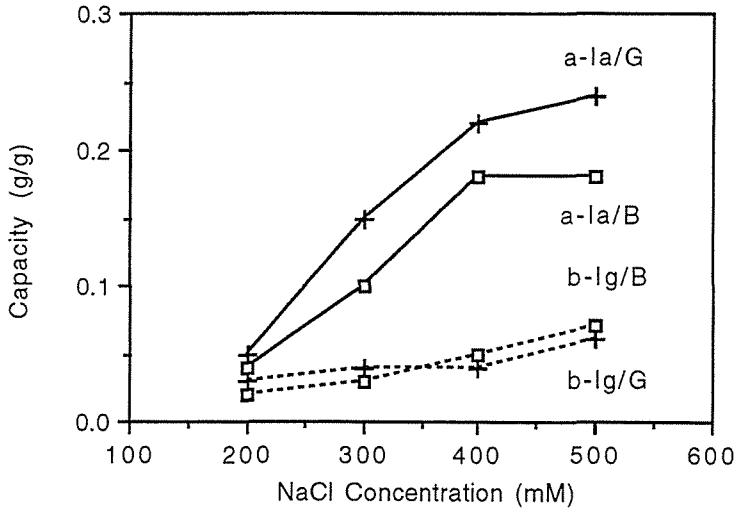
DMO-cellulose, prepared by activation with epichlorohydrin, has fairly short spacers, ie, a hydroxy propyl group, so that the alkyl groups are quite close the cellulose matrix. If the cellulose is not very porous in high salt concentration, it is difficult for protein molecules to access the alkyl groups. Therefore it was thought that a longer spacer might be advantageous to place the alkyl group further out from the cellulose matrix and avoid steric hindrance. Thus, a DMO-cellulose product with longer spacers was prepared by activating cellulose HP8-50 by 1,4-butanediol diglycidyl ether and coupling N,N-dimethyloctylamine. This exchanger placed the alkyl group 12 atoms out from the cellulose chains, compared with 3 when epichlorohydrin was used. Its substitution level was 0.55 meq/g. Although two epoxy groups in every ether molecule probably lead to higher cross-linking than when epichlorohydrin was used (shown by the increase of dry matter content to 20%), the exchanger made from it showed higher capacity for α-lactalbumin in 400 mM sodium chloride at pH 2.5 than that with a similar substitution level made from epichlorohydrin. If the curve of capacity vs substitution level of DMO-Cell activated by ECH was extrapolated to a substitution level to 0.55 meq/g in Figure 3.15, a capacity below 0.1 g/g would have been observed in 400 mM sodium chloride versus a value of 0.14 g/g obtained when 1,4-butanediol diglycidyl ether was used to prepare the 0.55 meq/g

product. Therefore long spacers are indeed advantageous for more protein molecules to access the alkyl group..

Because of the large structure of the ether, it was only possible to make a exchanger with a substitution level up to 0.55 meq/g by a single process.

The high cost of 1,4-butanediol diglycidyl ether also hinders its practical application.

Figure 3.16 Binding of a-lactalbumin and b-lactoglobulin at pH 2.5 to (B) beaded DMO-Cell and (G) ground DMO-Cell



3.2.7 Effect of Calcium Ion

This work has been carried out using WPI as the starting material for the isolation of α-lactalbumin. Since the purification of WPI by ion exchange is a fairly expensive process it would be preferable to use a UF retentate of whey as starting material. UF retentate of whey contains inorganic salts. Calcium , sodium and potassium ions are the main cations. The type of salt was important to the hydrophobicity of HIC because it has different contribution to the surface tension of proteins. Monovalent ions were recommended for HIC. K^+ ion was considered better than Na^+ ion. On the other hand, when whey protein was adsorbed by phenyl-Seph at pH 7.5 it could be eluted by even a low concentration of calcium (33 mM) (Lindahl L & Vogel H. J., 1984). On the other hand the precipitation of milk salt forming above pH 4.6 decreases resin efficiency. Further, the hydrophobicity of α-lactalbumin decreases after binding calcium ion. Unlike usual adsorption experiments usually done in neutral buffer, this work prefers to use DMO-Cell in high salt concentration at low pH, ie., 2.5. Therefore, it was essential to survey the effect of calcium chloride being abundant in UF retentate of whey on the adsorption of the proteins on DMO-Cell. Since 400 mM calcium chloride precipitated α-lactalbumin immediately two samples of 0.25 g of wet DMO-Cell were mixed with 10 ml of 0.25% of α-lactalbumin in 100 and 300 mM calcium chloride at pH 2.5. The capacities obtained were 0.04 and 0.22 g/g respectively. This indicates that calcium chloride in UF retentate does not have an adverse influence on the adsorption of α-lactalbumin at least.

The different influence of calcium on binding α-lactalbumin at pH 7.5 and 2.5 also confirm that pH indeed has a significant effect on the hydrophobicity of α-lactalbumin.

Because the adsorption occurred at low pH there was no risk of the precipitation of milk salt. The pretreatment to remove inorganic salt was able to be ignored in the adsorption of α-lactalbumin by DMO-Cell.

3.2.8 Effect of Washing on Elution of Protein

In Figure 3.13 DMO-Seph column (1 g) was saturated by 10% WPI in 500 mM NaCl at pH 2.5. The column was then washed by passing through 6 ml of 500 mM NaCl at pH 2.5 at a flow rate 0.1 ml/minute. Not only β-lactoglobulin but also 11 mg of α-lactalbumin were found in the washing. This indicates that an extensive washing process brings a large amount of α-lactalbumin, which binds to the DMO-Seph column reversibly, off the column. To remove most of the β-lactoglobulin and the least amount of α-lactalbumin from the exchanger a suitable washing process must be found.

Because of these reasons a fast and stepwise washing of DMO-Cell saturated with α-lactalbumin or β-lactoglobulin was tested. Thus, wet DMO-Cell (5 g) was mixed with 20 ml of 1% solution of the protein in 400 mM sodium chloride at pH 2.5 for 2 hours, then, the exchanger was filtered and sucked dry. α-Lactalbumin (154 of 156 mg) was adsorbed. Then, four of samples of the resin, 1.0 g each, were soaked in 1 ml of 400, 500, 600 and 700 mM sodium chloride at pH 2.5, respectively, for 1 min., These samples were then filtered. Air pressure was used to force as much liquid as possible to leave the exchangers. The drained-dry exchanger was treated in this way washing procedures 2-3 times with different soaking time. Unlike the slow and contiguous washing, there was no α-lactalbumin in the filtrates except 0.2 mg from that sample being soaked in 400 mM sodium chloride at pH 2.5 for 5 minutes. Thus, this way of washing was safe and retained α-lactalbumin on DMO-Cell

α-Lactalbumin was replaced with β-lactoglobulin and the process was repeated. Different results were observed. DMO-Cell (5 g) bound 32 of 166 mg of β-Lactoglobulin from 1% solution. β-Lactoglobulin was washed away from DMO-Cell each time it was washed (Table 3.12). Thus, this way of washing was necessary to remove some β-lactoglobulin which was bound reversibly under these condition.

