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%.

Acknowledgements

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.

List of Tables

Chapter1.

Chapter2

List of Figures

Chapter 1

Chapter 2

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).

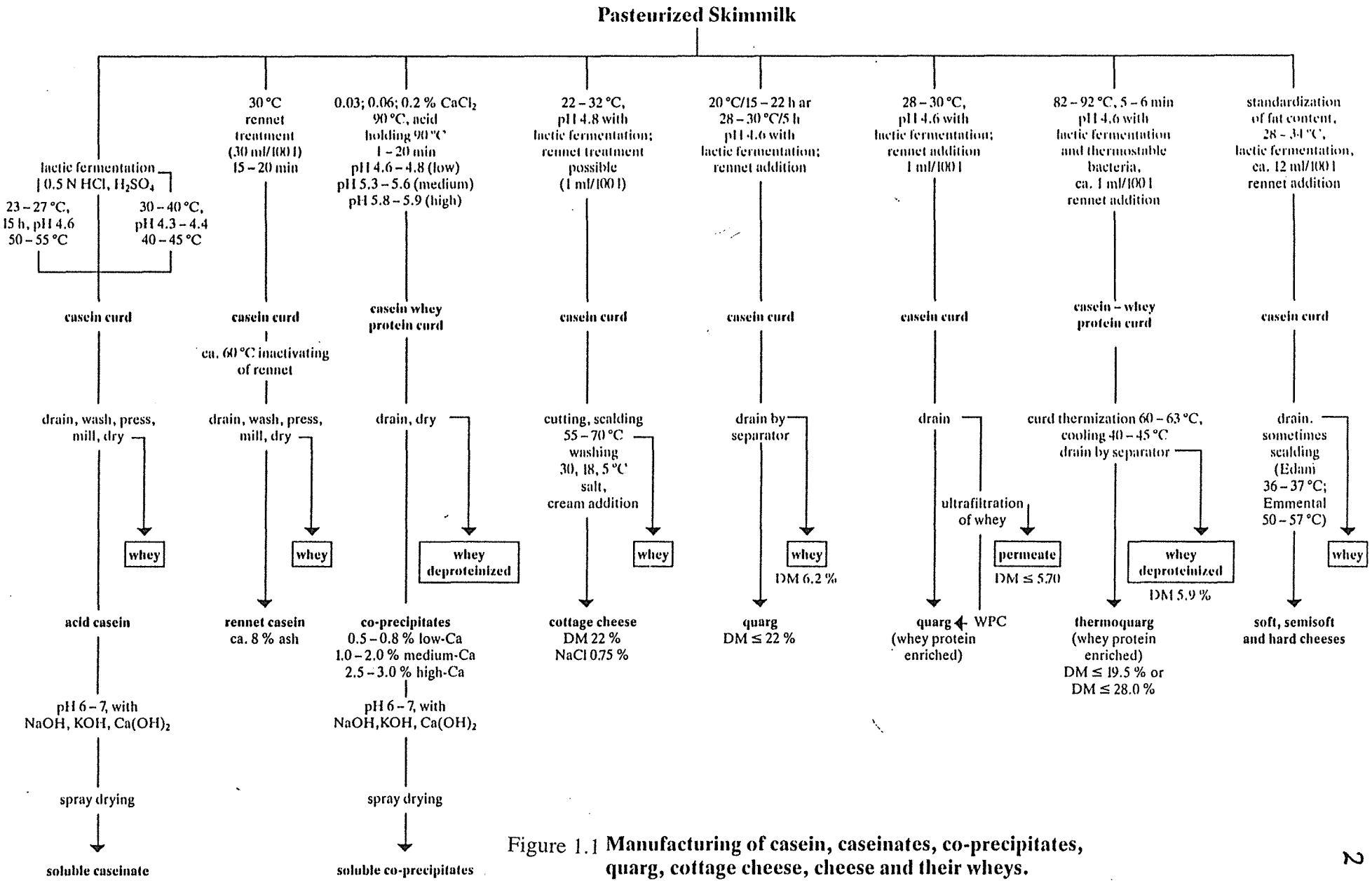


Figure 1.1 **Manufacturing of casein, caseinates, co-precipitates, quarg, cottage cheese, cheese and their wheys.**

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.

