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ISOLATION OF LACTOFERRIN FROM BOVINE COLOSTRUM BY CHROMATOGRAPHIC TECHNIQUES

BY

NORMAN TIANSHU ZHANG

A THESIS SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF SCIENCE

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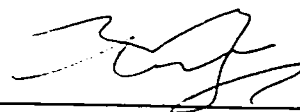

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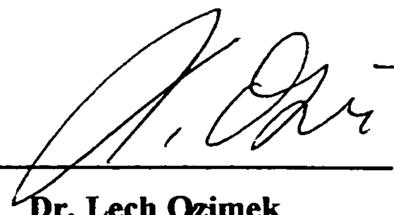

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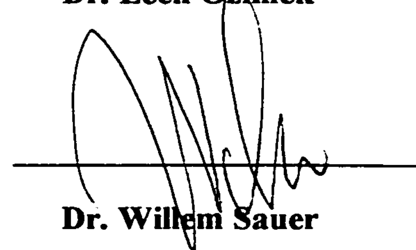

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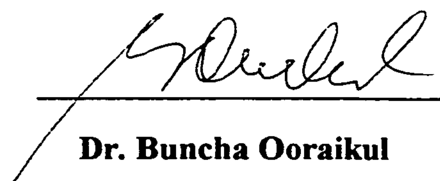

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ABSTRACT

Bovine lactoferrin is an iron-binding glycoprotein, which is claimed to possess many important biological functions, such as antimicrobial, iron binding and transportation. Some of the above biological functions of lactoferrin may lead to the development of novel functional ingredients and/or nutraceuticals. Therefore, the main objective of carried out research was to isolate lactoferrin from bovine colostrum by cation exchange chromatography on sp-sepharose big beads matrix (SSBB) and by affinity chromatography on single strand DNA agarose (SSDA) matrix.

The isolated lactoferrin was further analyzed by reverse-phase chromatography; size-exclusion chromatography; ion-exchange chromatography; SDS-PAGE; MALDI-MS and amino acid composition analysis.

Lactoferrin molecular weight was 78 KD as identified by SDS-PAGE and MALDI-MS. The binding capacity of lactoferrin was on average 39 mg/mL of sp-sepharose big beads gel and 12 mg/mL of single strand DNA agarose matrix, respectively. The recovery of standard LF to sp-sepharose big beads matrix was 80% compared to 45% of LF from colostrum whey. The purity of the isolated lactoferrin by cation exchange chromatography and by single strand DNA agarose affinity chromatography was, on average, 93%. The protocol to isolate lactoferrin from bovine colostrum was established at the laboratory scale and large-scale process is also proposed.

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Finally I would like to express my appreciation and love to my family, who always were here for me during the time of my study.

DEDICATION

This thesis is dedicated to my beloved parents Mrs. & Mr. Zhang, my beautiful wife Carol and my lovely daughters Michelle and Myra.

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LIST OF ABBREVIATION

β_2MG	β_2 - microglobulin
AC	Affinity chromatography
B_{12}BP	Vitamin B_{12} - binding protein
BSA	Bovine serum albumin
CEC	Cation exchange chromatography
DF	Dia-ultrafiltration
FBP	Folate-binding proteins
GI	Gastrointestine
GMP	Glycomicropeptide
GT	Galactosyl transferase
HIV	Human immunodeficiency virus
HIV-1	Human immunodeficiency virus-1
HSV-1	Herpes simples virus type-1
IEA	Ion exchange adsorption
IEC	Ion exchange chromatography
Igs	Immunoglobulins
IWPF	Individual whey protein fractions
K_α	Dissociation constant
α-LA	α - lactalbumin
LF	Lactoferrin
LFC	Lactoferricin
LFC-B	Bovine lactoferricin
β-LG	β - lactoglobulin
LPD	Lactoperoxidase
LPS	Lipopolysaccharide

MALDI-MS	Matrix assisted laser desorption/ionization mass spectrometry
MW	Molecular weight
pI	Isoelectric point
PP	Proteones peptones
RPC	Reverse phase chromatography
Rs	Resolution
SDS	Sodium dodecyl sulfate
SDS-PAGE	SDS-polyacrilamide gel electrophoresis
SEC	Size exclusion chromatography
SP	Suphopropyl
SSBB	Suphopropyl-Sepharose Big Beads
SSDA	Single strand DNA agarose
TFA	Trifluoroacetic acid
UF	Ultrafiltration
WP	Whey powder
WPC	Whey protein concentrate
WPI	Whey protein isolate

CHAPTER 1

INTRODUCTION

1.1. INTRODUCTION

From a physico-chemical point of view, milk is a colloidal system made up of an aqueous solution of lactose, salt and many other dissolved elements, and also proteins in suspension, and fat in emulsion (1). Milk is estimated to contain more than 100 thousand molecular species. However, milk's average composition can be simplified to 3.7-4.2% fat, 3.6% protein, 4.9% lactose and 0.7% ash, with the balance consisting of water (2). Milk's role in nature is to nourish and provide immunological protection for the mammal young. Milk has been one of the important food sources for humans since prehistoric times (3); it is not surprising, therefore, that milk's nutritional value is very high.

Bovine milk has up to 3.6% protein content, consisting of about 80% casein, and 20% whey protein. The principal casein fractions are α_{s1}, α_{s2}, β, and κ-caseins. The distinguishing properties of all caseins are their low solubility at pH 4.6 and high heating stability. The common compositional factor is that caseins are conjugated proteins, most with phosphate groups esterified to serine residues. These phosphate groups are important to the structure of the casein micelle, since most of the caseins are stabilized as casein micelles in bovine milk (3). The proteins appearing in the supernatant of milk after precipitation at pH 4.6 are collectively called whey proteins. Most of whey proteins in bovine milk are globular proteins, which are more water-soluble than caseins and are subject to heat denaturation. Native whey proteins have good gelling and whipping properties. Denaturation increases their water-holding capacity. The principal whey protein fractions in bovine milk are β-lactoglobulin (β-LG), α-lactalbumin (α-LA), bovine serum albumin (BSA) and immunoglobulins (Igs), and minor whey proteins such as lactoferrin (LF), lactoperoxidase (LPD), other enzymes, and proteose-peptones.

Casein isolation has been commercialized for at least 70 years; however, it was not until 1960s that isolated casein became an important food protein. Today, casein, produced by acid or rennet coagulation, is one of the principal functional food

proteins, with an annual world production of ~ 250,000 tonnes (4). The common method of isolating casein fractions from bovine milk includes the following steps: defating, coagulation, cooking, dewheying, washing, dewatering, and drying. In addition to the above method, several alternative methods for isolating casein or co-precipitates have been developed. These include the precipitation of milk protein by ethanol (5) and separation by ultrafiltration (UF), followed by rennet or acid precipitation or centrifugation (6,7). Whey is the liquid remaining after the removal of fat and casein from milk during the manufacture of cheese or acid and rennet casein. There are two principal types of whey: sweet whey (minimum pH 5.6) obtained from production of cheese or rennet caseins, and acidic whey (maximum pH 5.1) from production of acidic casein. Whey proteins represent only 10% of the total solids of whey; however, a number of processes have been developed to recover the whey proteins in more concentrated forms, for example, whey protein concentrate (WPC) and whey protein isolate (WPI) produced by membrane separation or by ion-exchange adsorption, followed by spray drying (8, 9, 10, 11). Major whey proteins, such as β-LG, α-LA, are separated by a combination of pH, ionic strength and membrane separation (12, 13). There is also considerable interest in the isolation of biologically-active proteins from whey, such as LF or LPD, since they might have specific biological functions that will benefit human health.

The utilization of milk protein products as food ingredient depends on their physico-chemical and functional properties. Traditionally, milk protein, especially caseins, is widely used in bakery, dairy, beverage, dessert, pasta, and meat products. However, whey is usually considered as a by-product of cheese or casein manufacture. It has been reported that some of the whey proteins have distinct physiological and biochemical functions; for example, LF binds and transports iron, and prossesses antimicrobial properties. α-LA is a constituent of lactose synthetase, lysozyme is an enzyme that destroys the bacterial cell wall, and Igs are part of the defense mechanism against gastrointestinal (GI) infections (14, 15, 16).

The bovine whey proteins have a broad spectrum of physiological and biomedical properties, and the present work has focused on bovine lactoferrin and its isolation by chromatographic methods. The main objectives of the current work were as follows:

1.To isolate LF from bovine colostrum whey by cation-exchange chromatography (CEC)

2. To isolate LF from bovine colostrum whey by affinity chromatography. Both isolation techniques were evaluated, and separated LF were further identified by other methods.

As a prelude to a better understanding of the experimental results obtained in the present work, CHAPTER 2 covers the structural properties of major milk proteins and their separation methods, with a major focus on bovine LF and its isolation techniques. The experimental results of bovine LF isolation by cation exchange chromatography and by affinity chromatography are then presented in CHAPTER 3 and CHAPTER 4, respectively. CHAPTER 5 summarizes the results and their relevance to the dairy industry, and outlines areas where future work would be desirable.

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CHAPTER 2

LITERATURE REVIEW

2.1 THE MILK PROTEIN SYSTEM

Bovine milk contains about 30-35 g of protein L^{-1}, which is very heterogeneous. The major milk proteins have been extensively characterized in terms of structure, physiological, genetic, nutritional, physico-chemical and functional properties (47, 48, 49, 50). Milk proteins are often subdivided into two major groups: caseins and whey proteins (Figure 2-1). Caseins constitute over 80% of total milk protein and consist of α_{s1}, α_{s2}, β, and κ-caseins. In bovine milk, the four species of casein together with a large fraction of the mineral component are associated into roughly spherical aggregates, with typical diameters of approximately 100 nm, termed casein micelles. Whey proteins make up about 20% of total milk protein, which falls into five types, β-lactoglobulin (β-LG), α-lactalbumin (α-LA), bovine serum albumin (BSA), immunoglobulins (Igs), and minor whey protein components; serum transferrin, lactoferrin (LF), and enzymes. In a normal milk environment, whey proteins have a more limited tendency to self-associate. The characteristics of caseins and whey proteins differ significantly, and details are shown in Table 2-1.

2.1.1 CASEIN PROTEINS

The four major casein proteins in bovine milk are α_{S1}-casein, α_{S2}-casein, β-casein and κ-casein, which represent about 45, 12, 33 and 10%, respectively, of total casein protein. The caseins occur as casein micelles containing calcium and inorganic phosphate and represent some of the few phosphateproteins. Caseins are not easily quantified due to their heterogeneity and micellar composition. They are, however, classically determined by acid precipitation at pH 4.6, which is the isoelectric point (pI) of casein. The α-casein is the dominant casein in bovine milk, which is calcium sensitive; in other words, it can be precipitated by calcium, and β-casein is also calcium-sensitive. The κ-casein is

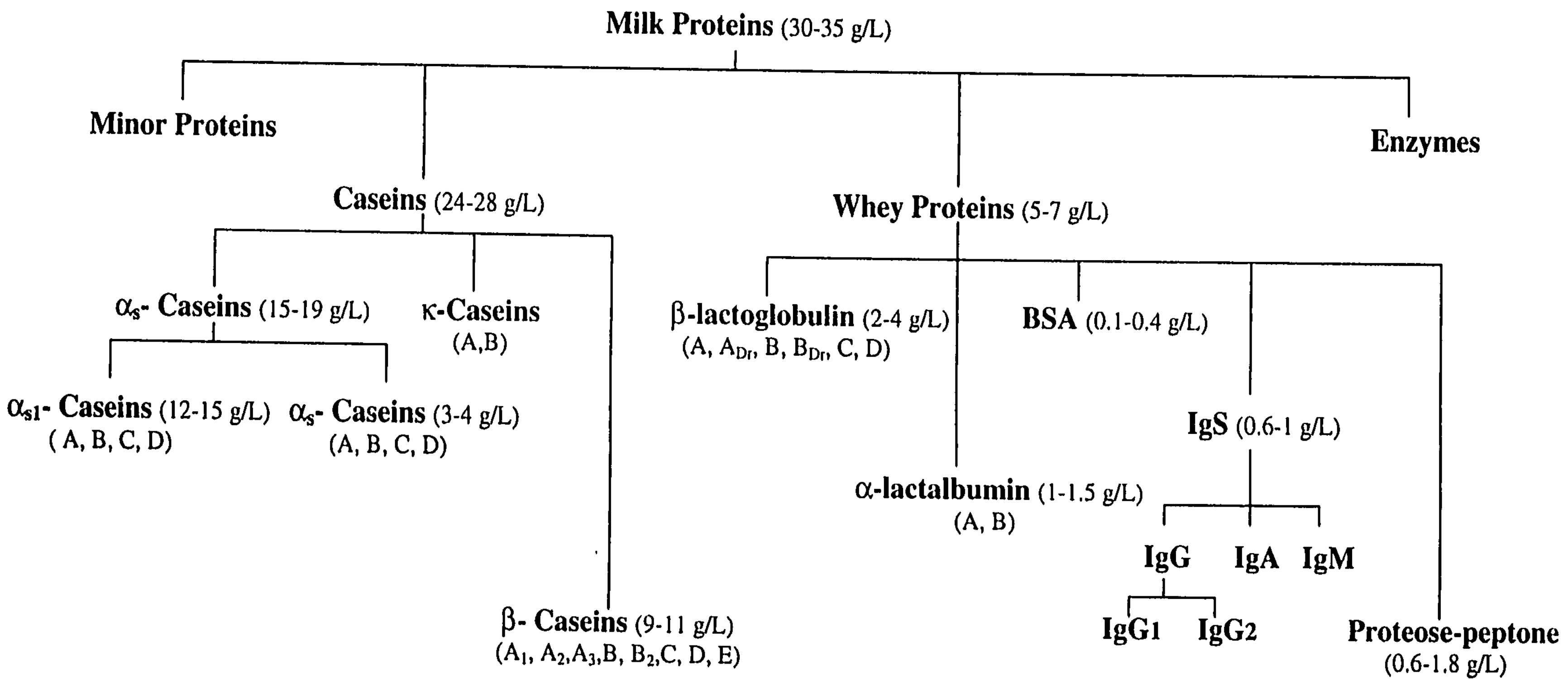


Figure 2-1. Distribution of fractions and proteins in bovine milk (adapted from reference 51).

Table 2-1. Principal differences between caseins and whey proteins

Characteristic	Caseins	Whey protein
Solubility at pH 4.6	N	Y
Rennet coagulation	Y	N
Heat stability	High	Low
Particle size	(micelles: MW ~10^8)	(molecules: MW ~ 10^4)

N- No, Y- Yes.

Adapted from reference 80.

unique since it remains soluble in calcium solution under conditions that precipitate all other casein components and can consequently occur in whey fractions. It stabilizes the other caseins in the presence of calcium and plays a key role in the formation of casein micelles. Casein is very heat-resistant but is precipitated at low pH values, resulting in curd formation. It is also precipitated by chymosin, which occurs in the stomach.

The primary function of caseins is to provide nutrition to the newborn. Caseins have this function not only due to the their role as the sources of amino acids but also because of their role as sources of calcium and inorganic phosphate.

2.1.1.1 α_{S1} - CASEIN

The α_{S1}-casein contains 199 amino acid residues and has an average molecular weight (MW) of about 23.6 Kdal. The mass varies slightly depending on the type of genetic variant and the degree of phosphorylation. Five genetic variants, A, B, C, D and E of α_{S1} - casein, have been identified in bovine milk. This protein exhibits progressive consecutive self-association to dimers, tetramers, etc., with the degree of association being strongly dependent upon the pH and ionic strength of the solution (51). Its primary structure, predicted secondary structure and physico-chemical properties are all consistent with an amphipathic structure composed of a hydrophobic and a polar domain. The anionic clusters in the polar domain are probably responsible for the sensitivity of its physico-chemical properties to pH and ion-strength (43).

2.1.1.2 α_{S2} - CASEIN

Bovine milk contains at least two genetic variants of α_{s2} - casein, termed A and D (52). Both variants contain 209 amino acid residues giving a MW of about 25.3 Kdal. The primary structure of α_{s2} - casein has been determined both chemically (53) and from sequencing of complementary DNA (54). The α_{s2}-caseins are more hydrophilic, in comparison to α_{s1}-caseins, containing three anionic clusters, may contain inter- or intramolecular disulfide bonds, and have 40% fewer prolyl residues. The presence of these clusters is responsible for the extreme sensitivity, to pH and ionic strength, of the self-association reactions of α_{s2}-casein.

2.1.1.3 β - CASEIN

Seven genetic variants of β-casein have been identified in bovine milk, referred to as A_1, A_2, A_3, B, C, D and E. The primary structure of at least the first five of these variants is known (55), each containing 209 amino acid residues, mostly hydrophobic residues. This characteristic makes β-casein be the most hydrophobic casein and to contain more prolyl residues than any other caseins. The self-association reactions of β-casein display a much greater sensitivity to temperature than other caseins. The cooling of milk to about 4 °C leads to the dissociation of β - casein from casein micelles due to a weakening of intermolecular hydrophobic forces as the temperature decreases (56, 57).