Table 3.12 Elution of β-Lactoglobulin by Salt

NaCl(mM)	400	500	600	700
β-lg Eluted (mg)				
1 min.	0.9	0.6	0.6	0.8
then, 7 minutes.	1.0	1.1	0.7	0.5
then, 5 minutes	0.1	0.4	0.5	0.4

3.2.9 Effect of Cellulose Particle Size

To investigate the effect of particle size of the cellulose, five more products of DMO-Cell with particle sizes ranging from 40 to 350 um were synthesised with the same procedure and chemical consumption as those for cellulose (150-210 um) (shown in Table 2.2.). The substitution levels of the products are shown in Table 3.13. For comparison DMO-Cell (150-210 um) made in the previous section is listed in this table.

Table 3.13 Substitution Levels of Cellulose with Particle Sizes Ranging from 40 to 350 um

Particle Size (um)	40-100	70-150	150-210	210-250	250-300	300-350
Sub.* (meq/g)	0.92	1.03	0.97	1.17	1.1	1.0

Sub.* =Substitution level

Samples of each of the five preparations (39 mg dry weight) were mixed with 10 ml of 0.25% solution of protein in 400 mM sodium chloride at pH 2.5 for 2 hours to determine their capacities. The results are shown in Figure 3.17. Particle size had significant influence on the adsorption capacity. The bigger the particle was, the less protein it bound. The products made from cellulose with particle size less 210 um had satisfactory capacities. The effect of particle size was the result of bigger particles having lower ratios of surface area to mass. It was also observed that although the product made from 40-100 um cellulose had similar size to DMO-Seph, its capacity was only about half of the latter's, 0.3 and 0.8 g/g, respectively. This difference is a result of the greater porosity of the agarose.

3.2.10 Rate of Protein Adsorption

The rate at which α-lactalbumin was adsorbed from 40 ml of 0.25% α-lactalbumin in 400 mM sodium chloride at pH 2.5 by DMO-Cell (144 mg of dry matter) on the smallest and largest particles was measured over 5 hours. The results are displayed in Figure 3.18. Although small size particles (40-100 um) adsorbed α-lactalbumin faster than the large one (300-350) in the first 10 minutes there was little difference beyond the point. Both of them required 180 minutes to reach their saturation.

To see whether this long adsorption time was the result of the short spacer holding the DMO group close to the cellulose surface DMO-Cell prepared by activation with 1,4-butanediol diglycidly ether (Section 3.2.5) was tested. This product presented a similar adsorption rate to those products with short spacer up to 90 minutes. It required 120 minutes to reach saturation. The close adsorption rates of these three products suggested that binding rate of α-lactalbumin to the DMO group is not related to the collision between the protein and the octyl group. That it took such a long time to reach saturation was probably related to the properties of hydrophobic interaction adsorption, ie., relatively weak interaction and a slow and reversible conformational change or reorientation of the

proteins on the alkyl group, which possibly is the rate-limiting step (Jennissn H. P., 986) (Wu et al, 1986).

Figure 3.17 Binding of a-lactalbumin and b-lactoglobulin in 400 mM NaCl at pH 2.5 to DMO-Cell (Size range from 40-350 um)

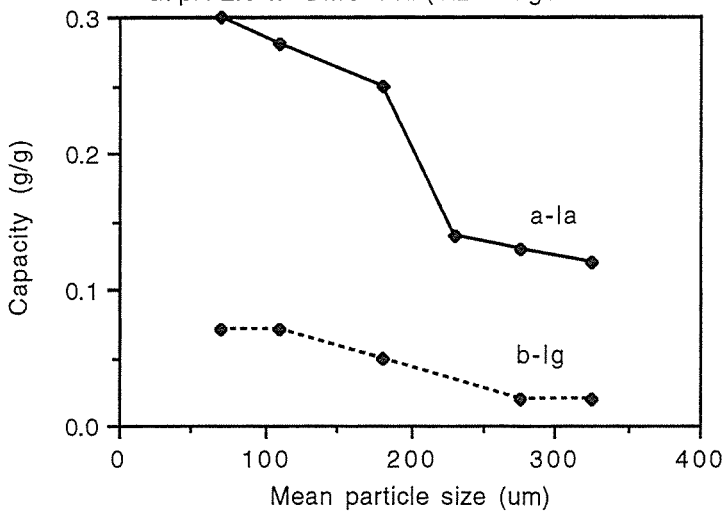
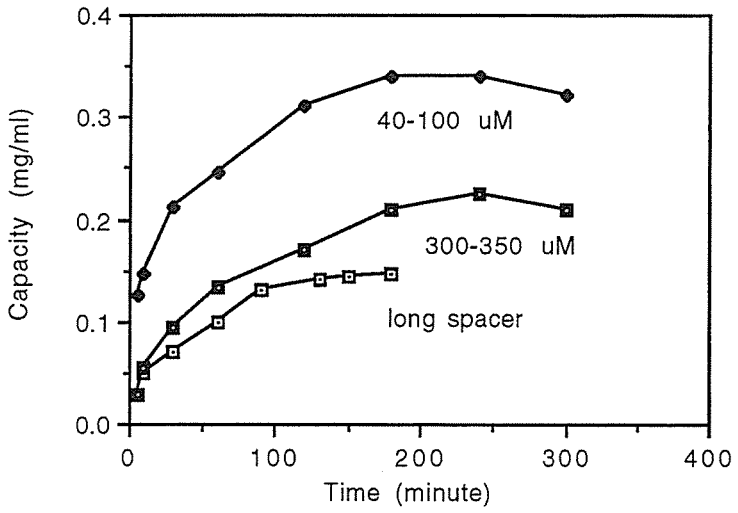


Figure 3.18 Kinetics of adsorption of a-lactalbumin in 400 mM NaCl at pH 2.5 to DMO-Cell



3.2.11 Effect of Ratio of Resin to Protein

Figure 3.19 shows the protein adsorbed by DMO-Cell (size 150-210 um) and DMO-Seph from 10 ml of 0.25% α-lactalbumin and β-lactoglobulin (containing 20 mg of protein) in 400 mM sodium chloride at pH 2.5 using different ratios of resins to the protein. The affect of the ratio on the binding of α-lactalbumin was much greater than that on β-lactoglobulin. The amount of α-lactalbumin bound increased by 8-9 mg, compared with about 2 mg of β-lactoglobulin, when increasing the wet resins used from 0.1 to 0.4 g. These results confirm that DMO-Seph and DMO-Cell interact with α-lactalbumin much stronger than with β-lactoglobulin and indicate that it is necessary to use a high ratio of resin to protein to separate α-lactalbumin from β-lactoglobulin.