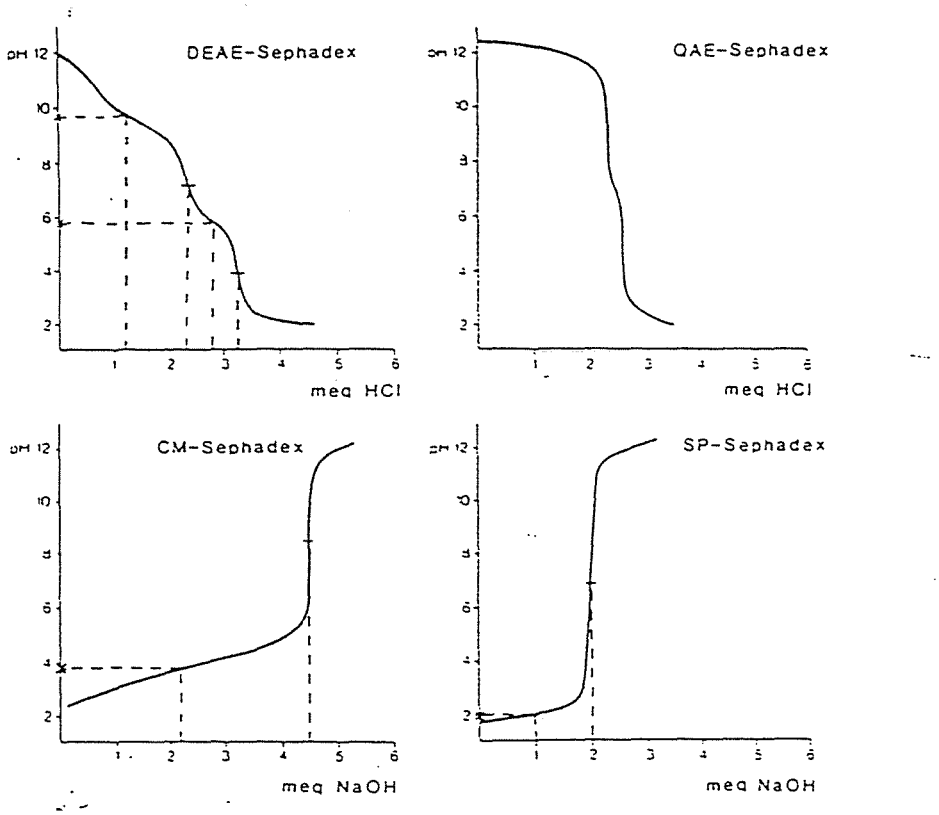
Although some of the fractions obtained by precipitation processes were rich in α-lactalbumin, its isolation with high purity from such fractions could not be achieved readily by salting-out procedures. Even the crystallised forms of α-lactalbumin and β-lactoglobulin were not completely homogeneous according 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

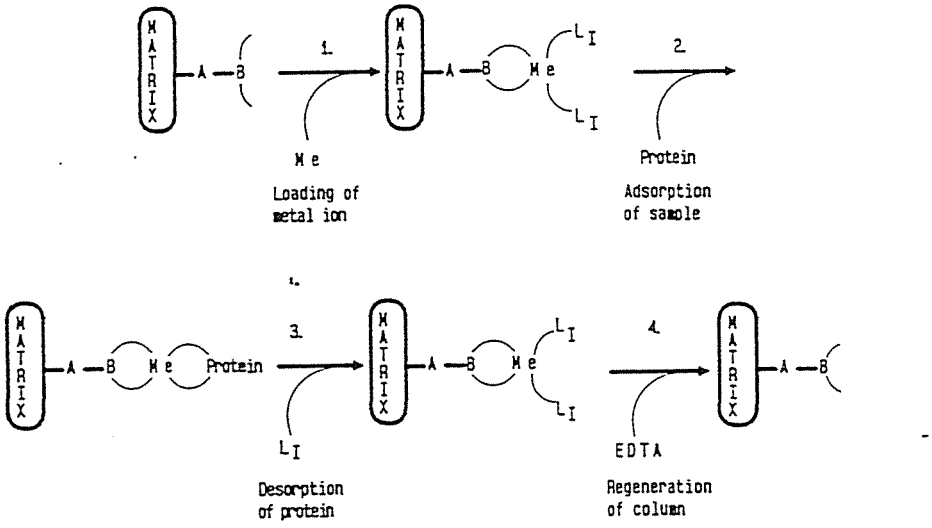


Figure 1.3. Principle of immobilized metal ion affinity chromatography. A = linkage (spacer) group, B = chelating group, Me = metal ion, L_I = solvent or buffer molecule.