2.1.1.4 κ - CASEIN

κ-casein contains 169 amino acids residues, equivalent to a MW of about 19 Kdal. It is the only glycosylated casein. Two genetic variants of κ-casein, A and B, have been recognized in bovine milk (58, 59). A physiological role of this protein is the stabilization of calcium-sensitive caseins in the presence of calcium salt in milk. Its structure is clearly amphipathic, but without the anionic phosphate cluster in its polar domain, κ-casein remains soluble in the presence of calcium at all temperatures. The hydrophobic domain of κ-casein, as compared to β-casein, is less hydrophobic, and has a lower frequency of prolyl residues and probably contains more secondary structure. The cleavage of κ-casein by chymosin at phe105-met106 splits the molecule into a glycomacropeptide (GMP), which contains the glycosyl groups and is therefore hydrophilic, and the remaining hydrophobic portion, called para-κ-casein. This is the fundamental ingredient of cheese production, in which the release of GMP from the surface of casein micelles leads to a loss of the steric and electrostatic repulsive forces, causing the micelles to aggregate into a network (60, 61).

2.1.2 WHEY PROTEINS

Whey proteins comprise about 20% of the total proteins in bovine milk. They are normally classified into major whey proteins, including β-LG, α-LA, BSA and Igs, and

minor whey proteins, such as LF, LPD, enzymes and proteose-peptones. Whey proteins are typically globular proteins that may be denatured by heating at temperatures above 65 °C; however, the whey proteins are relatively stable to pH variation (like pH 4.6) but more sensitive to heat treatment than casein (1). The biological functions of each whey protein are shown in Table2-2. Whey proteins are well known for their high nutritional value due to their high content of essential amino acids. Except for their nutritional values, the biological functions of individual whey protein have been recognized. For example, the globular structure of β-LG is remarkably stable against acid and proteolytic enzyme (2). The biological function of α-LA is to support biosynthesis of lactose, which is an important source of energy for the newborn. BSA could bind insoluble free fatty acids for transportation in blood and is probably an important source of glutathione (a peptide which has immunoenhancing activity for patients whose HIV tests are positive) (3). Furthermore, LF and LPD are well known bioactive minor whey proteins in terms of their antibacterial activities in the GI track (4).

2.1.2.1 β - LACTOGLOBULIN (β-LG)

The most abundant protein in bovine whey is β-LG, comprising about 50% of total bovine whey protein, and has been well characterized. The structural and physico-chemical properties of this protein were reviewed by Hambling et al. (62).

Six genetic variants of β-LG have been discovered in bovine milk: A, B, C, D, H, and D_R, which is the only β-LG known to be N-glycosylated (63, 64). Preliminary crystallographic studies on the dimeric forms of bovine β-LG have suggested that the interface between subunits may involve both hydrophobic interactions, and stacking of the symmetry-related residue (65).

The denaturation of bovine β-LG involves the dissociation of dimer to monomer, a major change in the confirmation of the polypeptide chain, and aggregation. The denaturant can be alkali, heat, organic compounds or heavy metal ions, and it is not clear how each of these acts to yield insoluble aggregates. Although a common mechanism is unlikely to be present throughout, the initial stages of unfolding by heat at neutral pH and by alkali do appear to follow a similar path (66).

Table 2-2. Major biological functions of bovine milk proteins

Proteins	Isoelectric point	Major biological functions
Caseins	4.6	calcium absorption
β -lactoglobulin	5.13	(pro)vitamin A transfer
α -lactalbumin	4.2-4.5	lactose synthesis
Bovine serum albumin	4.7-4.9	fatty acid transfer
Immunoglobulins	5.5-7.3	antigen – antiboby reaction
Lactoferrin	8.3-8.5	anti-microbial activity and iron binding
Lysozyme	N/A	anti-microbial activity
Proteose-peptones	N/A	opioid activity

Adapted from references 42 and 43.

Bovine β-LG is known to interact with α-LA, and several caseins, such as κ-casein, to form a complex by hydrophobic interactions. This complex is subsequently stabilized by covalent bonding and a conformational change that makes the disulfide bridges less susceptible to attack as a result of disulfide interchange (62).

To date, no biological function for β-LG has been discovered (62). The amino acid composition is such that the protein is of high nutritional value but the molecular properties, particularly the acid stability, lead to the suggestion that some other, more specific function exists.

2.1.2.2 α - LACTALBUMIN (α-LA)

The second most abundant whey protein is α-LA, consisting of about 20% of the total whey protein fractions. α-LA is a low MW (14.2 Kdal) globular whey protein that first was crystallized in the nineteenth century (62). The role of α-LA as a specific regulator in lactose synthase, the demonstration of its homology with type-c lysozymes, its recognition as a calcium metalloprotein, and its metal-binding properties have been reviewed by some workers (67, 68). Three genetic variants of α-LA, A, B and C, are known to be in bovine milk, with the B variant being the most common (69).

α-LA is a calcium-binding protein, which is also capable of binding manganese, potassium, sodium, zinc and possibly other minerals. The presence of calcium is essential to the maintenance of the native structure of α-LA, indicating that α-LA is a true metalloprotein (68).

The biological function of α-LA is to act as a co-enzyme for galactosyl transferase (GT). This enzyme is involved in the synthesis of glycoprotein and various di- and oligosaccharides, which contain galactose. In the absence of α-LA, GT has a poor binding affinity for glucose, but by associating with α-LA, its affinity for glucose increases, enabling synthesis of lactose in the golgi bodies of mammary cells. The synthesized lactose accumulates in the lumen of golgi bodies and generates an osmotic potential causing a flow of cellular water into the lumen; therefore, α-LA indirectly controls the water content of milk (69).

2.1.2.3 BOVINE SERUM ALBUMIN (BSA)

BSA represents about 10% of the serum protein in bovine milk and is identical to blood serum albumin. Only one genotype of BSA has been identified in bovine milk. One BSA molecule contains 582 amino acid residues, giving a calculated MW of about 66.3 Kdal, 17 intramolecular disulfide bonds and one free sulphydryl group at residue 34 (70).

BSA has been shown to bind calcium and other types of metal ions (67). Five to twelve binding sites for calcium have been identified at neutral pH, but it is not known how important calcium is in the maintenance of native structure. The hydrophobic nature of the interior of BSA allows it to strongly bind free fatty acids. A biological function of BSA is in the transport of free fatty acids through the blood circulatory system. No specific biological function has been identified for BSA in bovine milk.

2.1.2.4 IMMUNOGLOBULINS (Igs)

The Ig fraction of bovine milk is a complex mixture of large glycoproteins, which exhibit antibody activity. Their structures, mode of synthesis and transport, and role in immunology have been discussed by others (71, 72). The Igs are synthesized by plasma cells, which are located throughout the body, including the blood and the mammary glands. The Igs occur in bovine milk at low concentrations (0.06-0.1%, w/v) but are found in colostrum at much higher levels where they may constitute 80% of the whey protein fractions (73). In bovine milk, five classes of Ig have been recognized, namely, IgA, IgE, IgG_1, IgG_2 and IgM. In bovine colostrum or milk, IgG_1 is by far the most abundant Ig, comprising about 85% or 66% of total Ig fraction (72).

The biological function of bovine Ig fraction is to confer passive immunity *via* colostrum to the neonate shortly after birth. The absorption of Ig in the intestine of the neonate occurs for only about a day, after which, the intestinal cells undergo closure. Although Igs make up about 10% of total whey protein in milk, their contribution to the functional properties of milk and dairy products has been ignored.

2.1.2.5 LACTOFERRIN (LF)

Lactoferrin (LF), an iron-binding glycoprotein synthesized by neutrophils and glandular epithelial cells (87), is a major whey protein in human milk with a concentration range from 1 to 2 mg/ml, and a minor constituent of cow's milk with concentration from 0.01 to 0.1 mg/ml (88). LF concentration in colostrum or milk is variable both among and within species (Figure 2-2). LF concentration changes during lactation and is highest during the first week (colostrum) and increases again during the dry period (Figure 2-3). It also dramatically increases with mastitis, but response differs among cows (7, 8, 89). In addition to occurring in milk, LF also occurs in the saliva, bile, pancreatic fluid, and tears of all mammalian species except dogs and rats (6). A large range of LF molecular weight, from 76 to 85 Kdal, has been reported (45, 90), and this large MW difference of bovine LF may account for 1) different LF sources; for example, LF isolated from neutrophils differs from LF isolated from mammary secretions (36); and 2) differences in LF glycosylation (45).

The structure of bovine LF is similar to that of human LF. The single peptide chain with 691 amino acid residues is folded into two globular lobes, the N-lobe and the C-lobe, representing the N-terminal and C-terminal halves of LF molecule, and each lobe is further subdivided into two domains (N_1 and N_2, C_1 and C_2) with specific iron binding sites in the interdomain cleft of each lobe (Figure 2-4). However, bovine LF has a additional disulfide bridge, 160-183, which is present in bovine LF, but not in human LF. The other major differences in structure between bovine and human LF are that bovine LF has more relative orientations of lobes and domains, and a different amino acid residue sequence (44).

LF has been shown to inhibit the growth of several microbes, including *Escherichia coli*, *Salmonella typhimurium*, *Shigella dysenteria*, *Listeria monocytogenes*, *Bacillus stearothermophilus* and *Bacillus Subtilis* (91, 92, 93, 94, 95). A recent study by Turchany et al. (96) showed that human and bovine LF and their N-terminal peptides were giardicidal against *Giardia Lamblia* in *vitro*. It has been proposed that the antimicrobial effect of LF is based on its capacity to bind iron, which is essential for the growth of

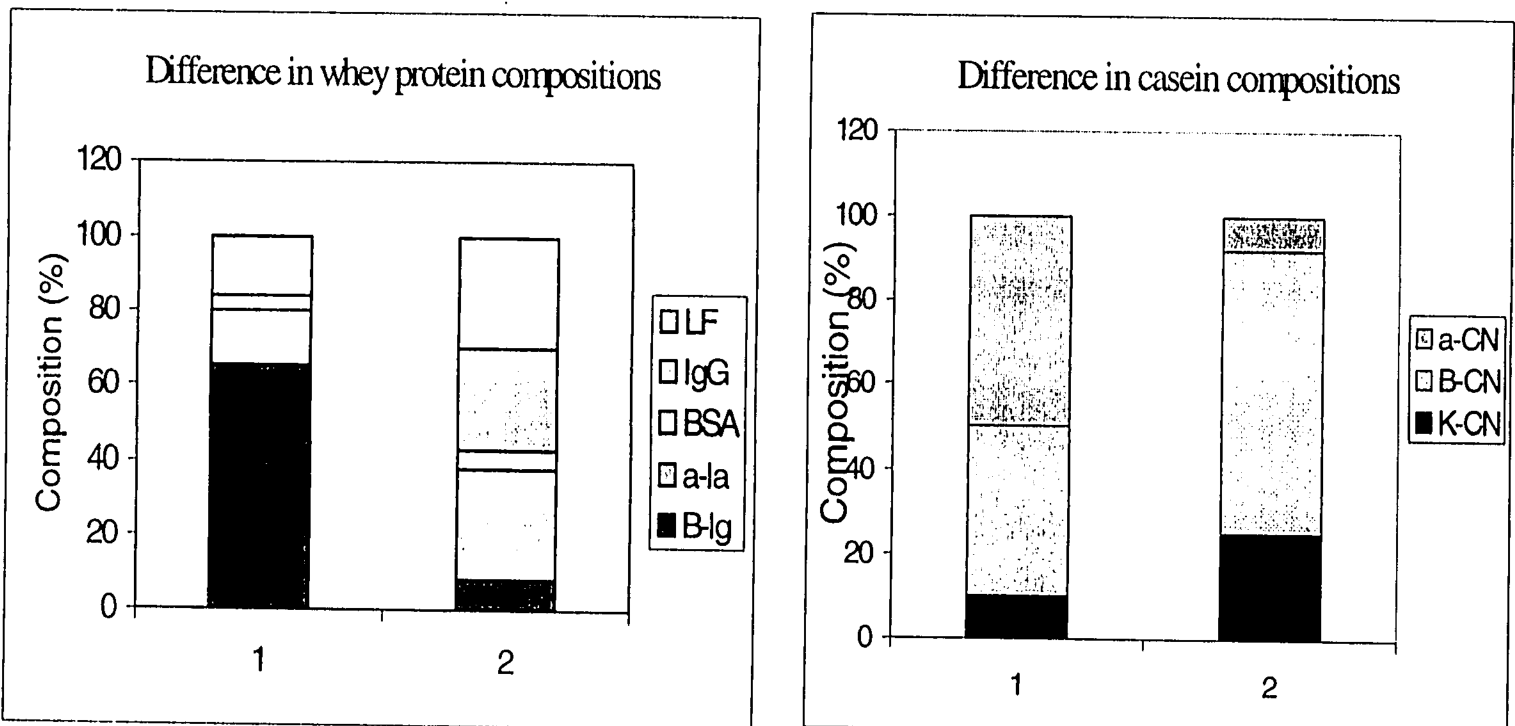


Figure 2-2. Difference in composition between casein and whey proteins of bovine and human milk (1: bovine milk, 2: human milk, data adapted from reference 1).

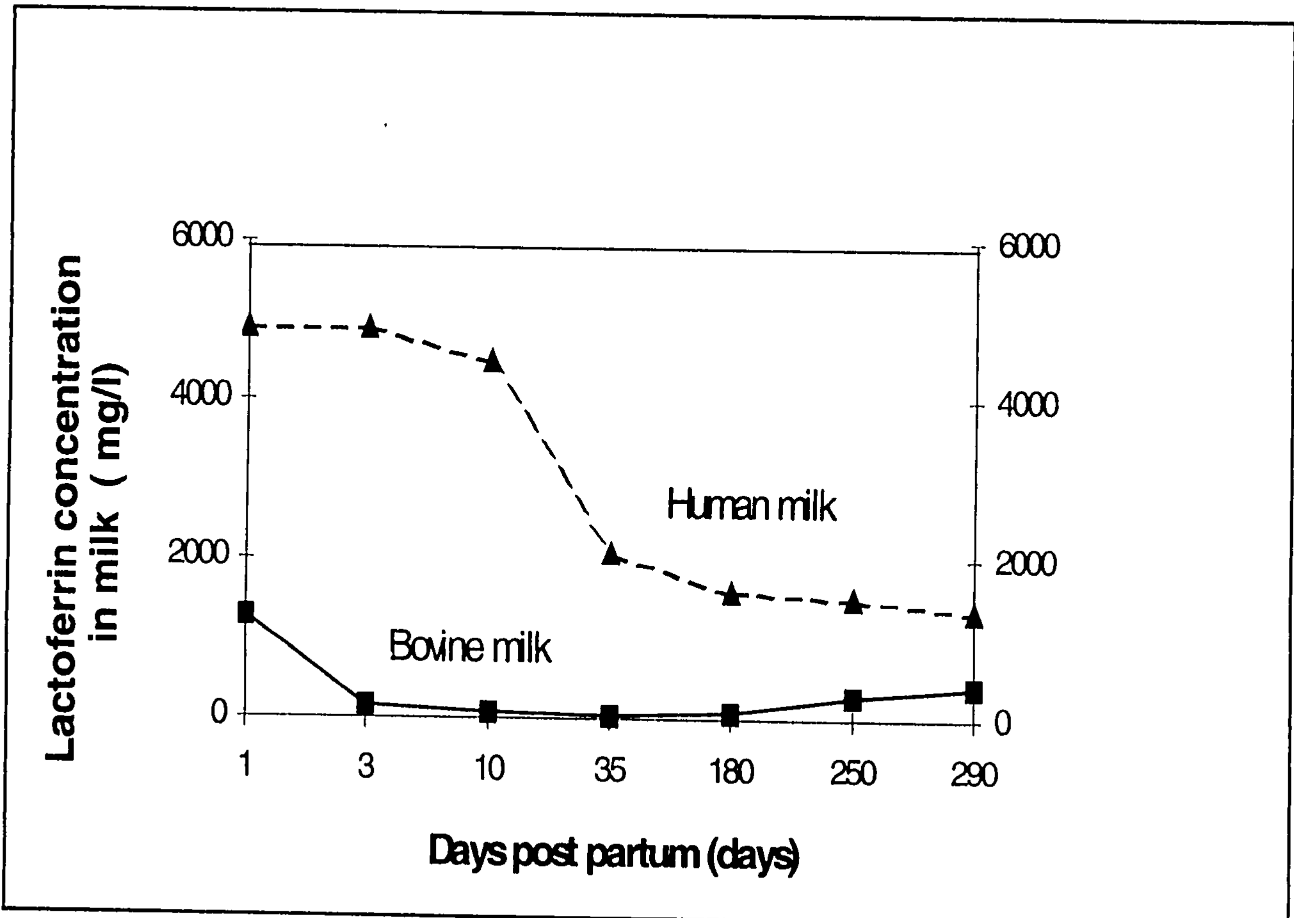


Figure 2-3. Human and bovine LF distributions at various postpartum time (adapted from reference 89).