Even with 0.5 g of DMO-Cell/20 mg of α-lactalbumin only about 12 mg of the α-lactalbumin was bound. This indicates that it is difficult to recover all of the α-lactalbumin from the solution in a batch method and that not all of the α-lactalbumin in WPI will be able to recover. Similarly for DMO-Seph except that its higher capacity allowed 80% of the α-lactalbumin to be recovered.

3.2.12 Column Binding of α-Lactalbumin at Two Concentrations

To investigate the binding property of DMO-Cell in a column process, α-lactalbumin (40 ml at 1.7 mg/ml) in 400 mM sodium chloride at pH 2.5 was passed through 2 g of wet DMO-Cell in 2 ml Pierce column at a flow rate 0.2 ml/min. The breakthrough fractions were collected and analysed by HPLC. The results are displayed in Figure 3.20. Although α-lactalbumin was bound completely in the very early fractions, only 59% of the protein was bound after 40 ml had been loaded (68 mg of α-lactalbumin). After elution of the column by dilute hydrochloric acid at pH 1.5, 48 mg (70%) of the protein was recovered.

When the experiment was repeated at the same flow rate but using 10 ml of α-lactalbumin at 7.1 mg/ml in 400 mM sodium chloride at pH 2.5, the column bound only 37% of the protein after 68 mg had been loaded (10 ml). After eluting the column with dilute hydrochloric acid at pH 1.5, 32 mg (47%) of the protein was recovered. The poor uptake of α-lactalbumin is probably a result of the slow adsorption kinetics shown in Figure 3.18.

These results suggest that the recovery of α-lactalbumin by a column process should be carried out on a low concentration for the protein solution unless a slower flow rate improves the adsorption efficieny. This was investigated further using WPI.

Figure 3.19 Effect of ratio of protein to
(C) DMO-Cell, (S) DMO-Seph

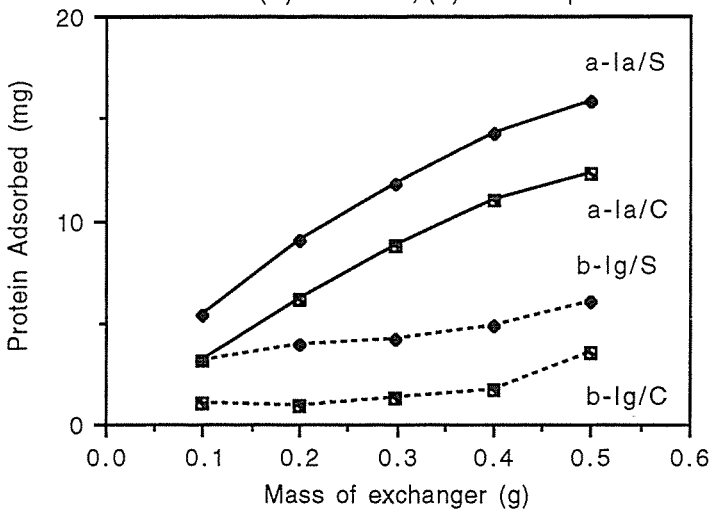
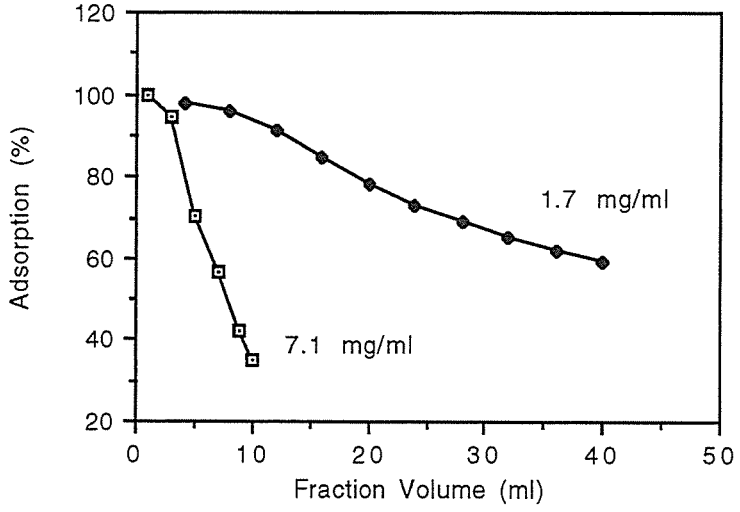


Figure 3. 20 Adsorption of a-lactalbumin at 1.7 and 7.1 mg/ml in
400 mM NaCl at pH 2.5 to DMO-Cell with column process



3.2.13 Recovery of α-Lactalbumin from Whey Protein Isolate.

3.2.13.1 By DMO-Cell Column

As mentioned in the previous section, the chromatographic adsorption of α-lactalbumin from a high concentration of α-lactalbumin by DMO-Cell was unsatisfactory. This was confirmed by the adsorption of the α-lactalbumin from a 4% solution of WPI containing α-lactalbumin and β-lactoglobulin at concentration of 6 and 25 mg/ml respectively. As seen in Figure 3.21, α-lactalbumin was found as early as in the first 3 ml of breakthrough. Slowing the flow rate to 2.4 BV/hour (0.04 ml/min) was not able to help the binding of this protein. The amount of α-lactalbumin in the breakthrough was almost same at two flow rates.

This is the result of the slow adsorption of α-lactalbumin by hydrophobic interaction as shown in batch process. Compared with the quick saturation of the column by β-lactoglobulin, the column still bound α-lactalbumin although it was not able to completely bind the protein.

3.2.13.2 By DMO-Cell Batch

To find the optimum amount of DMO-Cell (150-210 um) to use to recover α-lactalbumin from WPI, different ratios were applied to three WPI products, ie., WPI (MP0162), WPI (PT8253) and WPI freshly prepared in the laboratory. The latter was obtained by eluting GibcoCel Q2H used to treat the UF retentate of cheese whey as described in the Experimental Section. Major proteins in WPI (MP0162) were α-lactalbumin and β-lactoglobulin. WPI (PT8253) contained not only α-lactalbumin and β-lactoglobulin but also a large amount of GMP. The third WPI had a low ratio of α-lactalbumin to β-lactoglobulin compared with WPI (MP0162) and WPI (PT8253). The protein compositions of these WPI's are shown in Tables 3.14 to 3.16.