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^+)-$NHCH_2(CH_2)_6CH_3$

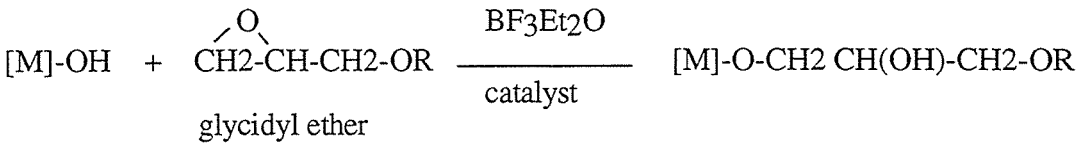
B [M]-O-CH_2-CH(OH)CH_2-O-$CH_2(CH_2)_6CH_3$

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).

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

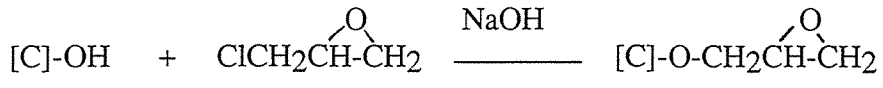
Strong base anion exchanger

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.

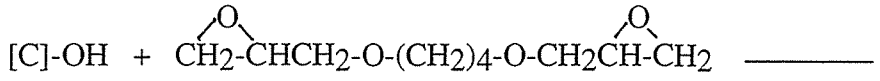
All of these methods put on secondary and tertiary amines first and then quaternized or then put on quaternary amino groups directly via an alkylating agent bearing the quaternary groups.

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:

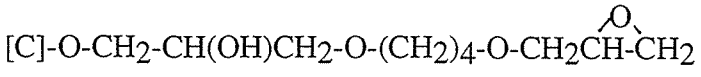
Activation:



Matrix Activated matrix



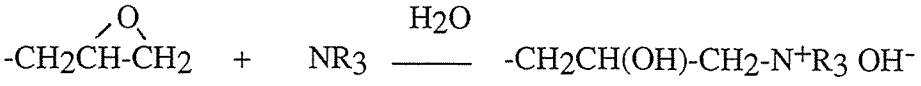
Matrix



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.

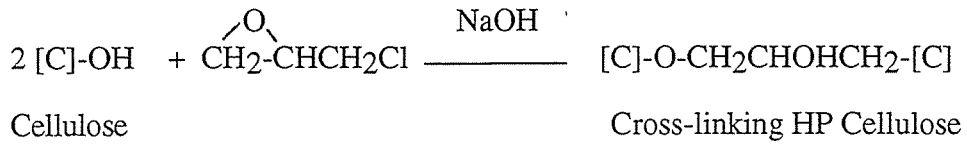
The cellulose ion exchangers for proteins were introduced in 1954 by Sober (1954) (1956) and Perterson (1956). These exchangers have been limitation because of their fibrous structure. They generally have low flow rates, become easily clogged by suspended particles and produce fines easily when handled. 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:

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

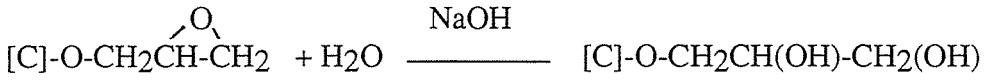
Cellulose

Hydroxypropyl group
HP Cellulose

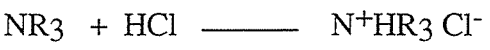


The swollen cellulose was then activated by epichlorohydrin. Finally, the activated cellulose was coupled with amines to make anion exchangers as shown in the activation and coupling reaction on the previous pages.

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.