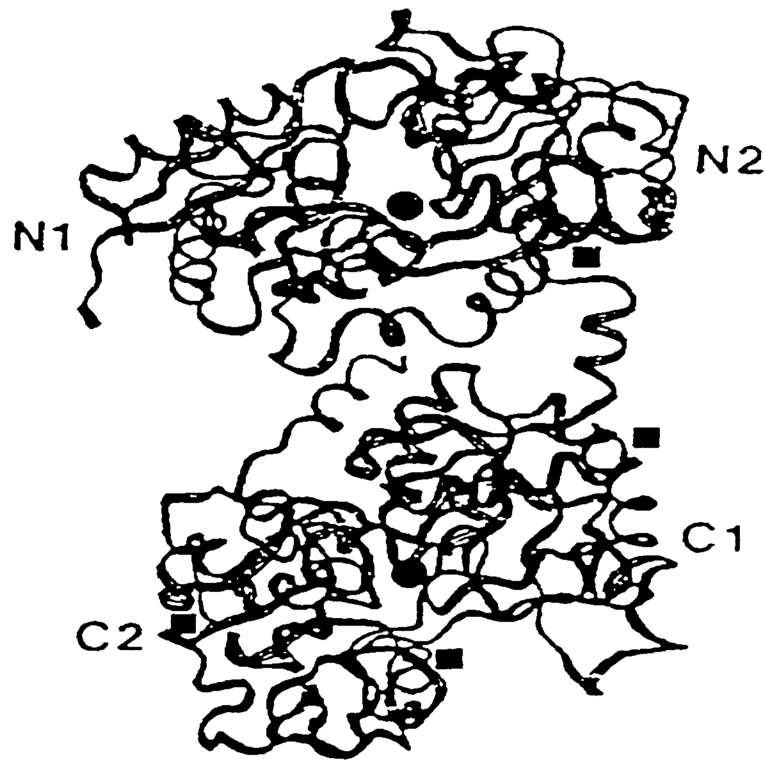


Figure 2-4. Three dimensional structure of bovine LF molecule (adapted from reference 44).

bacteria. However, recent studies have shown that in addition to iron chelating, other mechanisms might be also involved. In fact, an antimicrobial domain of bovine LF, distinct from the iron-binding region of the LF molecule, has been defined (91). Evidence has also shown that LF has a direct affinity for the cell wall of Gram-negative micro-organisms, and then releases significant levels of lipopolysaccharide (LPS) from the outer membrane (9, 10, 12). This release, comprising outer membrane permeability, increases bacterial susceptibility to other antimicrobial factors such as lysozyme (13, 14, 15). In addition, LF also binds to porin molecules in the outer membrane of *Escherichia coli* and *Salmonella typhimurium*, probably resulting in permeability changes as well (97). It has been also reported that LF has antiviral effects against herpes simples virus type-1 (HSV-1), human immunodeficiency virus-1 (HIV-1) and human cytomegalovirus *in vitro* (98, 99).

Studies have demonstrated that about 2-6% of ingested LF survives the GI tract, which suggests that intact LF has an important role to play in the antimicrobial defense of the neonatal GI tract. If 94-98% of LF is actually broken down in the GI tract to lactoferricin (LFC), peptide released by pepsin hydrolysis of the LF molecule may be a more important factor than native LF in the antimicrobial defense mechanism (103, 104). In this case, LF may serve as a precursor of LFC.

Some data indicate that LF stimulates cell growth and acts as a growth factor or iron carrier molecule (15, 16, 17). Evidence has also shown that LF could be a possible growth factor for the intestinal mucosa cells, and the role for LF in cellular proliferation has been recognized. Such evidence includes 1) better gastrointestinal development in newborn animals fed maternal milk as in comparison to newborns fed commercial formulas, 2) increased thymidine incorporation with LF supplementation of milk formulas, and 3) in vitro augmentation of thymidine incorporation into rat crypt cell DNA by LF. Recent studies showed that LF could bind DNA and activate transcription, which might explain the molecular basis of growth regulation (46).

The LF molecule consists of a single polypeptide chain folded into two globular lobes, each with one iron-binding site. Studies of iron distribution in human milk revealed that a major portion of iron (30-40%) is bound to LF. The higher bio-availability of iron, and a

higher concentration of LF in human milk compared to bovine milk lead to the hypothesis that LF might promote iron absorption in breast-fed infants (18). Indeed, this hypothesis is also supported by the following evidence: 1) human enterocytes can extract iron from LF (19); 2) enterocytes have a high LF uptake (19); 3) LF can transport iron across the intestinal brush border (6); and 4) iron from LF accumulates in brush border membrane vesicles (20).

In general, LF not only has antimicrobial properties but also a broad range of biological functions relating to the host defense system. Its biological activities include direct and indirect antibacterial action, such as inhibition of bacterial translocation in the gut, antiviral effects, regulation of cell function, immunomodulatory effects, regulation of inflammatory reactions, and stimulation or suppression of iron absorption in the gut (100, 101, 102).

Bovine LFC-B, a peptide derived from pepsin digestion of bovine LF, has more antimicrobial effects than LF against a large range of bacteria. Studies showed that LFC-B can be derived from the N-terminal region of the LF molecule and consists of 25 amino acid residues with the sequence of Phe-Lys-Cys-Arg-Arg-Trp-Gln-Trp-Arg-Met-Lys-Lys-Leu-Gly-Ala-Pro-Ser-Ile-Thr-Cys-Val-Agr-Arg-Ala-Phe, corresponding to the sequence of residues from 17 to 41 near the N-terminal end of the bovine LF molecule (105). This region has been identified as the structural domain responsible for the bactericidal properties of LF. The study also showed that the antimicrobial sequence of LFC-B consists mainly of 18 amino acid residues formed by a disulfide bond between residues of 19 and 36, located near the N-terminus of the LF molecule. The site of glycosylation and the metal-binding residues of LF are known to be located in other regions of the molecule. It seems that LFC-B represents the antimicrobial domain of LF or that LF acts as a precursor molecule releasing LFC-B (106). LFC-B's mode of action has not yet been clearly defined, but as it is a cationic peptide with a MW of around 3 Kdal, it may well kill organisms in a fashion similar to that of other cationic peptides such as defensins, the magainins and the antibiotic polymyxin (107). These cationic peptides display an affinity for biological membranes, and their ability of lethal effect against microorganisms is

thought to result from the ability to disrupt essential functions of the cytoplasmic membrane (105).

LFC-B has antimicrobial activity against a wide range of microorganisms. A physiologically diverse range of microorganisms has been tested and found to be susceptible to inhibition and inactivation by LFC-B. Among the susceptible microorganisms, for example, are *E. coli, Salmonella enteriditis, Klebsiella pneumoniae, Proteus vulgaris, Listeria monocytogenes, Clostridium perfringens,* and *yeast Candida albicans*. Recent studies have shown that LFC-B bound rapidly to the cells of Gram-positive, Gram-negative bacteria and yeast, and that the rate of binding was consistent with its rapid rate of killing (108). LFC-B can be obtained by pepsin digestion of LF *in vitro* suggesting the possibility that this protein may serve as a precursor of lactoferricins. Animal studies have been confirmed that LFC-B can be produced by gastric pepsin digestion of LF *in vivo* (107).

Bovine LF can be recovered from skim milk or cheese whey on an industrial scale and made available as a functional ingredient. Using LF as the starting material, the large-scale production of LF hydrolysate and LFC-B can be achieved. These new materials derived from bovine LF have potentially broad-spectrum antimicrobial properties and considerable potential for their widespread commercial use. Such material could be useful as a 'natural' preservative agent in foods or cosmetics, and as a functional component in new clinical foods and hygienic products for prevention or treatment of microbial infections and disease.

2.1.2.6 OTHER WHEY PROTEINS

Along with the whey proteins described above, folate-binding proteins (FBP), vitamin B_{12}-binding protein (B_{12}BP), β_2- microglobulin (β_2MG) and proteose peptones (PP) also occur in bovine milk.

The FBP in bovine milk occurs at a trace concentration consistent with the low concentration of folic acid, the latter determined as about 50-60μg/L milk (74). The physiological function of FBP in milk may be to regulate the transport of folate to the neonate. However, it is not clear whether the purpose of FBP is to actively promote the

transport of folate across the small intestine or to regulate and possibly slow down its transport (75).

In bovine milk, almost all of vitamin B_{12} is bound to B_{12}BP (76). The B_{12}BP may be involved in sequestering vitamin B_{12} from blood into the mammary cells and hence into milk. It may also inhibit the growth of undesirable bacteria in the intestine of neonates by reducing the bio-availability of vitamin B_{12} (75).

The β_{12}MG contain 98 amino acid residues, giving a MW of 11.6 Kdal. β_2 MG displays self-association reactions, the extent of which increases with increasing protein concentration (77).

The PP fraction of bovine milk is a heterogeneous mixture of polypeptides, of which many are phosphorylated and are the results of proteolysis of the casein by plasmin, an indigenous milk enzyme. At least 38 PP components have been identified in bovine milk, of which 25 are due to casein hydrolysis by plasmin (78). The most abundant type of PP in milk is PP_3, which represents about 25% of total PP. No biological function has been identified for PP_3, though it has been suggested that its purpose could be to help prevention of the precipitation of calcium in milk and in mammary cells, its multiple phosphorylation enabling it to bind strongly with calcium (79).

2.2 ISOLATION OF MILK PROTEINS

Industrial production of major milk proteins, such as casein or co-precipitate, has been well reviewed by others (79, 80). There is a trend towards harvesting individual milk protein fractions for value added and unique properties. Therefore, the isolation methods of individual milk proteins are briefly discussed below.

2.2.1 ISOLATION OF CASEINS

Casein has been produced commercially for at least 70 years; however, isolated casein did not become an important food protein until 1960s. Today, casein, produced by acid or rennet coagulation, is one of the principal functional proteins, with an annual world production of about 250,000 tonnes (80). The procedure of isolating casein from milk is shown in Figure 2-5. The first step in isolating casein fraction from milk is to remove fat

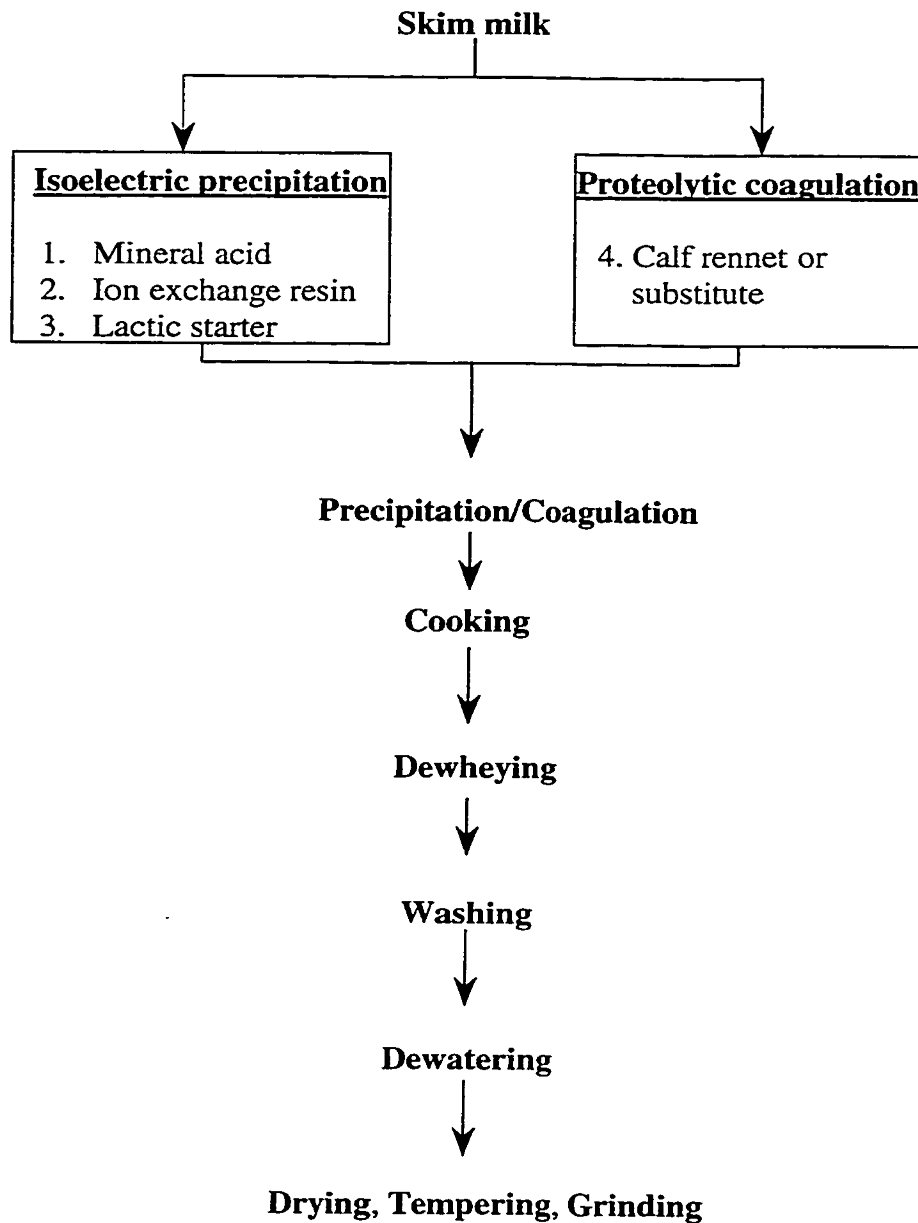


Figure 2-5. Casein manufacture (adapted from reference 43).

by centrifugation to yield a skim milk from which the casein is isolated after destabilizing it and rendering it insoluble. Caseins can be precipitated by either isoelectric precipitation caused by mineral acid, ion-exchange resin and lactic starter, or proteolytic coagulation by calf rennet or its substitute (Figure 2-6). Following precipitation of casein, the precipitated curd is separated from whey, then is washed by water to remove residual whey constitute (lactose, whey proteins, salts) and free acids. Finally, casein is mechanically dewatered, dried, and ground.

A number of methods for fractionating casein into β- casein-rich and α_s-/ κ- casein-rich fractions on a potentially industrial scale have been developed (81). A method for fractionation of a diluted sodium caseinate solution by UF into a β- casein-rich permeate and a α_s-/ κ- casein-rich retentate is shown in Figure 2-7. This method is based on the association characteristics of the caseins that are dependent on ionic strength and /or temperature. At low temperature (4°C) β- casein exist in solution as monomers. In other words, under these conditions, β- casein remains soluble while α_s-casein and para- κ-caseins coagulate. A method for isolation of β- casein by microfiltration of calcium caseinate at 5°C was also reported (80).

2.2.2 ISOLATION OF WHEY PROTEINS

Whey proteins represent only 10% of total solids of whey; however, a number of whey processes have been developed, and are now being exploited commercially to recover the whey proteins in more concentrated forms. Figure 2-8 shows what the potentially industrial-available whey protein products are: whey powder (WP), whey protein concentrate (WPC), whey protein isolate (WPI), and individual whey protein fractions (IWPF).

Whole whey powder that contains less than 15% protein can be produced by concentrating whey by evaporation or a combination of membrane processing and evaporation, followed by spray drying. For modified whey powder production, demineralization by electrodialysis or ion-exchange and /or lactose crystallization is needed to reduce mineral and / or lactose concentration.

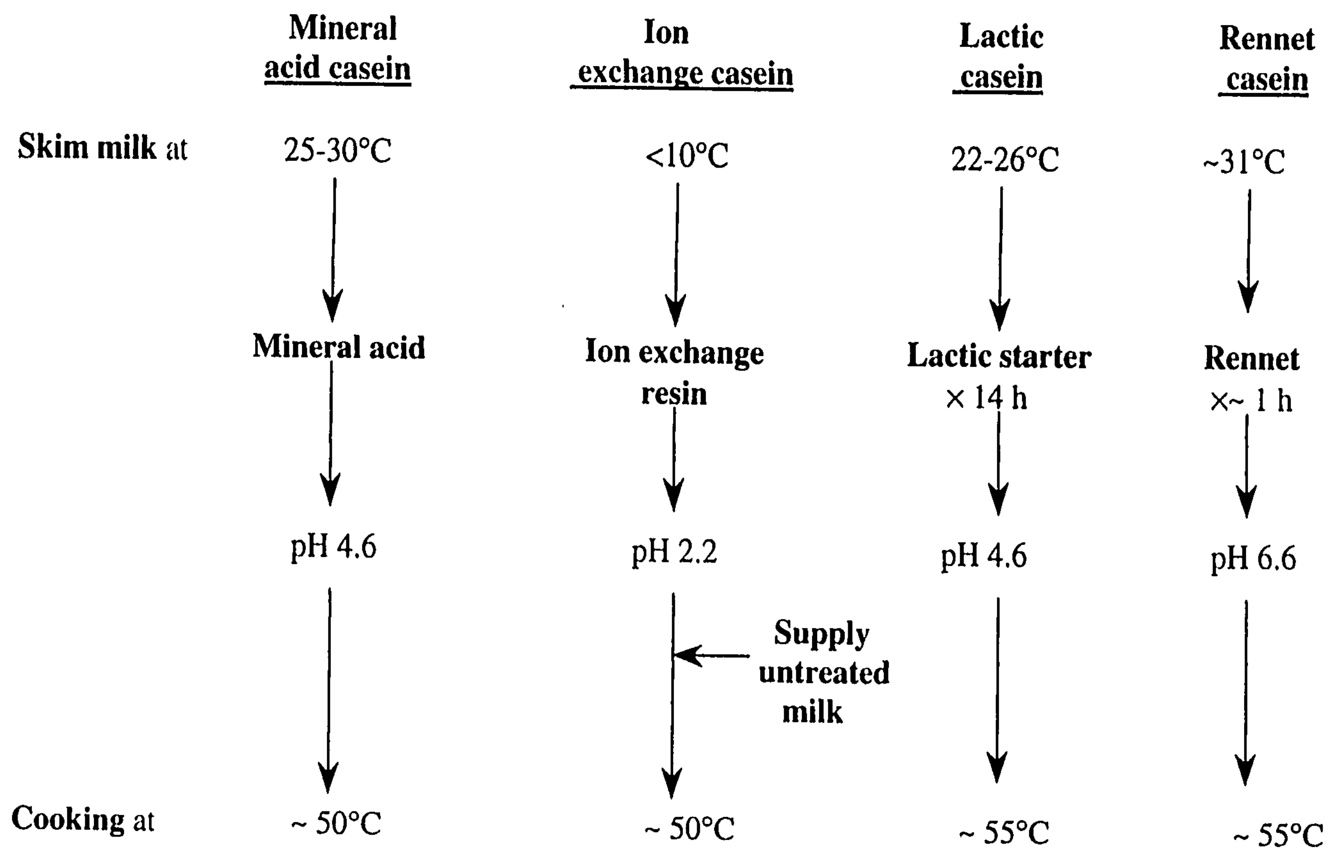


Figure 2-6. Different casein precipitation conditions (adapted from reference 123).