The recovery of α-lactalbumin from these WPI by DMO-Cell (0.94 meq/g) in high salt concentration at low pH were carried out using a batch method. The results are shown in Tables 3.14-3.16.

A WPI (MP0162)

MP0162 (10 ml of 4%) in 400 mM sodium chloride at pH 2.5 was tested with 1.0, 2.0, 2.4 and 3.0 g of wet DMO-Cell (150-210 um). Table 3.14 shows the results. When 1.0 g

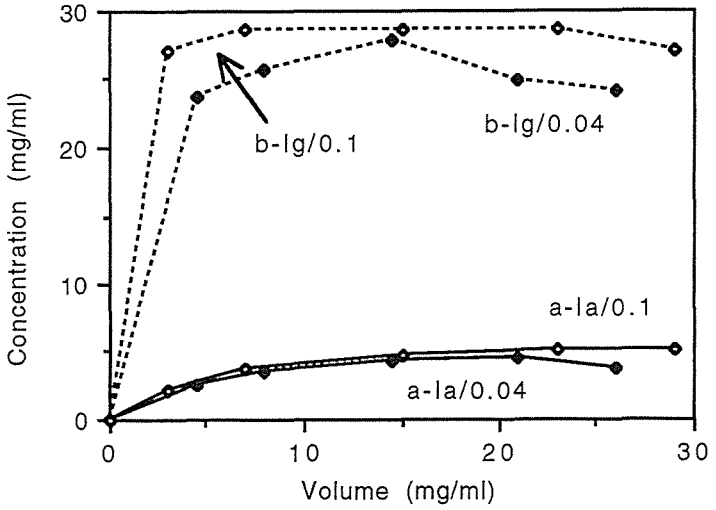
of resin was used 67% of the α-lactalbumin was bound by DMO-Cell. In contrast, only 1.4% of the β-lactoglobulin and 7% of the BSA were recovered by the exchanger. Because of GMP's strong hydrophilicity and the pH of 2.5 was below isoelectric point (pH 2.5-4.2), all of the GMP was left in the filtrate. When the amount of DMO-Cell was increased from 1.0 to 3.0, relatively constant amount of α-lactalbumin (40-44.5 mg) was bound by all of these exchangers. Excess exchanger caused more binding of BSA and β-lactoglobulin. This led to the decrease in purity of the recovered α-lactalbumin from 88 to 75%. Thus, by selecting a ratio of α-lactalbumin to exchanger of 60 mg/1.0-2.0 g an excellent purity, 84-88% of the recovered α-lactalbumin, was achieved. The capacity was also reasonable, 16-32 mg/ml, when 1-2 g of exchanger was used for 60 mg of α-lactalbumin contained in the WPI. The weakness of DMO-Cell was that it took 3 hours to bind the protein.

To observe the effect of resin particle size 5 ml of 4% WPI (MP0162) solution in 400 mM sodium chloride at pH 2.5 was tested with 0.6, 0.9, 1.5 g of wet DMO-Cell with a particle size of 40-100 um (Table 3.14). DMO-Cell with small particles showed a better selectivity than that with 150-210 um particles. It hardly bound any β-lactoglobulin. Almost pure α-lactalbumin (96%) was able to be obtained by a proper ratio of WPI to resin. This finer product also gave a better recovery of α-lactalbumin. By using 1.5 g of resin with 5 ml of 4% WPI solution an 85% yield of α-lactalbumin was obtained with a purity of 84%.

B WPI (PT8253)

Only one ratio of resin to α-lactalbumin was tested using WPI (PT8253) (Table 3.15). In spite of the presence of large amount of GMP in the WPI, DMO-Cell (150-210 um) showed similar property of binding of all components with that shown with WPI (MP0162). A purity and a yield of α-lactalbumin of 84 and 67% respectively were achieved at the ratio of 125 mg/5.0 g (α-lactalbumin/wet exchanger).

Figure 3.21 WPI (4%) solution in 400 mM NaCl at pH 2.5 processed by DMO-Cell column at flowrates 0.04 and 0.1 ml/min



C Eluate from Q2H used to treat the UF Retentate of Cheese Whey (a low α-lactalbumin content WPI)

The recovery of α-lactalbumin from 10-15 ml of this eluate (about 4% protein) in 400 mM sodium chloride at pH 2.5 with 1-5 g of wet DMO-Cell (150-210 um) was carried out at 21 and 26 °C. A two hour adsorption time was used in both cases. The results are displayed in Table 3.16.

(a) Adsorption at 26 °C

When 1.0 g of DMO-Cell was applied to 20 ml of the WPI solution containing 45 mg of α-lactalbumin it was only able to recover 49% of the α-lactalbumin efficiently. α-Lactalbumin (77%) with purity of 69% was recovered from 15 ml of WPI solution containing 33.8 mg α-lactalbumin by 2.0 g DMO-Cell (Table 3.14). A ratio of α-lactalbumin/exchanger lower than this 17 mg/g was not able to increase the binding of α-lactalbumin significantly. Compared with the results shown in Table 3.16 more exchanger was needed to achieve similar yield of α-lactalbumin from this WPI product than those from WPI (MP0162). This was the result of this WPI product having a much lower ratio of α-lactalbumin to β-lactoglobulin (0.13-0.17/1) than WPI (MP0162) (0.21/1) and WPI (PT8253) (0.31/1)

Insufficient washing before elution by dilute hydrochloric acid at pH 1.5 caused the presence of large amount of the contaminants, β-lactoglobulin and GMP, which should not have been in the eluate (see footnote to Table 3.16).

Table 3.14 Recovery of α-Lactalbumin from WPI (MP0162) by DMO-Cell

Mass of wet resin (g)	1.0	2.0	2.4	3.0	0.6	0.9	1.5
Particle size (um)	150-210	150-210	150-210	150-210	40-100	40-100	40-100
Volume of WPI (ml)	10	10	10	10	5	5	5
Load (mg)							
α-la	59.2	59.2	59.2	59.2	31.5	27	27
β-lg	316	316	316	316	146	126	126
BSA	12.3	12.3	12.3	12.3	4.8	4.0	4.0
GMP	4.8	4.8	4.8	4.8	2	0	0
Filtrate (mg)							
α-la	18.6	7.2	7.5	7.5	8	6.6	5.2
β-lg	319	243	301	234	134	127	125
BSA	11.4	6.9	7.8	5.1	3.2	3.4	2.6
GMP	4.8	3.9	3.9	3.9	1.8	0	0
Eluate (mg)							
α-la	40	40	44.4	44.5	21	21	23
β-lg	4.6	5.9	9.1	11.4	0.2	1.5	1.6
BSA	0.8	1.7	2.9	3.3	0.7	1.8	2.9
GMP	0	0	0	0	0	0	0
Purity of α-la (%)	88	84	78	75	96	86	84
Yield of α-la (%)	67	67	75	75	68	78	85
Capacity for α-la (mg/ml)	32	16	15	12	27	17.5	11.5

* Particle sizes were medium (150-210 um) and fine (40-100 um) and adsorption times were 3 and 2 hours respectively.