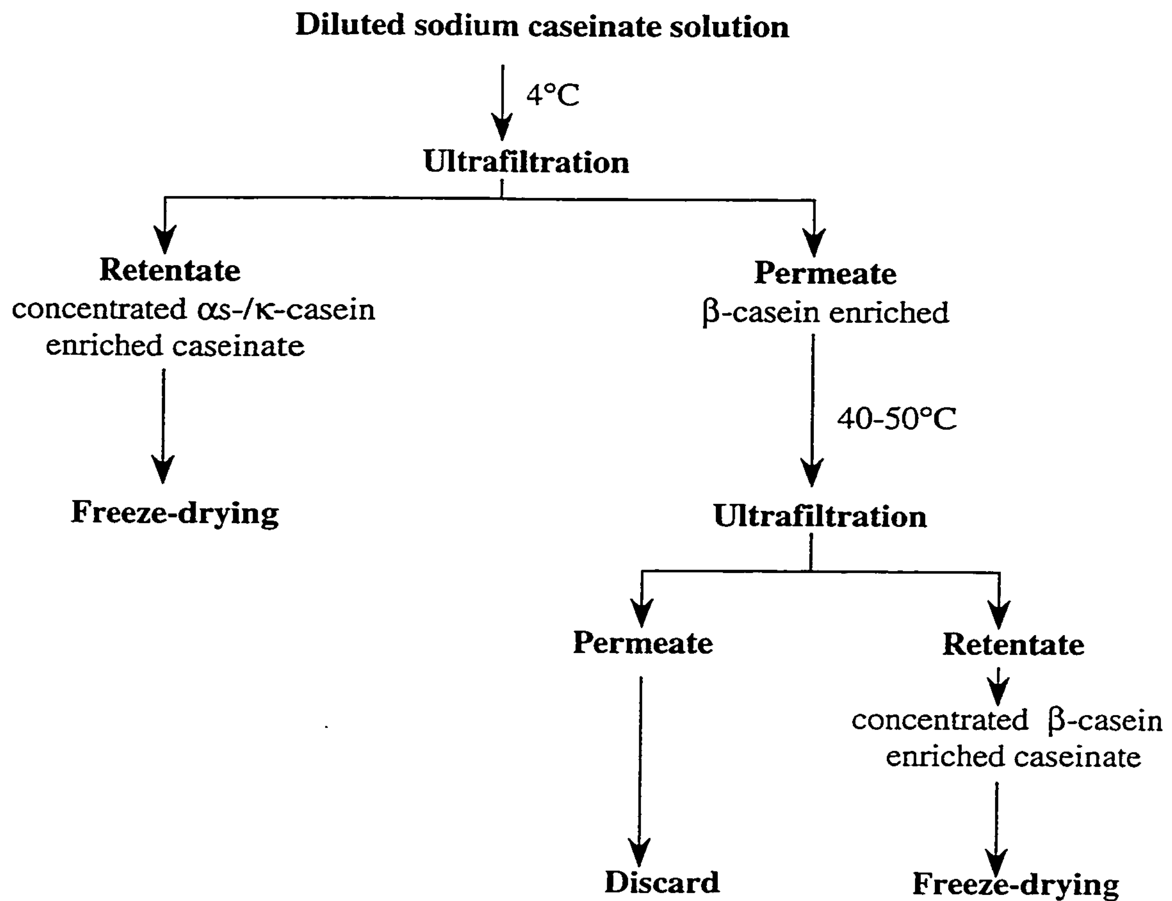


Figure 2-7. Method for preparing α_s-/κ- and β-casein enriched casein (adapted from reference 81).

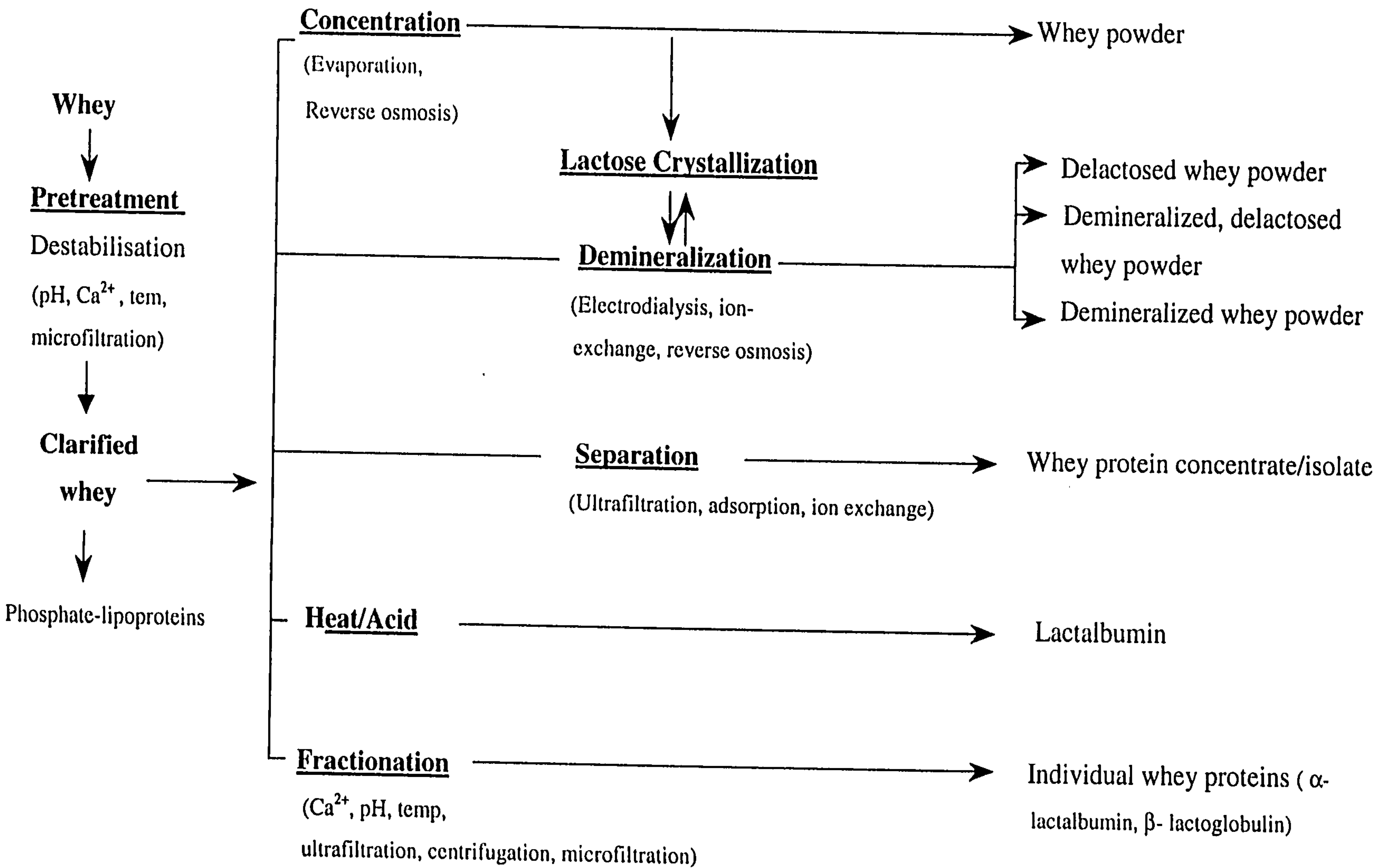


Figure 2-8. Industrial isolation of whey protein products (adapted from reference 124).

UF is currently the method of choice for commercial manufacture of WPC. UF is a pressure membrane filtration process facilitating the selective separation of whey proteins from lactose, salts and water under mild conditions of temperature and pH. It is a physico-chemical separation technique in which a pressurized solution flows over a porous membrane allowing the passage of only relatively small molecules (Figure 2-9) (80). The protein is retained by the membrane and is concentrated relative to other solutes in retentate. The limit for whey concentration by UF is about 24% total solids, with a protein/total solids ration limit of about 0.72:1 (80).

Ion exchange adsorption (IEA) is widely used to commercially produce WPI (Figure 2-10). Whey proteins are amphoteric molecules. At pH values lower than their pIs, whey proteins have the net positive charges and can be adsorbed on cation exchangers. In contrast, at pH values higher than their pIs, whey proteins have negative charges and can be adsorbed by anion exchangers. Media with suitable pore size and surface characteristics have been developed specifically for recovery of whey proteins. Based on the above principle, two major ion-exchange fractionation processes have been commercialized for the manufacture of WPI: Vistec process and Spherosil process (82, 83).

A number of methods have been developed that may have commercial production potential to fractionate β-LG, α-LA and to produce WPC enriched in these fractions (Figure 2-11). These methods depend on the following conditions: 1) either mild heat treatments of a whey concentrate or a clarified whey under controlled pH and ionic conditions; and 2) demineralization of whey concentrate under controlled pH conditions, selectively reversible precipitation of β-LG or α-LA enriched fractions and the separation of the precipitate from β-LG or α-LA enriched solutions. The precipitate is re-solubilized by water and pH adjustment and then dried while the soluble protein is further concentrated by UF/DF prior to drying (84, 85, 86).

2.2.3 ISOLATION OF LACTOFERRIN (LF)

Chromatography has been established as the pre-eminent method for the purification of proteins and other bio-molecules. While many reasons exist for its widespread use, its

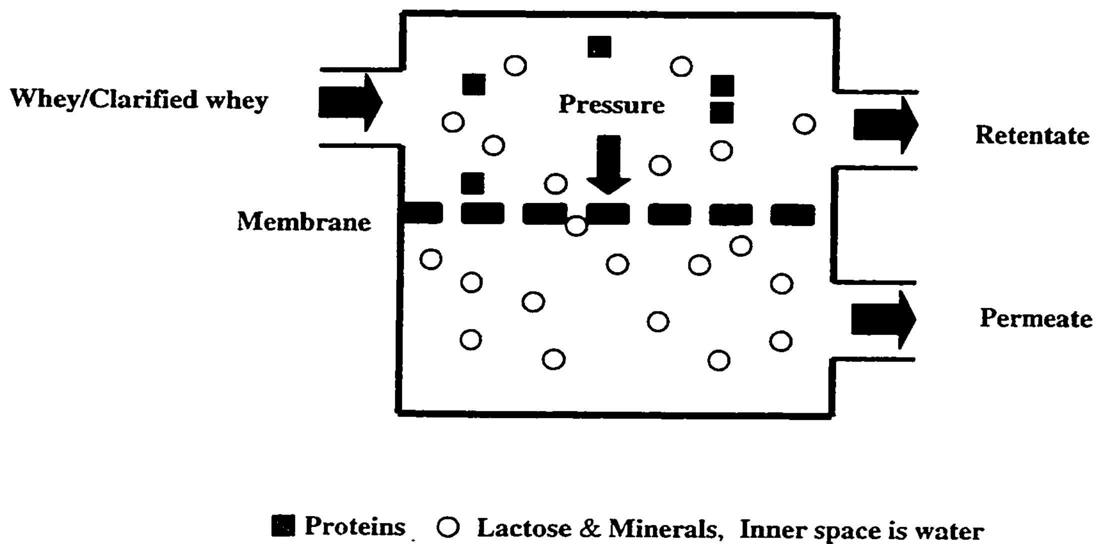


Figure 2-9. Production of whey protein concentrate by ultrafiltration (adapted from reference 80).

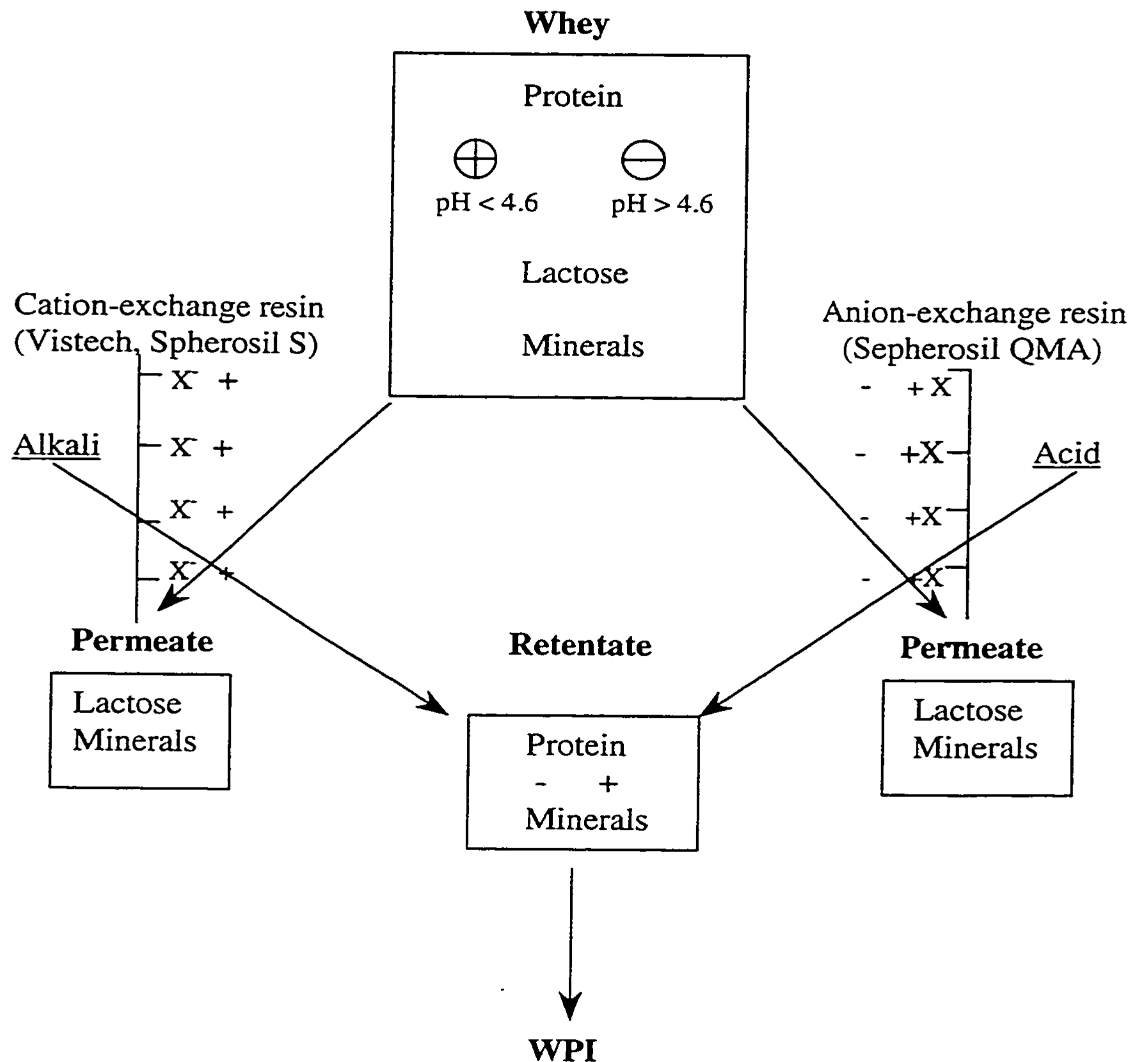


Figure 2-10. Production of whey protein isolate by ion exchange adsorption (adapted from reference 125).