Table 3.15 Purification of α-lactalbumin from WPI(PT8253) by DMO-Cell (150-210 um)

Proteins	Load (mg)	Filtrate (mg)	Eluate (mg)	Purity (%)	Yield (%)	Capacity (mg/ml)
α-la	125	35	84.3	84	67	13.5
β-lg	407	410	9.8			
BSA	6.3	7	3.9			
GMP	87	98	2			

*DMO-Cell (5 g) was mixed with 20 ml of 4% WPI (PT8253) for two hours.

(b) Adsorption at 21 °C

It can be seen from Table 3.16 that insufficient exchanger (1g per 60 mg of α-lactalbumin) resulted in a low yield of α-lactalbumin. Using 2 g of the exchanger per 45 mg of α-lactalbumin increased the yield to 68% with excellent purity (91%). It required a big increase in the amount of DMO-Cell to further increase the yield and purity suffered. To recover 70% of α-lactalbumin with high purity a ratio of α-lactalbumin (contained in WPI) to exchanger of 22 mg/g should be selected.

Sufficient washing with binding buffer before elution by dilute hydrochloric acid at pH 1.5 gave good purity of the recovered α-lactalbumin (> 90%).

The results of the recovery of α-lactalbumin from these three WPI products by DMO-Cell indicates that DMO-Cell shows relatively high selectivity for α-lactalbumin by hydrophobic interaction in high salt concentration at low pH. DMO-Cell could be useful to purify α-lactalbumin from WPI products.

After the WPI solution had been mixed with DMO-Cell for 2-3 hours in the summer the filtrate was cloudy. This was probably the result of irreversible denaturation of part of the WPI. So the recovery of α-lactalbumin from WPI by DMO-Cell should be carried out at 10-20 °C.

Table 3.16 Purification of α-Lactalbumin from an Anion WPI with a low α-lactalbumin content by DMO-Cell

Mass of resin (g/g)	1.0	2.0	3.0	4.0	1.0	2.0	3.0	4.0	5.0
Temperature (°C)	26	26	26	26	21	21	21	21	21
Load (mg)									
α-la	45	33.8	33.8	33.8	60	45	45	45	36
β-lg	334	250	250	250	360	270	270	270	216
BSA	0	0	0	0	0	0	0	0	0
GMP	132	99	99	99	115	86	86	86	69
Filtrate (mg)									
α-la	20.5	5.0	3.5	3.5	27	7.5	5	3.5	2
β-lg	326	228	171	234	346	259	257	260	204
BSA	0	0	0	1.5	0	0	0	0	0
GMP	118	86.5	66	88	109	81	82	82	67
Eluate (mg)									
α-la	22	26.1	26.7	28.2	25.5	30.8	33	34	28
β-lg	3	6.4	6.4	9.7	1.5	2.4	5.5	5.5	7.6
BSA	0.3	0.3	0.5	1.4	0	0	0	0	0
GMP	3.7	5.2	3 0	5.6	0.7	0.8	1.2	1.1	1.2
Purity of α-la (%)	76	69	73	63	93	91	83	84	76
Yield of α-la (%)	49	77	79	83	43	68	73	73	78
Capacity for α-la (mg/ml)	18	10.4	7.1	5.6	20	12.3	8.8	6.8	4.5
Load α-la/exchanger (mg/g)	45	16.9	11.3	8.5	60	22.5	15	11.2	7.2

1. The tests at 21 °C used air pressure to expel the remaining WPI from the exchanger between draining the filtrate and washing with 400 mM NaCl at pH 2.5.
2. The tests at 26 °C only used washing with 400 mM NaCl at pH 2.5 without using air pressure to expel the remaining WPI from the exchanger.

4. Conclusion

Q-Seph-ff, DMEA-Seph, DMH-Seph, and DMO-Seph bound both of proteins, α-lactalbumin and β-lactoglobulin in 25 mM NaCl., at pH > IEP by ion exchange. Q-Seph-ff, DMEA-Seph, DMH-Seph did not bind either of the proteins at pH < IEP. DMO-Seph, DMD-Seph, DMDo-Seph, and Do-Seph bound both of the proteins, especially α-lactalbumin, by hydrophobic interaction at and below the proteins' IEP.The proteins bound by DMD-Seph, DMDo-Seph were not able to be eluted by dilute hydrochloric acid at pH 2.5. Dilute hydrochloric acid at pH 2.5 could be used to elute these proteins from DMO-Seph.

DMO-Seph,DMD-Seph,DMDo-Seph and Do-Seph showed strong hydrophobic affinity for α-lactalbumin but not β-lactoglobulin in 200-500 mM NaCl.

Recovery of native α-lactalbumin from WPI in 400 mM NaCl at pH 2.5 by DMO-Seph gave an α-lactalbumin yield and purity of 79 and 73% and a capacity of DMO-Seph 0.73 g/g in 400 mM NaCl at pH 2.5, compared to 67%, 84% and 16 mg/g of DMO-Cell.

DMO-Cell, activated by 1,4-butanediol diglycidyl ether, with substitution level 0.55 meq/g showed a better binding capacity than DMO-Cell activated by epichlorohydrin.

Recovery of native α-lactalbumin from different WPI in 400 mM NaCl at pH 2.5 by DMO-Cell showed good selectivety of DMO-Cell for α-lactalbumin from all of three WPI. From a low α-lactalbumin content WPI this gave an α-lactalbumin yield and purity of 70 and 91%.

Chapter 2 Osteopontin Recovery by DMEA-Cell

Introduction

Proteose peptone is the whey protein fraction that remains in solution after heat treatment at 90 °C for 30 minutes followed by removing casein at pH 4.7 (Rowlands, 1937 &1938). It represents about 10% of total whey protein (Nejjar et al. 1986). It is a complex mixture of glycoproteins, phosphoproteins and peptide, containing at least 38 components.(Andrews & Alichanidis, 1983) (Paquet, 1989)

Osteopontin is one of three major proteins present in the proteose peptone fraction of bovine milk. It is present in a concentration of 3-10 mg/litre of milk. The molecular weight of osteopontin of bovine milk is 60 Kd. It is an acidic glycoprotein rich in aspartic acid, glutamic acid and serine and contains carbohydrate and large amounts of phosphate (Sorensen & Petersen, 1993).