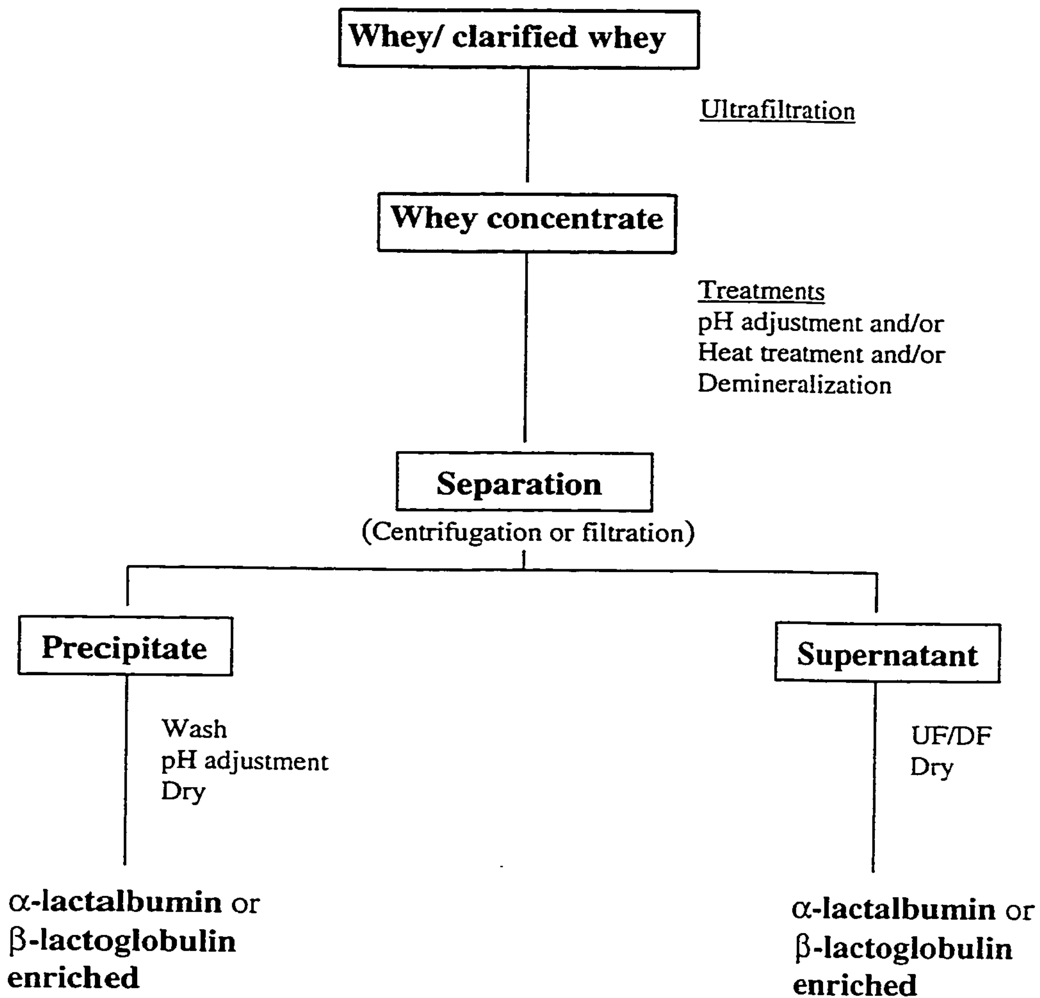


Figure 2-11. Fractionation of whey proteins (adapted from reference 126).

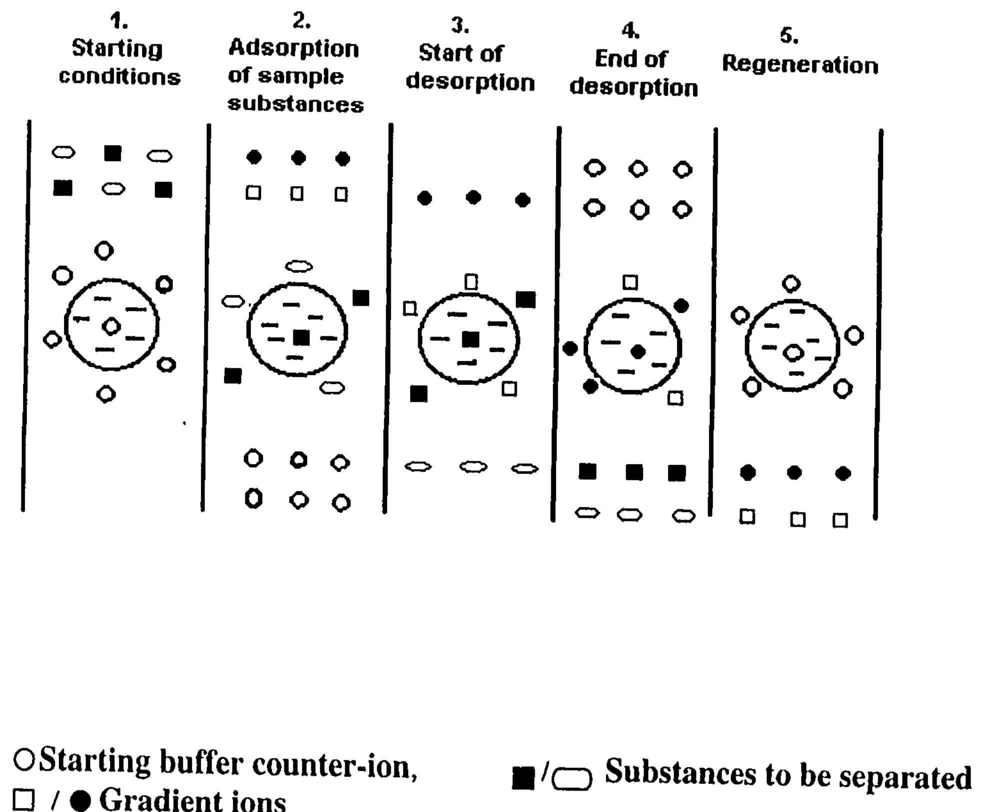
rich variety of absorbents and ability to resolve closely related proteins are two important factors contributing to its popularity. Gel filtration, ion exchange, and hydrophobic chromatography were the first types of chromatography routinely used for protein purification in the late 1950s and 1960s. Early in 1970s, bio-specific affinity chromatography was introduced to purify proteins; however, at that time, some problems have prevented its widespread use during process scale-up. These problems included the unavailability and expense of biological ligands, the lack of biological stability and activity of immobilized ligand, ligand leakage, and low binding capacity (109).

LF was first isolated from human milk by B. Johansson in 1960 (5). Since then, LF has been partially purified or isolated from mammary gland secretions and infant fecal and urine sources by chromatography techniques at laboratory level. In general, LF chromatographic isolation methods can be classified into four categories: the ion-exchange chromatography method, the affinity chromatography method, the size-exclusion chromatography method and the batch chromatography method. However, no well-documented industrial scale of LF purification exists, for any LF purification procedure is considered proprietary by the company that developed it. Most of these industrial scale methods are carried out by ion-exchange chromatography or by affinity chromatography.

2.2.3.1 ISOLATION OF LACTOFERRIN (LF) BY ION EXCHANGE CHROMATOGRAPHY (IEC)

2.2.3.1.1 *Theory of ion-exchange*

Separation by IEC depends upon the reversible adsorption of charged solute molecules to immobilized ion exchange groups of opposite charge. Most ion exchange chromatography techniques are performed in five main stages. These steps are illustrated schematically in Figure 2-12. The first stage is equilibration, in which the ion exchanger is brought to a starting state, in terms of pH and ionic strength, allowing the binding of the desired solute molecules. The exchanger groups are associated at this time with exchangeable counter-ions. The second stage is sample application and adsorption, in which solute molecules carrying the appropriate charge displace counter-ion and bind



○ Starting buffer counter-ion, □ / ● Gradient ions ■/▭ Substances to be separated

Figure 2-12. The theory of isolating protein by cation exchange chromatography.

reversibly to the gel. Unbound substances can be washed out from the exchanger bed starting buffer. In the third stage, the substances are removed from the column by changing to elution conditions unfavorable for ionic binding of the solute molecules. This process normally involves increasing the ionic strength of the eluting buffer or changing its pH. As shown in Figure 2-12, the desorption is achieved by increasing salt concentration gradient, and solute molecules are released from the column in the order of their strengths of binding, the most weakly bound substances being eluted first. The fourth and fifth stages are the removal from the column of substances not eluted under the previous experimental conditions, and equilibration at the starting conditions for the next purification. Furthermore, the separation is completed since different substances have different degrees of interaction with the ion exchanger due to differences in their charges, charge densities and distribution of charges on their surfaces (118).

2.2.3.1.2 *The matrix*

An ion exchanger consists of an insoluble matrix to which charged groups have been covalently bound. The charged groups are associated with mobile counter-ions. These counter-ions can be reversibly exchanged with other ions of the same charge without altering the matrix. The exchangers with positive charges, having negatively charged counter-ions available for exchange, are called anion exchangers. Negatively charged exchangers with positively charged counter-ions are termed cation exchangers. The matrix can be based on inorganic compounds, synthetic resins or polysaccharides.

The first exchangers designed for use with biological substances were cellulose ion exchangers developed by Peterson and Sober (110). Because of the hydrophilic nature of cellulose, these exchangers have little tendency to denature proteins. Also, many cellulose ion exchangers have low capacities and poor flow properties due to their irregular shapes. The ion exchangers based on dextran (Sephadex), followed by those based on agarose (Sepharose) and cross-linked cellulose (DEAE Sephacel), are more practical exchangers either in analytical or in preparative scales. Developments in gel technology enabled researchers to obtain highly cross-linked, agarose-based media such as Sepharose Fast

Flow and Sepharose Big Beads. These new media enable developing of fast, high-capacity, high-resolution IEC for the isolation of proteins.

2.2.3.1.3 *Factors affecting IEC separation*

The result of an ion exchange chromatography, as with other chromatographic separation, is often expressed as the resolution between the peaks of interest. The resolution is defined as the distance between peak maximum compared with the average base-width at half of the two peaks. Rs is a measurement of the relative separation between two peaks and can be used to determine if further optimization of the chromatographic procedure is necessary. The resolution achievable in a chromatographic system is proportional to the product of selectivity, and the efficiency and the capacity of the chromatographic system; furthermore, resolution is the most important parameter to control in column chromatography. The analytical expression for Rs is

$$Rs = (1/4)\,[(\alpha - 1)/\alpha] * (\sqrt{N}) * (k/1+k)$$

α - Selectivity N - Efficiency k - Capacity factor

2.2.3.1.3.1 Selectivity

The selectivity (α) is defined as the ability of the chromatographic system to separate peaks, i.e., the distance between peaks. Good selectivity is a more important factor than high efficiency in determining resolution, since Rs is linearly related to selectivity but quadratically related to efficiency. The selectivity factor can be calculated from the chromatogram, using the expression

$$\alpha = (Vr_2 - V_0) / (Vr_1 - V_0) \cong Vr_2 / Vr_1$$

V_0 - void volume, Vr_1 - $peak_1$ elution volume, Vr_2 - peak $_2$ elution volume

2.2.3.1.3.2 Efficiency

The column efficiency is expressed as the number of the theoretical plate (N) for the column under specified experimental conditions. The column efficiency is related to the zone broadening that occurs on the column and can be calculated from the following equations:

$$N = 5.54\,(V_r/W_h)^2 \quad \text{or} \quad H = L / N$$

V_r - peak elution volume, W_h - peak width at half peak height,

H - height equivalent to a theoretical plate, L - column bed length,

N - theoretical plate numbers

The main cause of zone broadening in a chromatography bed is longitudinal diffusion of the solute molecules. In practice, better efficiency could be achieved by using smaller bead sizes. However, unevenly packed chromatography beds and air bubbles will lead to channeling, zone broadening, and loss of resolution.

2.2.3.1.3.3 Capacity

The capacity of an ion exchanger is a quantitative measure of its ability to take up exchangeable counter-ions. The capacity may be expressed as total ionic capacity, available capacity or dynamic capacity. The total ionic capacity is the number of charged substituent groups per gram dry ion exchanger or per mL swollen gel. The available capacity is the actual amount of protein that can be bound to an ion exchanger, under defined experimental conditions. The available capacity is used practically to determine the capacity of the matrix, which is defined as follows:

$$\text{Capacity, (mg/mL)} = P_{max} / V_{matrix}$$

Where: P_{max} - maximum amount of protein (mg) bound to matrix;

V_{matrix}- matrix bed volume (mL)

The experimental conditions affecting the capacity are pH, the ionic strength of the buffer, the nature of the counter-ion, the flow rate and temperature.

2.2.3.1.4 *Isolation of LF by ion exchange chromatography*

2.2.3.1.4.1. Isolation of LF by IEC at laboratory scale

Proteins are high-molecular-weight compounds of amino acids linearly polymerized by peptide bonds. Therefore, they posses a large number of dissociable groups, that is, the terminal amino and carboxyl groups, carboxyl groups of asparatyl and glutamyl side chains, an amino group with lysine side chain, a guanidinium group with an arginine side chain, and an imidazole group contained in histidine (112). For example, LF has about

236 dissociable groups with pI at pH 8.5. These dissociable groups are distributed on the surface of LF molecules. The pK values of these groups differ depending on the micromolecular environment. The net charge on LF molecules is zero at the isoelectric point. The number of net negative charges increases with pH above 8.5. Similarly, the net positive charge increases with a decrease in pH below 8.5, Figure 2-13 demonstrates the protein net charge and protein adsorption behavior in ion exchanges as a function of pH. Therefore, LF can be looked upon as polyvalent amphoteric ions; in the other words, LF contains both positive and negative charges; the positive charges result from the ionization of 51 lysine and 36 arginine residues, and the negative charges from 66 aspartic and 73 glutamic acid residues. The charges originate from the ionization of weak acid or weak carboxyl-based groups of these amino acid residues, such as the carboxyl groups of aspartic acid and glutamic acid residues, and guanidino groups of lysine and arginine residues (111). Since the ionization of such groups is pH dependent, the net charges on LF molecules will be a function of the pH of their environment. LF molecules are charged positively while the pH is within the range from 7 to 8; in contrast, the other whey proteins are charged negatively at the same pH condition, since the pI of LF is about 8.5. This is much higher than the pIs (Table 2-2) of other whey proteins.

The matrix used in our experiment to isolate LF was Suphopropyl (SP) - sepharose big beads, purchased from Pharmacia Biotech Inc. Sp-sepharose big beads (SSBB) ion exchangers are based on 100-300 μm agarose beads. A higher degree of cross-linking, compared to other ion-exchangers, is used to give the media greatly improved physical and chemical stability. Due to its excellent physical stability and large bead size, SSBB can be run at high flow rates even with viscous samples. This ability is an important advantage for further industrial scale-up of LF production. Suphopropyl (SP) is the functional group on the SSBB matrix, which carries negative charges (Figure 2-14). When prepared, the whey sample is applied to the SSBB matrix. SP on the SSBB can interact with or retain LF molecule by electrostatic interaction at pH 7.5; thus, the unbound whey proteins and other components quickly pass through the matrix since they carry negative charges at pH 7.5 due to their lower pIs or do not interact with SP. After washing the column, desorption of LF by ion gradient is carried out.

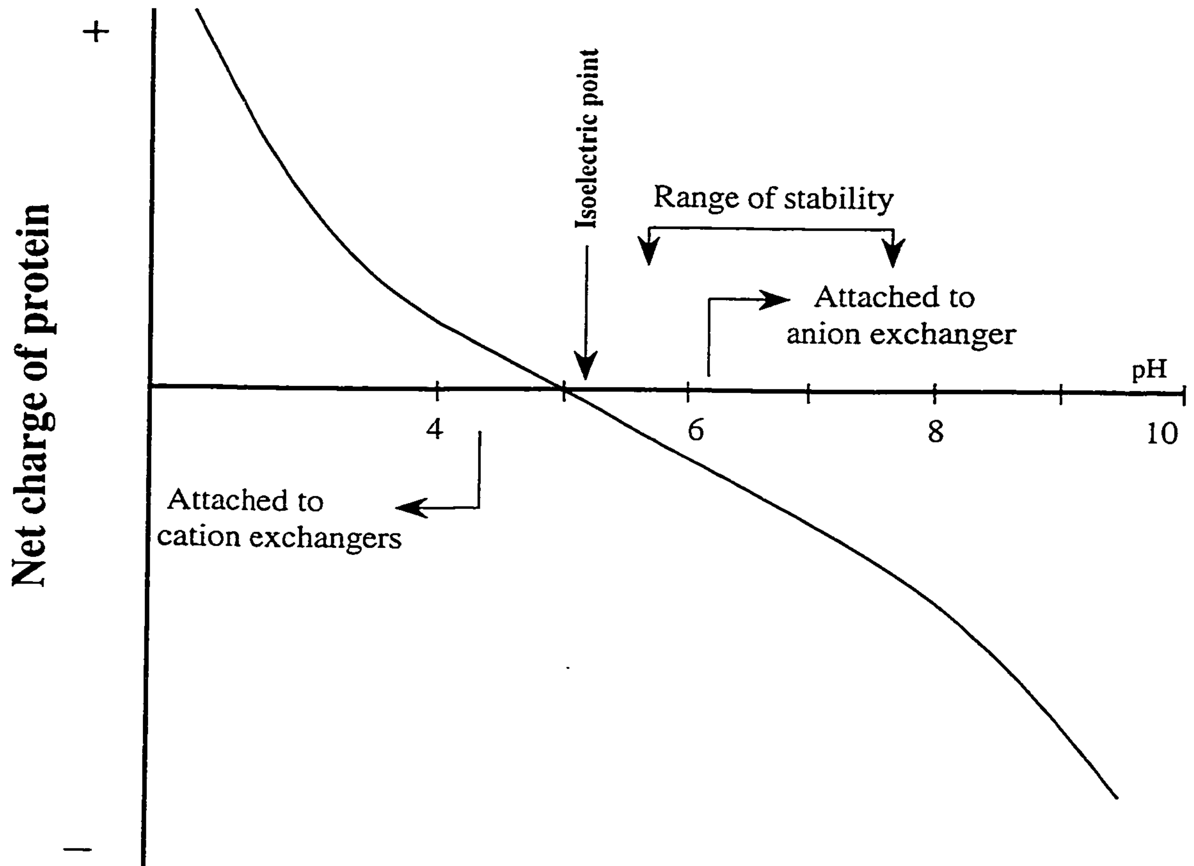


Figure 2-13. The protein net charge and protein adsorption behavior to ion-exchangers as a function of pH .

Sulphopropyl (SP)

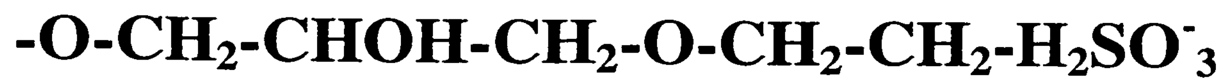


Figure 2-14. The functional charge group of the SSBB matrix.

2.3.1.4.2. Isolation of LF by IEC at large-scale

Most industry-scale chromatographic processes are directly scaled up from the laboratory scale isolation methods. The objective of small-scale work is to gain a physical and chemical understanding of the chromatographic process to allow the separation steps to be defined, and the range of operating parameters to be established. Differing from the purpose of the small-scale system, the goal of industry-scale production is economical recovery of desired proteins, so that the production yield and processing rate become significant, rather than to have good resolution of products. Consequently, these two purposes have to be kept in mind throughout the design of an industry-scale purification process.

IEC is perhaps the most generally useful of all the chromatographic techniques available for protein purification. It offers several times better resolution than gel chromatography, and it can be applied at a large scale using a 40 liters or larger column without difficulty. Most of the large-scale protein purification procedures described in the literature have been used the conventional ion-exchange cellulose, although in some instances, ion-exchange Sephadex have been used (113, 114). The more recently available cross-linked agarose ion exchangers such as sp-sepharose big beads have not been used in industrial-scale chromatography in protein separation as yet. However, SSBB gels do offer a number of potential advantages in protein purification; for example, they have high flow rates, are virtually non-compressible under ordinary conditions, have a high capacity for proteins at reasonable ionic strengths, and do not shrink and swell on changes in ion strength and pH. For these reasons, these gels can be regenerate in a column by washing with high salt and alkali and acidic solutions. In addition, they can be sterilized by autoclaving, which may offer a significant advantage in the purification of pharmaceutical products.

A systematic approach to design and scale up the chromatographic process is shown in Figure 2-15. Using the proposed model, the product concentrations and purity, cycle time, and through output for a given setup can be predicted. Furthermore, they allow for some predictions of how the separation will be affected by changing sorbent particle size, flow rate, pH, ionic strength, and loading, thus minimizing the number of experiments needed

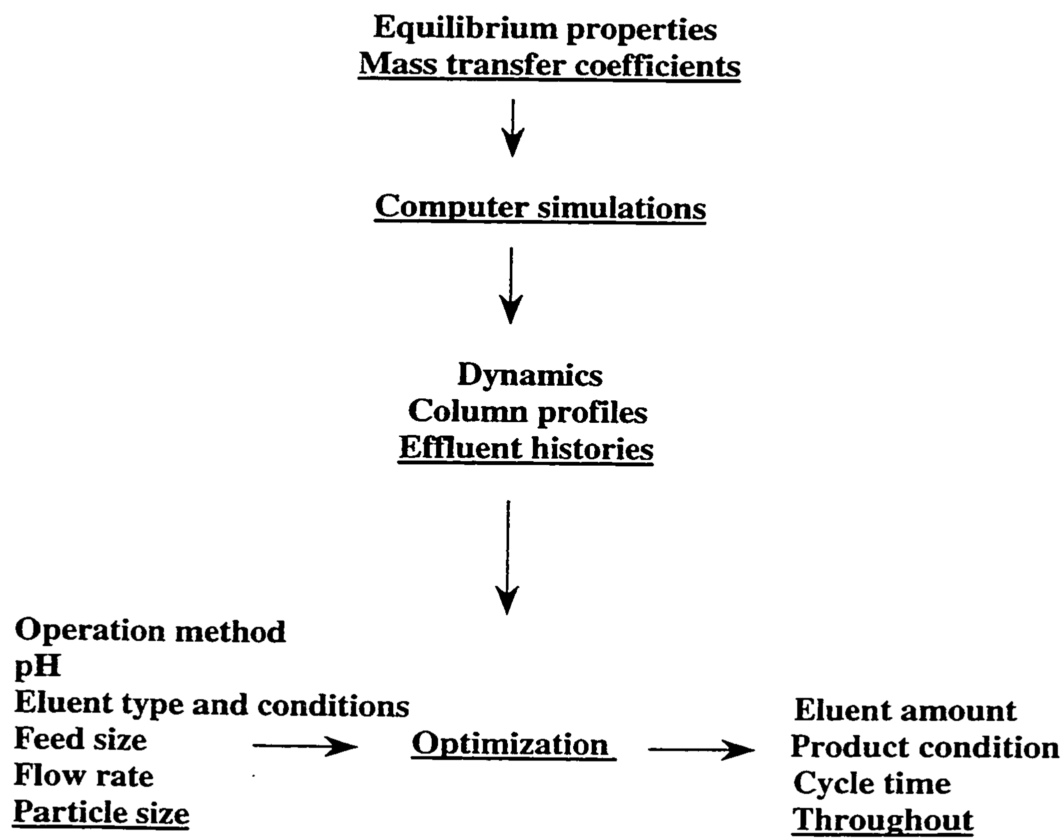


Figure 2-15. Methodology of scale-up for large-scale chromatographic process (adapted from reference 120).

for efficient design. The design and operating parameters can also be adjusted properly to achieve the desired efficiency, product concentration, or purity. To improve adsorbent utilization in large-scale chromatography of proteins, a concentrated feed or a large feed size is often used. For such systems, interference phenomena must be considered for design and scale up.

Many protein-purification methods prove satisfactory at the laboratory level but reveal inherent problems on scale-up and must be modified for production use. Scale-up is universally recognized as an extremely difficult area of applied science, since it is essential for protein purification procedures, taking into account not only the yields but also the rate at which each operation is carried out, together with the effects of changes in operating conditions. An attempt is made here to outline the basic principles involved and to emphasize the critically necessary approach. Table 2-3 summarizes those factors affecting scale-up in this context. Overall, the subject can be divided into two categories: those factors common to all aspects of the scale-up of protein purification, and those aspects characteristic of, but not necessarily restricted to, particular unit operations within the overall process.

Time is one of the major factors influencing the effectiveness of any scale-up attempt. Extended processing time can prove especially troublesome during the initial separation stages. At this point, one must accept that the susceptibility of proteins to degradation and /or inactivation is legendary and that loss of activity occurs via a wide range of factors including pH, temperature, ionic strength, metal ions, improper mixing, irreversible adsorption and microbial contamination. Obviously, processing time and exposure to adverse elements must be reduced to a minimum level.

Handling problems result from the volumes of material involved, the necessity for containment of biological material and the type of equipment to be used, and personnel requirements owing to large-scale purification.

Temperature variations of 10-15°C can occur during large-scale purification if insufficient attention is paid to their control. An adequate approach to scale-up requires that temperature must be considered during processing, in terms of refrigeration capacity, heating and cooling cycles, and heat generation.

Table 2-3. Summary of factors affecting scale up of protein extraction and purification procedures.

(1) General factors
a. Extended processing times
b. Handling problems
c. Increased variation in process liquid temperature
d. Requirement for alternative equipment
e. Equipment capacity and compatibility
f. Economics
g. Personnel requirements

(2) Specific factors
a. Protein extraction and recovery
b. Preliminary fraction
c. Column chromatography
(i) Column dimensions
(ii) Particle size of matrix
(iii) Particle size distribution of matrix
(iv) Flow rate
(v) Ratio of sample volume to total column volume
(vi) Mechanical strength (Compressibility) of stationary phase

Adapted from reference 119.

In addition to these factors above, alternative equipment design, optimization of equipment capacity and compatibility, and process economics also need to be considered carefully from the initial stages of development. In the specification of a large-scale column chromatography process, some factors are also essential to achieve successful protein purification, such as column dimensions, matrix particle size, flow rate, and ratio of sample volume to total column volume.

In practical aspects, the basic principles of laboratory-scale column chromatography are equally important in large-scale chromatography. Thus, the column should be constructed properly to have minimum dead volume above and below the packing material, and the end pieces should be designed so as to ensure an even distribution of material over the column's entire surface area. The packing of large-scale columns needs to be carried out with care so as to avoid particles of varying sizes or the inclusions of cavities in the gel bed. In large-scale protein purification, the columns can be run under gravity flow, but pumped flow is often used to reduce the process time, particularly if salt gradient elution is used. Large-scale gradient makers can be readily constructed from pairs of identical plastic tanks of the desired capacity, connected either by tubing at the base or by siphon. The collection of fractions from the large-scale chromatographic columns requires large-volume collectors.

So far, no well-documented literature exists on large-scale purification of bovine LF, especially on commercialized scale manufacturing of LF, even though there are two successfully commercialized LF producers: DMV International Inc. in the Netherlands and AgriCell Ltd. in the United States. The processing protocols are considered as commercial secrets.

2.2.3.2 ISOLATION OF LF BY AFFINITY CHROMATOGRAPHY (AC)

2.2.3.2.1 *Theoretical aspects of affinity chromatography*

Affinity chromatography is one of the most powerful procedures that can be applied to protein purification, and the principle of affinity chromatography is shown in Figure 2-16. A successful affinity separation requires that a biospecific ligand is available that can be covalently attached to a chromatographic bed material, called the matrix. It is also

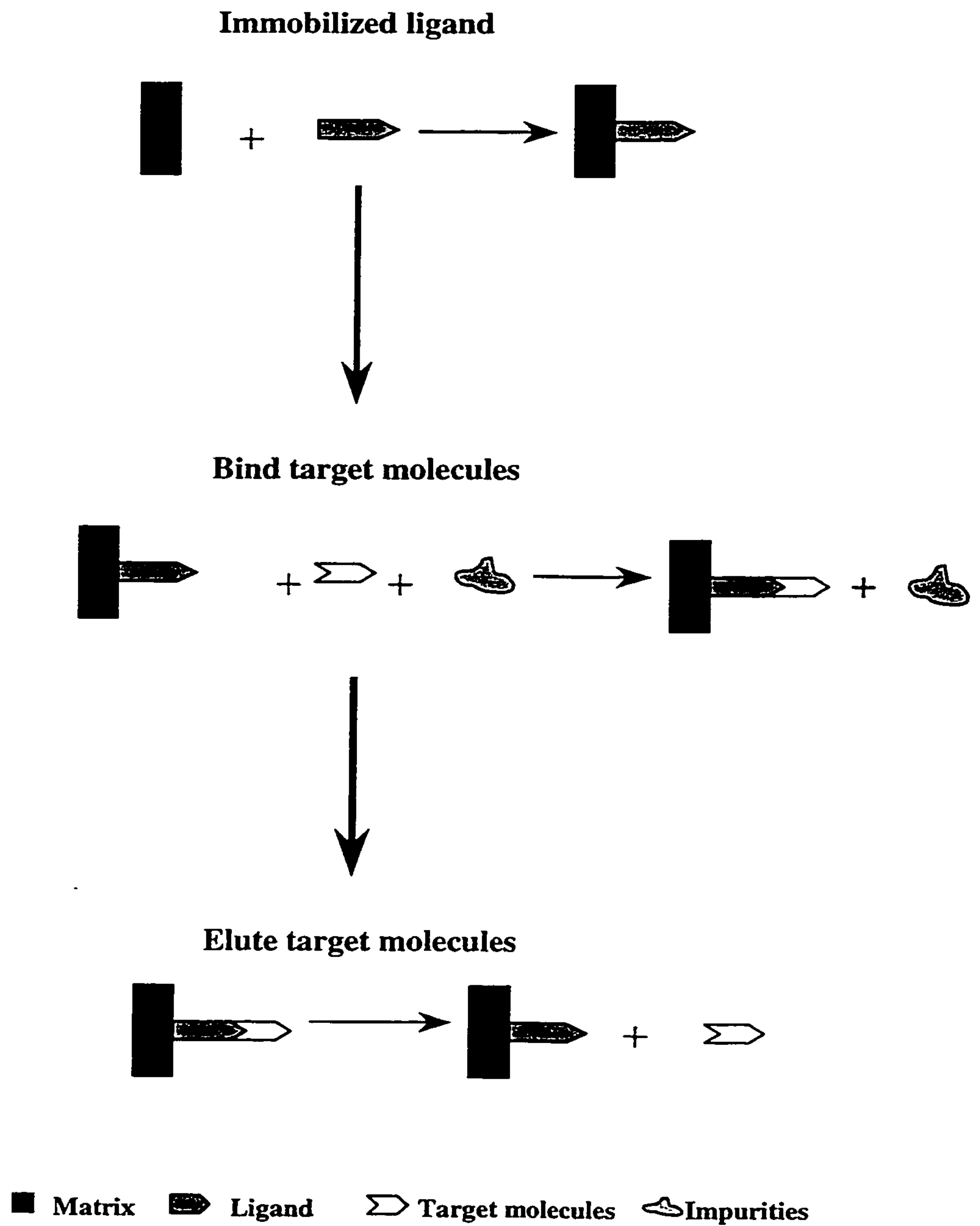


Figure 2-16. The principle of affinity chromatography to isolate protein.

important that the immobilized ligand retains its specific binding affinity for the substance of interest and that method is available for selective desorption of the bound substances in an active form by changing the ionic strength, pH, and polarity of the buffer after washing away unbound material.

Affinity chromatography as a biospecific technique began only about 30 years ago, even though it had been used as an experimental separation procedure for many years. This procedure takes advantage of one or more biological properties of the molecules being purified. These interactions are not due to the general properties of the molecules such as electrostatic interaction, hydrophobicity, or molecular size. This highly specific separation utilizes the specific reversible interactions between bio-molecules such as ligand and interesting protein molecules (Figure 2-17). In this context, "ligand" refers to a substrate, product, inhibitor, coenzyme, allosteric effect, or any other molecule that interacts specifically and reversibly with protein or other macromolecules to be purified.

Affinity chromatography appears to have a number of inherent advantages over the classical methods of protein isolation. Firstly, an adsorbent is designed and constructed specifically for protein to be purified, and secondly, the specific binding permits a rapid separation of the desired protein from other contaminants including proteolytic enzymes. Thirdly, the affinity chromatography technique might operate as a single-step procedure leading to a high yield of purified protein because of the reduced time involved and protection of the protein from denaturation by stabilization of tertiary structure. Fourthly, the technique's dependence on biological specificity rather than non-physico-chemical properties make the technique ideally suited for the isolation of proteins at very low concentration.

2.2.3.2.2 *Matrix and affinity ligand*

An essential prerequisite for affinity chromatography is the availability of appropriate chromatographic matrixes with the covalently bound specific ligands. In many cases the matrix can be used for a specific purification step only, so the correct choice of matrix support and the covalent linkage between the matrix and the bioaffinity ligand may be essential for the successful application of affinity chromatography.

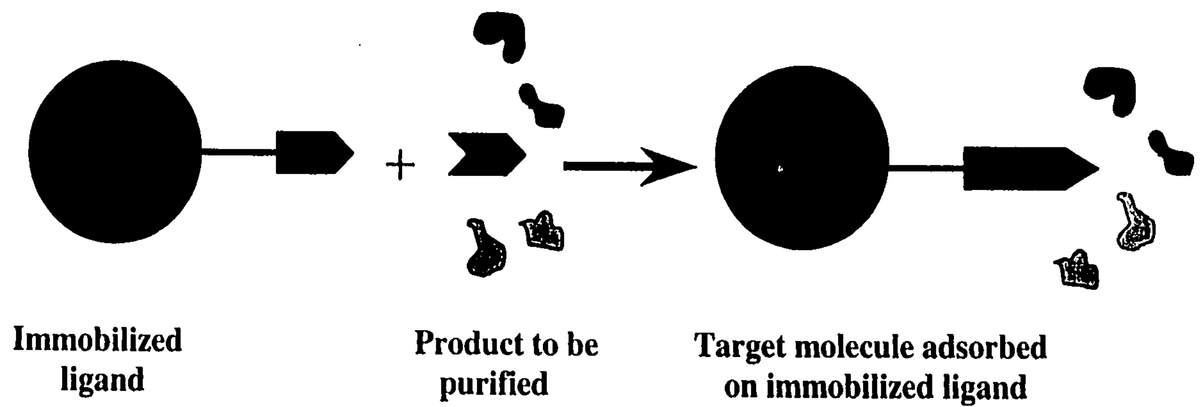


Figure 2-17. Specific binding of affinity chromatography

The affinity matrix should have properties generally required for a chromatographic matrix and, therefore, qualities derived from the specificity of the affinity chromatography. A good matrix for affinity chromatography should have the following properties:

- Hydrophilicity: reduces the nonspecific interactions.
- Large pores: allow all areas of the matrix to be available to most of the molecules in the mixture.
- Rigidity: the matrix must withstand the pressures of packing and solvent flow during elution and washing.
- Inertness: the matrix should not contribute to the separation.
- Chemical stability: the matrix must be stable to all solvents used in the separation.

Table 2-4 shows examples of commercially available matrix supports for affinity chromatography, and Table 2-5 illustrates the ligands used in affinity chromatography and their specificity. A ligand with a very narrow specificity - or mono-specificity - has a high selectivity for purification of a particular substance, but only for that substance, not for any other compound.