Osteopontin is an important component in the formation of bone. It might function as a cell attachment factor (Oldberg et al, 1986). It may contribute to the oncogenic phenotype (Craig et al, 1989, 1990) and to early resistance to bacterial infections (Fet et al, 1989)

Osteopontin has been isolated from human milk by DEAE-Trisacryl chromatography, barium citrate affinity chromatography and reversed-phase HPLC (Senger et al., 1989). It has also been purified from bovine milk by Sephadex G-75 gel chromatography, Q-Sepharose ion-exchange and additional Sephadex G-75 gel chromatography in the presence of urea (Sorence & Petersen, 1993).

Osteopontin is very acidic and thus tightly binds to anion exchangers such as DEAE-Trisacryl. When the proteins were eluted from anion exchangers after milk had been passed through, osteopontin was eluted last.

The purpose of the research was to prepare an anion exchanger (DMEA-Cell) with relatively low substitution levels by coupling different amounts of N,N-dimethylethanolamine to cellulose. The objective was to try to bind and concentrate osteopontin from raw skim milk without adsorbing too many other components of skim milk, particularly the major whey proteins α-lactalbumin and β-lactoglobulin. It was hoped that at a sufficiently low substitution level the ionic strength present in milk would prevent the binding of these major whey proteins but not that of osteopontin.

Experiment.

1 Synthesis of DMEA-Cell.

The synthesis of DMEA-Cell consists of the modification of cellulose, activation of the modified cellulose HP8-50 and coupling with N,N-dimethylethanolamine (DMEA). The procedure of preparing HP8-50 had been introduced by Lilly M. (1988). Thus, cellulose with size range from 150 to 210 um (40 g) was added to a stainless bomb cooled to below 5 °C. NaOH (60 ml of 30%), cooled to below 5 °C, and a mixture containing 3.2 ml of epichlorohydrin and 20 ml of propylene oxide were added into the bomb simultaneously. The mixture was mixed evenly until the cellulose had swelled. The bomb was sealed and heated in a water bath at 45 °C for 1.5 hour. The bomb was then cooled to room temperature within 0.5 hour. The cellulose was transferred to a filter. The large excess NaOH in the HP8-50 was washed away by water. The resin was then drained of excess water on a sintered glass filter and divided into eight equal parts. Water was added to each part again to make a slurry. NaOH (30%) was added to the slurry to adjust the concentration of alkali to 0.25, 0.5, 0.75 1.0, 2.5 3.0, 4.0, and 5.0%, respectively. These slurries were cooled to below 5 °C and 25% molar excess of epichlorohydrin (ECH) to NaOH was added. The slurries were placed on a roller in a cool room (4 °C) overnight, followed by 3 hours at 25 °C The celluloses were then washed with large amount of water to remove the residual epichlorohydrin and drained of excess water on a sintered glass filter. Water was added to make slurries again and about a 10 excess moles of N,N-dimethylethanolamine to epoxy group were added into the slurries. The dimethylethanolamine was 10% neutralised with HCl prior to use. These slurries were mixed on a roller at room temperature overnight, followed by being heated at 70 °C for 3 hours. The exchangers were then cooled to room temperature and collected on filters. The exchangers were then washed with 1 M HCl, followed by water until the washing was neutral. The exchangers were drained of excess water on sintered glass filters before being stored in a fridge.

All chemicals for the synthesis procedure are shown in Table 1.

Table 1 Synthesis of DMEA-Cell

	Modification			Activation				coupling		
Cellulose	ECH	PO	NaOH	ECH	H_2O	NaOH		HCl	Amine	Subst.
(g)	(ml)	(ml)	(ml)	(ml)	(ml)	(ml)		(ml)	(ml)	(meq/g)
5	0.4	2.5	7.5	0.43	25	0.5	0.5	0.7		0.04
5	0.4	2.5	7.5	0.85	25	1.0	1.0	1.5		0.15
5	0.4	2.5	7.5	1.46	35	2.3	1.5	2.2		0.30
5	0.4	2.5	7.5	3.40	35	2.0	2.1	3.2		0.36
5	0.4	2.5	7.5	5.60	45	6.7	5.0	5.0		0.88
5	0.4	2.5	7.5	6.80	45	8.2	5.0	5.0		0.87
5	0.4	2.5	7.5	9.50	45	11.4	5.0	5.0		1.07
5	0.4	2.5	7.5	12.3	45	14.8	5.0	5.0		1.17

Subst. = substitution level
Amine = N,N-dimethylethanolamine
NaOH = 30% aqueous solution
HCl = 1 mole/litre aqueous solution

2 Analysis of Substitution Level

This procedure was the same as that for DMO-Cell. Thus, an accurately weighed amount of moist DMEA-Cell (about 10 g for those with substitution levels below 0.4 meq/g and 2-3 g for those with substitution levels above 0.87 meq/g) was soaked in 1 M NaOH for 10 minutes. The exchanger was transferred to a sintered glass filter and then washed with large amount of water until the washing was neutral to pH indicator paper. The exchanger was completely transferred back into a clean titration cup and then 10 ml of 500 mM NaCl was added. The exchanger was then titrated with 0.1 M HCl to pH 4.0. The substitution level was calculated using the following equation:

$$\text{Substitution level (meq/g)} = N * V / (D * M)$$

N: Concentration of HCl (moles/litre)
V: Volume of HCl (ml)
D: Dry matter content of DMEA-Cell (%/100)
M: Mass of wet DMEA-Cell (g)

3 Recovery of Osteopontin by DMEA-Cell

Whole milk was obtained from a local farm. The fat in the milk was removed by centrifugation at the speed of 4300 rpm. for 10 minutes.

3.1 Adsorption of Osteopontin by a Single DMEA-Cell Column

DMEA-Cell, equivalent to a volume of 10 ml, was mixed with 50 mM NaCl and stirred to remove air bubbles. The slurry was then transferred to a 10 ml column (Pierce Chemical Company, Illinois, USA) and the resin bed was packed with the flow of additional 50 mM NaCl through the column under gravity. A peristaltic pump was then used to set the flow through the column at 5 BV/hr for 1 hour. Skim milk (100 ml) was then loaded through the column at this flow rate. The last 20 ml of breakthrough was collected. After the skim milk had been passed through the column, the resin was rinsed with further 50 mM NaCl until all traces of milk disappeared.

NaCl (10 ml of 0.35 M) was then passed through the column. The eluate was collected. The exchanger was left sitting in the NaCl for 1 hour. Another 10 ml of 0.35 M NaCl was then passed through the column and the eluates were combined and made up to 20 ml. The eluate was diluted 3 : 1 with 20 mM Tris at pH 8.0 and analysed by a Pharmacia Smart System using a Mono Q Column.

3.2 Batch Process

DMEA-Cell (0.5, 2.0 and 5.0 ml, respectively) was mixed with 50 ml of skim milk in a 80 ml beaker at 17-20 °C for 2 hours under gentle stirring. The exchanger was then collected on a filter and washed with 50 mM NaCl until there were no traces of milk in the breakthrough. Adsorbed proteins were eluted as described in Section 3.1 above. The eluate was diluted 3 :1 with 20 mM NaAc at pH 4.6 and analysed by the Smart System.

3.3 Double Column Process

The DMEA-Cell column in section 3.1 was replaced with two 5 ml DMEA-Cell columns connected in series. NaCl (50 ml of 50 mM) was passed through the columns for 2 hours to equilibrate them. Skim milk (50 ml) was then loaded through the columns. The breakthrough was collected from the outlet of the second column (Column2). After loading the milk 50 mM NaCl was loaded to clean the columns until the traces of milk disappeared.

The columns were then disconnected and eluted separately with 10 ml of 350 mM NaCl for 1 hour. The eluates as well as the breakthrough were analysed by Smart System.

The same experimental procedure was repeated using columns respectively packed with 0.5 and 2.0 ml of DMEA-Cell.

4 Whey Preparation

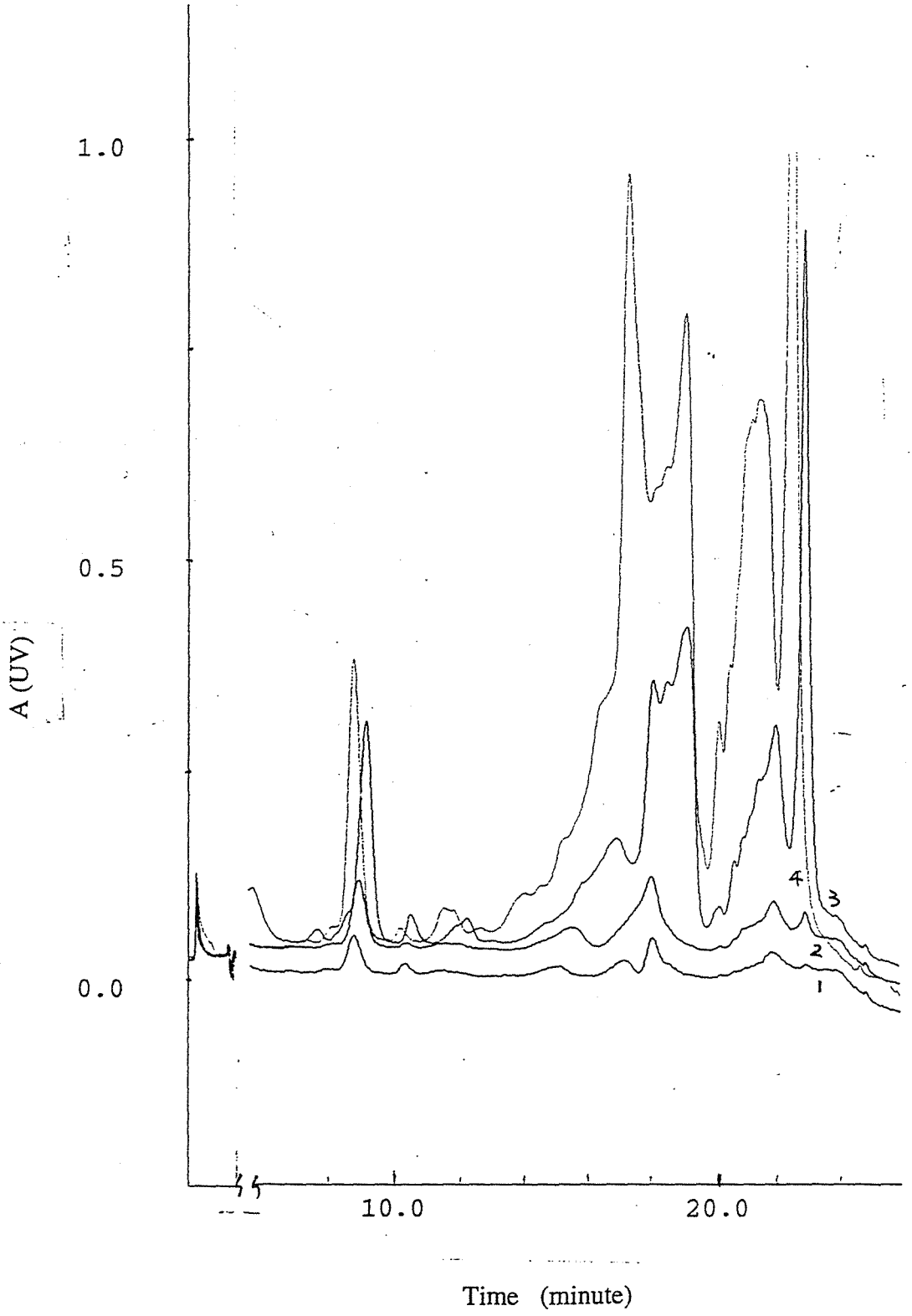
The pH of skim milk was adjusted to 4.6 by slowly adding 5 and then 1 M HCl. The precipitate was allowed to settle for half hour before centrifugation. The supernatant was analysed by Smart System.