2.2.3.2.3 *Some theoretical and practical considerations*

2.2.3.2.3.1 Some theoretical considerations

Affinity chromatography represents the ultimate extension of adsorption chromatography since it comprises many different types of interactions in the binding of a protein to a ligand (e.g. hydrophobic, steric, and electrostatic interactions). The interaction between a protein and a ligand has been described by the term of "dissociation constant" (K_α). Here, the following equation defines equilibrium of protein with adsorbent:

$$K_\alpha = C\,P / P_b$$

K_α - dissociation constant, C - the concentration of binding sites free for binding,

P - the concentration of protein in free solution at equilibrium with adsorbent,

P_b - the concentration of protein bound on the adsorbent

Table 2-4. Examples of matrix support for the affinity chromatography.

Type	Chemical structure
Biopolymers (Polysaccharides)	Agarose (cross-linked, beads, macro Beads); cross-linked dextran; cellulose
Synthetic copolymers	Polyacrylamide; poly (hydroxyethyl)-Methacrylate; polystyrene
Inorganic material	Porous glass, iron oxide (magnetogels)
Biopolymers / synthetic copolymers	Agarose polyacrylamide
Inorganic materials / organic copolymers	Silica / hydrophilic copolymers

Adapted from reference 121.

Table 2-5. Ligand specificity

Ligand	Specificity
NAD, NADP	Dehydrogenases
Lectins	Polysaccharides
Poly (U)	Poly (A)
Histones	DNA
Protein A	Fc antibody
Protein G	Antibodies
Lysine	rRNA, dsDNA, plasminogen
Arginine	Fibronectin, prothrombin
Heparin	Lipoproteins, DNA, RNA
Blue F3G-A	NAD+
Red HE-3B	NADP+
Orange A	Lactate dehydrogenase
Benzamidine	Serine protease
Gelatin	Fibronectin
Polymixin	Endotoxins
Calmodulin	Kinase
Blue B	Kinases, dehydrogenases, nucleic acids- binding proteins

Adapted from reference 122.

2.2.3.2.3.2 Some practical considerations

The procedures, and equipment necessary to establish and run affinity chromatographic columns have been discussed in detail in reviews (115, 116). In addition to column packing, the following factors may also be critical to achieve a good affinity adsorption.

- The choice of equilibration buffer

The buffer used to equilibrate the adsorbent should reflect the optimum pH, ionic strength, temperature and chemical composition necessary to achieve a strong interaction between the insolubilized ligand and the protein. In this context, it is important to realize that optimal conditions for catalytic activity may not necessarily be optimal for binding of the affinity ligand.

- The sample volume, flow rate and equilibration time

In general, the volume of the sample applied to an affinity adsorbent is not critical if the substance of interest has a high affinity for the immobilized ligand. The substance will form a narrow zone at the top of the gel bed.

- The effect of flow rate

The adsorption equilibrium between the immobilized ligand and the macromolecule to be purified is often reached at a very slow rate. The sample should be applied to the column at the lowest flow rate acceptable from a practical point of view. The time dependence of the interaction between the enzyme and the immobilized ligand is also reflected in the effect of incubation time.

- The effect of protein concentration

There is no apparent effect of complementary protein concentration on the capacity of an affinity adsorbent under column conditions. The conclusion may be drawn that the interaction between the complementary protein and the immobilized ligand appears to be almost entirely independent of the concentration of inert protein in the sample except at very high flow rates.

- The effect of the temperature

In general, the strength of adsorption to an affinity gel decreases with increasing temperature, since the adsorption is exothermic. The more exothermic the interaction, the more temperature sensitive is the adsorption. Under chromatographic conditions, a

decrease of temperature will shift the equilibrium to lower concentrations in the mobile phase, and lower temperatures increase the adsorption. Thus, for protein purification by affinity chromatography, the temperature must be carefully controlled.

- The capacity of affinity adsorbents

The capacity of selective adsorbent is determined by two interdependent sets of parameters: 1) the correct choice of matrix, spacer molecule and ligand to optimize the protein ligand interaction; and 2) the way in which the capacity is determined by some dynamic factors such as flow rate, equilibration time and adsorption technique. When the defined quantity of adsorbent *m* is incubated with a defined quantity of the protein in question at concentration C_0, and after establishing the equilibrium, the concentration of unbound protein C can be determined. The capacity is then calculated from the following equation:

$$\text{Capacity} = (C_0 - C) / m$$

However, practically the capacity is defined as follows:

$$\text{Capacity (mg/mL)} = P_{max}/\text{mL Gel}$$

P_{max} - maximum amount of protein bound to matrix (mg)

2.2.3.2.4 *Isolation of LF by affinity chromatography*

In affinity chromatography, the target protein molecules are specifically and reversibly bound by immobilized ligand, whereas the unbound material stays or passes through the affinity column. An eluent is chosen that has a greater affinity for the ligand than the protein in question, and it displaces the bound protein from the ligand. The eluent can then be removed from the matrix by other methods, for example, membrane processing. The isolation of bovine LF by single strand DNA-agarose (SSDA) affinity chromatography is based on the specific and reversible binding of LF to DNA. It has been suggested that there are probably three binding sites on a LF molecule to DNA (46), as is shown in Figure 2-18.

Calf SSDA, an DNA affinity matrix, has been used to purify LF from human milk and infant urine at the laboratory scale (27, 28, 29, 30). Metal-chelate affinity chromatography (30, 31, 32), antibody affinity chromatography (34), dye-affinity chromatography (37),

(1). GGCACTT(G/A)C

(2). TAGA(A/G)GATCAAA

(3). ACTACAGTCTACA

Figure 2-18. Three DNA sequence sites for lactoferrin binding (adapted from reference 46).

and other affinity chromatography techniques (35, 36, 38, 39) have been reported to successfully isolate either human or bovine LF at the laboratory scale.

The scale-up purification process may mean going from microgram to milligram or from milligram to kilogram quantities. The first general consideration in scaling-up affinity chromatography is that the column diameter should be increased while the column height should remain constant. Increasing the column diameter achieves the larger column volume necessary when larger sample volumes are applied. If the bed height is held constant, the kinetics involved in the binding of the proteins to a ligand will be unaffected.

The flow rate is the next parameter for consideration when scaling up an affinity separation. The linear flow rate should be maintained in going from a smaller to a larger column. If linear flow rate is decreased, the residence time of the protein increases. If the flow rate is increased, the kinetics of binding may be shifted in such a way as to prevent adsorption and are especially critical in case of weak binding ($K_d = 10^{-4} - 10^{-5}$ M). The bound molecules are in equilibrium with a small amount of unbound material. The free agent competes with the ligand on the matrix for free molecules. The rate of elution is limited by the initial dissociation of the bound substance from the gel. Increasing the flow rate will affect this dissociation. Any change in linear flow rate will change the binding and elution characteristics of a protein, with a concomitant change in the resulting separation.

The protein sample is the next parameter to be considered in scaling up. Maintaining a constant ratio of sample volume to column volume while maintaining a constant sample concentration is essential. Changes in sample concentration will alter the size and shape of the eluted protein peaks and can affect the sample's binding characteristics. Altering the sample-to-column volume ratio can also have an effect in the case of a weak binding interaction. Large sample volumes can potentially lead to co-elution of a sample with non-adsorbed material.

The last important factor in scaling-up an affinity chromatography separation is the maintenance of the buffer volume to column volume ratio. The effects of variation in this parameter are evident when elution involves the development of a gradient.

In addition to the above, other factors such as the matrix's mechanical stability of the matrix, must be considered as well. It is also advisable to clean up the sample before application to the column. In the case of a larger column, disposal is often too costly, and cleaning may not be as expeditious. This clean-up will also benefit and simplify the column maintenance procedure. Currently, no large-scale purification of LF by affinity chromatography exists.

2.2.3.3 ISOLATION OF LF BY SIZE-EXCLUSION CHROMATOGRAPHY (SEC)

The basic principle of SEC is that molecules are partitioned between solvent and a stationary phase of defined porosity. The separation process is carried out using a porous gel matrix (in bed form) packed in a column and surrounded by solvent. The smaller molecules can enter the matrix pores and hence move more slowly through the column, appearing as the last component in the chromatogram. The larger molecules are excluded from the stationary phase and hence elute first from the column. Molecules intermediate in size can enter the stationary phase, but spend less time within it than smaller molecules do, and thus, all the molecules are eluted in order of their decreasing size (116).

Out of the many gels that exist, only relatively few are suitable for SEC. In addition to the chromatographic requirements, some practical demands should also be met: for example, the matrix must be inert, chemically stable, and have a low content of ionic groups to avoid ion exchange effects; the particle size and the particle size distribution must be controlled, and the mechanical rigidity of the gel grains should be as high as possible (115). Practically, the gels for SEC are dextran gels, polyacrylamide gels, agarose gels, cross-linked polymethylmetacrylate gels.

Generally, SEC can be qualitatively segregated into two categories: group separation and fraction. Group separation involves the separation of mixtures into two groups, which differ widely in molecular size. In such cases, the best separation is obtained when the large molecules are excluded from the gel matrix while the small molecules can penetrate the matrix to a considerable degree. Fraction is the separation or partial separation of

solutes from mixtures in which the molecular-size differences are much smaller than those in group separation (117).

The factors affecting the scale-up of SEC separation are identical to those affecting the separations themselves. The resolution obtained on a column depends upon column length, particle size (and distribution) of the bed packing material, the flow rate, and the ratio between sample volume and total column volume (117). In scaling-up a SEC operation, all of the foregoing, individually and collectively must be considered, and from a commercial viewpoint, the capacity/economy of the process must also be taken into account. The isolation of LF by SEC is frequently combined with other chromatographic methods such as IEC or affinity chromatography (AC) (23, 39, 40, 41, 45).

2.3 CONCLUSION

Colostrum whey or cheese whey is a mixture of lactose, protein, fat and minerals; therefore, the isolation of a specific whey protein such as LF is still a challenge. The potential and/or final application of LF must also be considered when designing the isolation protocol. The criteria for LF separation from milk and milk-derived by-product should be 1) the LF must retain a reasonable recovery rate and purity; 2) the use of organic solvents and other non-food grade chemicals must be avoided because of the potential application as nutraceutical and functional foods; and 3) the separation procedures must have a potential for commercialization.

SSBB ion-exchange chromatography and SSDA affinity chromatography were chosen as the LF extraction methods to be investigated by applying the above separation criteria. SSBB ion-exchange chromatography is established based on the electrostatic interaction of LF molecule to cation exchanger under a given pH condition, while SSDA affinity chromatography is based on specific DNA binding to LF under properly defined experimental conditions.

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CHAPTER 3

ISOLATION OF LACTOFERRIN FROM BOVINE COLOSTRUM BY CATION EXCHANGE CHROMATOGRAPHY

3.1 INTRODUCTION

Bovine milk is desirable for the nutrition, growth stimulation, and immunological protection of young calves. Since prehistoric times, bovine milk has been consumed by human beings either as fluid milk or as other dairy products until today because of its high nutritional value and versatile biologically functional properties. Milk proteins can be classified into two major categories: 1) casein and 2) whey proteins. Lactoferrin (LF) is a minor whey protein, whose concentration varies depending on lactation stage and species (1). Human milk is rich in LF (>2mg/ml), as compared to the milk of other species (2). In a bovine colostrum or late lactation milk, the LF concentration is about 5 to 10 times higher than in mature milk. LF has been shown to inhibit the growth of several microbes, including *Escherichia coli*, *Salmonella typhimurium*, *Shigella dysenteria*, *Listeria monocytogenes*, *Bacillus stearothermophilus* and *Bacillus Subtilis* (26,27,28,29,30,31). A recent study by Turchany et al. (31) showed that human and bovine LF and their N-terminal peptides were giardicidal against *Giardia Lamblia* in *vitro*. It has been proposed that the antimicrobial effect of LF is based on its capacity to bind iron, which is essential for the growth of bacteria. However, recent studies have shown that in addition to iron chelating, other mechanisms might also be involved. In fact, an antimicrobial domain of bovine LF, distinct from the iron-binding region of LF molecule, has been identified (26). The evidence also has shown that LF has a direct affinity to the cell-wall of Gram-negative micro-organisms and releases significant levels of lipopolysaccharide (LPS) from the outer membrane (32, 33, 34). This release, comprising outer-membrane permeability, increases bacterial susceptibility to other antimicrobial factors such as lysozyme (35,36,37). Studies of iron distribution in human

milk revealed that a major portion of iron (30-40%) is bound to LF. The higher bio-availability of iron and the higher concentration of LF in human milk compared to bovine milk lead to hypothesis that LF might promote iron absorption in breast-fed infants (38). Indeed, this hypothesis is also supported by the following evidence: 1) human enterocytes can extract iron from LF (39); 2) LF has a high uptake by enterocytes (39); 3) LF can transport iron across the intestinal brush border (6); and 4) iron accumulation from LF in the brush border membrane vesicles (40).

In general, LF not only has antimicrobial properties but also a broad range of biological functions relating to the host defense system. Its biological activities include direct and indirect antibacterial action, such as inhibition of bacterial translocation in the gut, antiviral effects, regulation of cell function, immunomodulatory effects, regulation of inflammatory reactions, and stimulation or suppression of iron absorption in the gut (41, 42, 43).

Many methods were reported for LF isolation, such as affinity chromatography (4, 5,6,7,8), size-exclusion chromatography (9,10), and other alterations of the above methods (3, 11,12,13). Thus, interest in LF research is increasing; however, LF still remains commercially expensive and difficult to isolate in significant quantities and quality. Bovine LF can be recovered from skim milk or cheese whey on an industrial scale and made available as a functional ingredient. Using LF as the starting material, the large-scale production of LF hydrolysate and LFC-B is achievable. These new materials derived from bovine LF have potentially broad-spectrum antimicrobial properties, and considerable potential appears to exist for their widespread commercial use. Such material could be useful as a 'natural' preservative agent in foods or cosmetics, and as a functional component in new clinical foods and hygienic products for the prevention or treatment of microbial infection and disease. In the research reported here, LF was isolated from bovine colostrum whey. The primary objective was to establish the parameters of LF separation by cation exchange chromatography using sp-sepharose big beads (SSBB).

The isolated LF was further analyzed by reverse phase chromatography, size-exclusion chromatography, MALDI-MS, SDS-PAGE and amino acid composition analysis. In addition, the ion-exchanger capacity, yield and purity of isolated LF were also studied.

3.2 MATERIALS AND METHODS

3.2.1 BOVINE COLOSTRUM TREATMENT

Bovine colostrum was collected from the Dairy Technology Center, University of Alberta Research Station, and stored at –20°C until use. Frozen colostrum was thawed at ambient temperature, and the procedure of bovine colostrum treatment is shown in Figure 3-1. Fat was removed by centrifugation at 15,000 × g at 4°C for 35 minutes, and thereafter, colostrum temperature was raised to 50 °C, and casein was precipitated by adjusting the pH to 4.6 by adding 1 M HCl. The casein curd was removed by secondary centrifugation at 25,000 × g at 4°C for 30 minutes, and colostrum whey was adjusted to pH 7.5 and ion strength adjusted to 50 mM Na_2HPO_4, followed by filtration through a Millipore Millex–GS 0.22 μm membrane (Millipore Corporation, USA) before application to a SSBB cation-exchange column.

3.2.2 CATION EXCHANGER, COLUMN PREPARATION AND ISOLATION PARAMETERS

SSBB matrix was purchased from Pharmacia Biotech Inc. (Uppsala, Sweden); LF standard (purity of 93%) was from DMV International Inc. (DMV International Inc., the Netherlands). Acetonitrile, formic acid, and sodium chloride were purchased from Fisher Scientific Inc. (Hampton, USA).

The SSBB matrix was packed into a Pharmacia HR 5/5 column (i.d. 0.5 × 5 cm) to 1ml bed volume; the ion capacity was 180-250 μM/mL gel. The column was washed with 20 bed volumes of HPLC grade water and regenerated with 5 bed volumes of 2 M NaCl in 50 mM Na_2HPO_4 (pH 7.5), followed by several bed volumes of HPLC grade H_2O and 50 mM Na_2HPO_4 (pH 7.5).