5 Mono Q Column analysis using Phamacia Smart System

All collections were analysed by mono Q column using Pharmacia Smart System at a flow rate of 80 ul/minute (this was used for pre-equilibration, gradient and re-equilibration) at pH 4.6 or pH 8.0 at 20 °C by the methods developed by Massey University. The sample volume of each collection analysed was 20 ul. The details of these two methods are shown as follows:

	At pH 4.6:	At pH 8.0
Buffer A	20 mM NaAc at pH 4.6	20 mM Tris at pH 8.0
Buffer B	20 mM NaAc in 1 M NaCl at pH 4.6	20 mM Tris in 1 M NaCl at pH 8.0
Gradient (min)		
0	Concentration of B =10%	Concentration of B =10%
5	Concentration of B =10%	Concentration of B =10%
19	Concentration of B =30%	Concentration of B =30%
23	Concentration of B =100%	Concentration of B =50%
25	Concentration of B =100%	Concentration of B =100%
26	Concentration of B =100%	Concentration of B =100%
28	Concentration of B =10%	Concentration of B =100%
30	Concentration of B =10%	Concentration of B =10%

Figure 1. Eluates from DMEA-Cell column loaded with skim milk
1. DMEA-Cell (0.04 meq/g) 2. DMEA-Cell (0.15 meq/g)
3. DMEA-Cell (0.30 meq/g) 4. DMEA-Cell (0.36 meq/g)



Result and discussion

1 Synthesis of DMEA-Cell

To obtain a macroporous structure for the matrix cellulose was modified by epichlorohydrin and propylene oxide in the presence of sodium hydroxide. The modified cellulose (HP8-50) has a large swollen volume and suitable rigidity for chromatographic analysis. The detailed discussion of the synthesis of HP5-80 was described in Chapter 1.

HP8-50 was activated with epichlorohydrin in the presence of sodium hydroxide. The low concentration of sodium hydroxide significantly affected the activation levels (Table 1). Therefore, to obtain anion exchangers with low substitution levels (below 0.88 meq/g) the large excess sodium hydroxide in the HP8-50 must be washed away by water completely. The influence of sodium hydroxide levelled off at high concentration (above 4%). To obtain high substitution levels (above 0.8 meq/g) sodium hydroxide concentrations above 2.5% in the slurries were needed. Therefore the excess sodium hydroxide in HP8-50 need not have been washed away and could have been used in the activation step when DMEA-Cells with high substitution levels (above 0.88 meq/g) were made.

2 Effect of Substitution Level of DMEA-Cell

Skim milk (100 ml) was passed through a column packed with 10 ml of DMEA-cell with substitution levels 0.04, 0.15, 0.30, and 0.36 meq/g. After the last trace of milk was washed from the column the protein was eluted. The eluates was analysed by Smart System at pH 8.0. The results are shown in Figure 1. The single peak with retention time of 10 minutes represents α-lactalbumin. The forκ-like double peaks between 16 and 20 minutes, which covered peaks of some small peptides, represents the two variants of β-lactoglobulin. The last major peak at about 22-23 minutes represents osteopontin. The substitution levels of DMEA-Cell had crucial influence on its capacity for osteopontin. The exchangers with substitution levels 0.04 and 0.15 meq/g hardly bound any osteopontin but at 0.3 meq/g osteopontin appeared in the eluate. When substitution was increased by 0.06 meq/g from

0.30 to 0.36 meq/g much more osteopontin was bound. This suggested that a minium substitution level of 0.3 meq/g for DMEA-Cell is necessary to achieve capacitis for the protein in the presence of skim milk. However higher substitution levels are needed to achieve good capacity.

As compared with that in whey, where the peak of osteopontin was hardly seen in the presence of large amount of α-lactalbumin and β-lactoglobulin, the proportion of osteopontin in the eluates was high and a significant purification has been achieved (Figure 1).

Because of the electrostatic interaction between resin and other proteins α-lactalbumin, β-lactogobulin and osteopontin were bound simultaneously. However, because α-lactalbumin, β-lactogobulin had less charge than osteopontin the binding of α-lactalbumin and β-lactogobulin to DMEA-Cell with low substitution levels (below 0.36 meq/g) were weak. On the other hand, the minerals in skim milk, approximately equivalent to 50 mM NaCl, weakened the attraction interaction between the exchanger and α-lactalbumin and β-lactoglobulin. Most α-lactalbumin and β-lactogobulin were consequently passed straight through the DMEA-Cell column when milk was loaded.

3 Batch Process.

To find out an optimal ratio of exchanger to milk 0.5, 2.0, and 5.0 ml lots of DMEA-Cell (0.36 meq/g) were mixed with 50 ml of skimmed milk in batches. The mixture was then filtered. The whey prepared from the breakthroughs were analysed by Smart System at pH 4.6. The exchanger was eluted and the eluates were also analysed. The results are displayed in Table 2. Little osteopontin was found in the eluates. DMEA-Cell (0.5 ml) bound only 6% osteopontin in the milk. Increasing the amount of DMEA-Cell used was not successful in improving the recovery of the protein significantly. Therefore recovering osteopontin by batch was not a useful method. This possibly was the result of the low concentration of osteopontin in the milk.

Table 2 Batch Binding of Osteopontin from 50 ml of Skimmed Milk by DMEA-Cell

Volume of wet DMEA-Cell (ml)	0.5	2.0	5.0
Osteopontin in breakthrough (%)	92	91	81
Osteopontin in eluate (%)	6	7	12

4 Double Column process

To compare with the batch process two columns were packed, each having same amount of DMEA-Cell (0.36 meq/g) as that used in batch processing, ie., 0.5, 2.0, and 5.0 ml. The columns were then connected in series. Skim milk (100 ml) was passed through two columns. In each case the breakthrough and the eluates from each column were analysed. The results are displayed in Table 3. Although column process presented higher capacities than batch process (Table 2), the recovery of osteopontin was still very low. All of the columns were saturated. Most osteopontin was passed straight through the columns. To test DMEA-Cell with high substitution levels the DMEA-Cell (0.36 meq/g) packed in the columns was replaced with 5 ml of DMEA-Cell (1.17 meq/g) and the experiment was repeated. The first column bound 53% of osteopontin from the milk. No osteopontin was found in the eluate from the second column. The breakthrough contained 26% of the osteopontin in the milk. That the osteopontin in the breakthrough was not able to be bound by the underloaded second column suggests that this osteopontin was not in the milk serum. It has been suggested by Palmono. K. (Private Communication, 1999) that some of the osteopontin is tied up in the casein micelles and not liberated until the casein is precipitated.

Table 3 Distribution of Osteopontin in DMEA-Cell Columns and Breakthrough

Volume of DMEA-Cell in each column (ml)	0.5	2.0	5.0
Osteopontin in eluate from Column1 (%)	8	17	18
Osteopontin in eluate of Column2 (%)	7	14	9
Osteopontin in breakthrough (%)	78	47	50

Conclusion:

DMEA-Cell was able to recover osteopontin from raw skimmed milk. A high substitution level of charged groups on the exchanger was essential. Because osteopontin may also be held in the casein micelles, a large proportion of the osteopontin (50%) in milk could not be bound by DMEA-Cell (1.17 meq/g)

It was not successful to obtain osteopontin with satisfactory purity because some α-lactalbumin and β-lactoglobulin were bound simultaneously when using this DMEA-Cell with a standard substitution level.

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