The high-pressure liquid chromatography (HPLC) consisted of the following Shimadzu LC-6A HPLC components: LC-6A binary pump system, CTO-6A column oven, SCL-6A

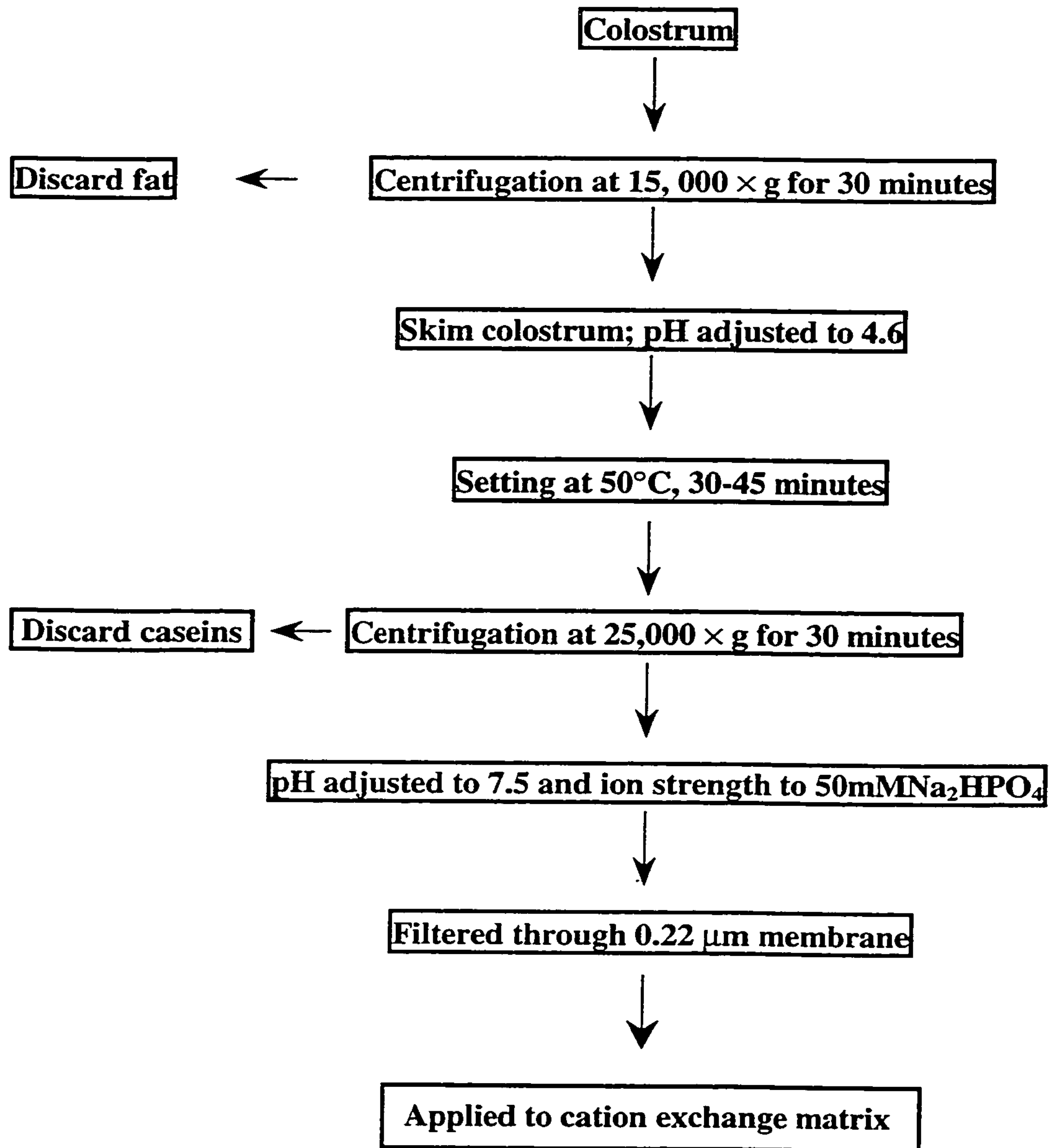

Figure 3-1. Flow diagram of bovine colostrum treatment

system controller, SIL-6A auto-sample injector, C-R6A integrator and SPD-10A UV spectrophotometric detector (Shimadzu, Kyoto, Japan), and Fractomtte® a-200 fraction collector from Bruchler Instrument Inc. (NJ, USA).

3.2.3 ISOLATION PROCEDURE OF LF FROM BOVINE COLOSTRUM WHEY

The bovine LF purification procedure is illustrated in Figure 3-2. The cation exchange column was equilibrated in 50 mM Na_2HPO_4 (buffer A, pH 7.5) until the absorbance at 280 nm was 0, and then 20 mL of colostrum whey was injected. Bound LF was eluted by stepwise NaCl gradient (Table 3-1) in an equilibration buffer for about 65 minutes. The peak fractions from SSBB column were pooled, dialyzed in Spectra/Por membrane (MWCO 12,000 -14,000, Spectrum Medical Industries Inc., Houston, USA) against MilliQ-H_2O for 24 hours, followed by lyophilization. The freeze-dried LF was stored at –20°C for further analysis. After each purification, the SSBB column was washed extensively with 2 M NaCl in 50 mM Na_2HPO_4, followed by 20% ethanol in H_2O, and washed with MilliQ-H_2O. When not in use, the SSBB matrix was stored in 20% ethanol at 4°C .

3.2.4 SP-SEPHAROSE BIG BEADS (SSBB) CATION-EXCHANGE MATRIX CAPACITY AND LF RECOVERY

SSBB matrix capacity was investigated by loading known amounts of standard LF or LF isolated from colostrum whey onto a column, and bound LF was measured by following equation:

$$\text{Capacity, (mg/mL)} = Lf_{max} / V_{matrix}$$

Where: Lf_{max} - maximum amount of LF (mg) binding to SSBB matrix;

V_{matrix}- SSBB matrix volume (mL)

LF recovery was expressed by the ratio of isolated LF (dried weight; mg) to total amount applied (dried weight; mg).

$$\text{Recovery, (\%)} = W_{lf} / W_{applied} \times 100\%$$

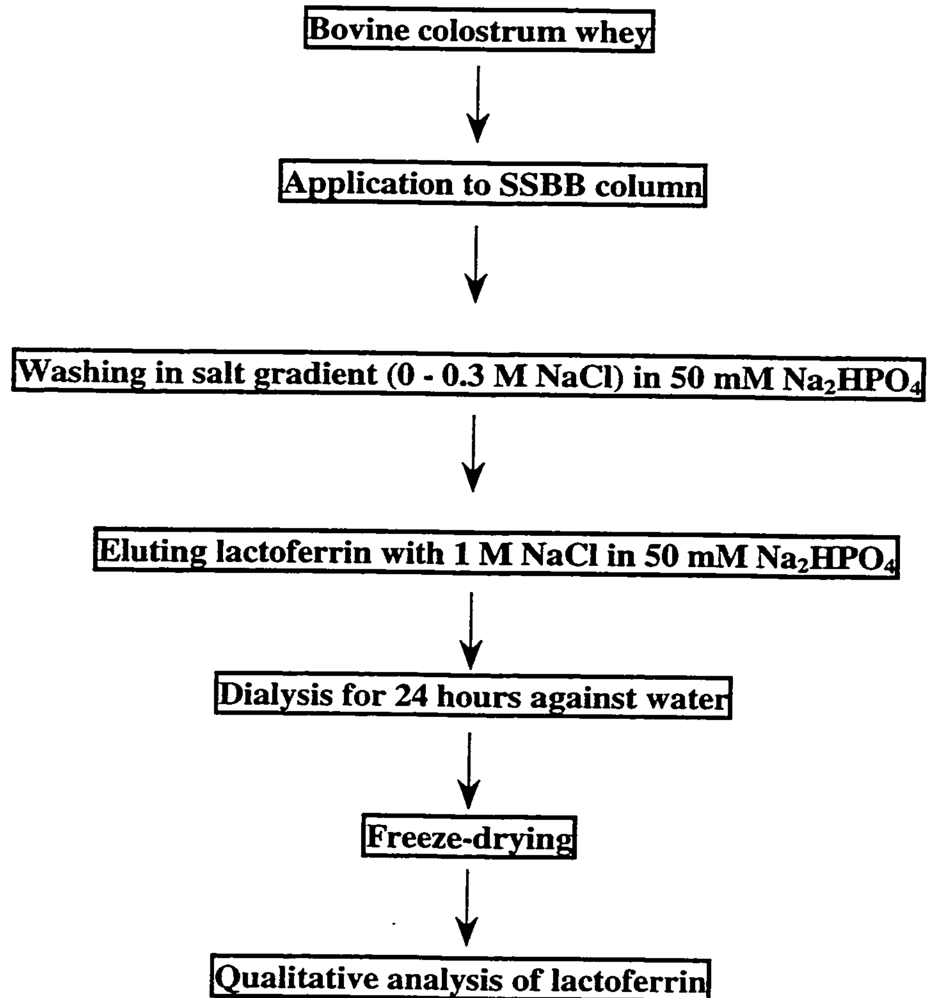

Figure 3-2. Flow diagram of bovine LF isolation by sp-sepharose big beads (SSBB) cation exchange chromatography.

Table 3-1. NaCl gradient used to isolate bovine lactoferrin by SSBB cation exchange chromatography

Time (Minutes)	Buffer B* (%)
0	0
25	0
25.1	30
40	30
40.1	100
70	100

* Buffer A: 50 mM Na_2HPO_4; pH 7.5;
Buffer B: 1 M NaCl in 50 mM Na_2HPO_4; pH 7.5;

Where: W_{lf} - Dried weight of isolated LF (mg), $W_{applied}$ - Dried weight of total amount applied (mg)

3.2.5 REVERSE PHASE CHROMATOGRAPHY OF LF ISOLATED FROM COLOSTRUM WHEY

An aliquot (10 μL) of purified LF or 10 μl LF standard (2.0 mg/mL) was applied to a Pharmacia PepRPC C_{18} reverse-phase column (Pharmacia Biotech Inc., Sweden). The buffer system was established as follows: Buffer A: 10% (v/v) mixture (10% (w/v) NaCl in 1% (v/v) CH_2O_2), 10% (v/v) acetonitrile, 80% (v/v) H_2O; Buffer B: 10% (v/v) mixture (10% (w/v) NaCl in 1% (v/v) CH_2O_2), 80% (v/v) acetonitrile, 10% (v/v) H_2O. The gradient used to analyze isolated LF is illustrated in Table 3-2. The flow rate was 1ml/min, and eluted protein was monitored by UV absorbance at 280 nm.

3.2.6 SIZE EXCLUSION CHROMATOGRAPHY OF LF ISOLATED FROM COLOSTRUM WHEY

An aliquots of purified LF collected from the SSBB cation exchanger column was applied to a TosoHAAS TSK-Gel 3000SW column (i.d. 0.78 × 30 cm, TosoHAAS Inc., Japan), and the elution buffer consisted of 20 mM sodium phosphate in 0.4 M KCl (pH 6.8). Samples were eluted at a constant flow rate of 1 ml/min, and protein was detected by UV absorbance at 280 nm.

3.2.7 MATRIX ASSISTED LASER DESORPTION/IONIZATION MASS SPECTROMETRY (MALDI-MS) OF ISOLATED LF

The MALDI-MS system was calibrated with a BSA dimer (MW_1 =66.4 Kdal, MW_2 =132.9 Kdal). Isolated LF from colostrum whey or standard LF was dissolved in 0.1% TFA to obtain the concentration of 1.5 mg/mL. Sinapinic acid (MW 224.21 dal) was chosen to be the matrix in MALDI-MS analysis. Saturated solutions of sinapinic acid matrix were prepared by dissolving sinapinic acid in 70% trifluoroacetic acid (TFA) / 30% acetonitrile solution at room temperature. Samples for MALDI-MS analysis were

Table 3-2. Mobile phase gradient used in analysis of isolated LF by reverse phase HPLC

Time (min)	Buffer B* (%)
0	25
20	70
25	70
35	25
40	25

* Buffer A: 10% (v/v) (10% (w/v) NaCl in 1% (v/v) CH_2O_2); 10% (v/v) acetonitrile; 80% (v/v) H_2O;
Buffer B: 10% (v/v) (10% (w/v) NaCl in 1% (v/v) CH_2O_2); 80% (v/v) acetonitrile, 10% (v/v) H_2O;

prepared by mixing 10 μL of the LF solution with 10 μL of sinapinic acid matrix solution, and then a 2 μl mixture was applied to a stainless steel insertion probe tip. Prepared samples were allowed to air-dry before they were applied into the vacuum system of the mass spectrometer. Mass spectrometry was performed on a Bruker Proflex MALDI mass spectrometer (Bruker Analytical Systems Inc., USA) operating in the positive-ion mode of detection. All MALDI mass spectra were recorded with a Bruker Reflex II Instrument (Bruker Analytical Systems Inc., USA). Ionization was achieved using a conventional nitrogen laser (337 nm beam, 3 ns pulse width, 5 Hz) set at an attenuation between 15 and 20. In the linear model of operation, LF molecules were accelerated at 20 kV.

3.2.8 ELECTROPHORETIC ANALYSIS OF LF ISOLATED FROM COLOSTRUM WHEY

An aliqouts of isolated LF was mixed with an equal volume of solubilization buffer (containing 2% sodium dodecyl sulfate, 3% mercaptoethanol), heated in a boiling water bath for 90 seconds, and electrophoresed on a 12.5% polyacrylamide gel with stacking gel, as described by Laemmli (18). After electrophoresis, the gel was fixed in a 10% acetic acid solution with 50% methanol. The gel was stained with Coomassie Blue (0.1% Coomassie blue and 10% acetic acid), according to Laemmli (18).

3.2.9 AMINO ACID COMPOSITION OF ISOLATED LF FROM COLOSTRUM WHEY

The amino acid composition of isolated LF by SSBB cation exchange chromatography was determined with a Beckman System 6300 amino acid analyzer, system gold version 6.01. The sample was hydrolyzed in 5.7 N HCl for 24 hours at 110°C before analysis. However, methionine, cysteine and tryptophan were not determined by this method.

3.2.10 SCALE-UP ISOLATION OF LF FORM COLOSTRUM WHEY BY SSBB MATRIX

The purity of isolated LF from colostrum whey was evaluated by reverse phase chromatography, electrophoresis using SDS-PAGE, and amino acid composition analysis. The yield of isolated LF was estimated by following equation:

$$\text{Yield (mg)} = W_{fraction} \times Purity_{lf}$$

$$\text{Purity, (\%)} = W_{lf} / W_{fraction} \times 100\%$$

Where: $W_{fraction}$ - Fraction dried weight per matrix volume (mg/mL);

W_{lf} - LF dried weight per matrix volume (mg/mL);

$Purity_{lf,}$ (%) - Purity of isolated LF

3.3 RESULTS AND DISCUSSION

3.3.1 BOVINE COLOSTRUM TREATMENT

Bovine colostrum is a mammary gland secretion produced by cows during the first 24-48 hours after calf birth. Colostrum is not only a source of nutrients such as proteins, carbohydrates, fat, vitamins and minerals, but also contains several biologically active molecules essential for specific biological functions. In colostrum, the concentrations of minor bovine whey proteins are relatively high; however, the colostrum is not industrially processed due to the small volume available and its unpleasant flavor and taste. The concentration of biological components such as LF in bovine colostrum is significantly higher than in milk (Figure 2-3), but the colostrum is not yet used as a source of these components on the industrial scale. The development of novel functional ingredients and nutraceuticals derived from milk requires development of technological processes for their isolation and purification. Therefore, colostrum or milk with physiologically increased level of bioactive proteins may become a primary source for their isolation. The separation of individual whey proteins from colostrum may provide an incentive to traditional dairy-industry processing sector for the development of a nutraceutical industry that will provide customers with functional and healthy food ingredients.

As described in Chapter 2, whey proteins are relatively sensitive to heat but more

stable to acid treatment; in contrast, caseins are very heat-stable, but become insoluble at pH 4.6. Therefore, casein was separated from whey proteins in colostrum by isoelectric precipitation at pH 4.6. The benefits of this procedure are 1) isoelectric precipitation does not introduce additional components interferring with LF isolation; 2) isoelectric precipitation is cheaper than enzymatic precipitation by rennet; and 3) the use of rennet might hydrolyze LF and damage its structure. The pH of colostrum whey, after casein removal, was adjusted to pH 7.5 with 1M sodium hydroxide and ionic strength was adjusted to 50 mM of Na_2HPO_4 by adding solid Na_2HPO_4. Filtration of colostrum whey through 0.22 μm membrane is essential for the removal of any protein particles and phospholipids. Phospholipid removal from cheese whey by microfiltration is recommended before further processing, because phospholipids interfere with the membranes and the chromatographic processing of whey.

3.3.2 CATION EXCHANGER, COLOMN PREPARATION

The sp-sepharose big beads (SSBB) cation exchangers used in this study are based on 100-300 μm agarose beads, which have higher degree of cross-linking, compared to other cation exchangers. This highly cross-linked nature of the matrix means that the bead size and bed volumes do not change with the changes in ionic strength or pH and thus provide high physical stability. The SSBB matrix is loaded with strong ion exchange groups, and these groups remain charged and maintain a consistently high capacity over the broad working pH range of 4-13. These characteristics allowed for the selection of a buffer system that best suited sample preparation and treatment. The SSBB matrix is also characterized by low compression and high flow rate (50 cm/h). These properties were the most important ones in the selection of a matrix for both laboratory and potential industrial scale lactoferrin isolation by ion exchange chromatography processing.

3.3.3 ISOLATION OF LF FROM BOVINE COLOSTRUM WHEY

LF was first isolated on DEAE-cellulose ion-exchanger from human milk by Johansson in 1960 (19). Since then, some isolation chromatography methods have been proposed and discussed. In early 1978, Banyard isolated LF from bovine tears by DEAE-Sephadex

A-50 anion-exchange chromatography combined with size-exclusion chromatography (13). Later, Ekstrand and Bjork explored LF separation on CM-cellulose from bovine milk (20), and in 1986 Foley and his co-workers successfully used cellulose-phosphate, followed by Sephadex G-100 gel-filtration chromatography (21), for the isolation of LF from human milk. CM-Sephadex was quite often used to purify LF by Tsuji (14), Buchta (11), and Yoshida (22).

Isolating bovine LF by IEC has been explored by Rejman, Banyard (12,13), Tsuji (14) and Moguilevsky (15); however, most methods involved multi-step operations. One-step LF isolation using a Mono-S matrix (23) and CM-cellulose ion exchanger was reported (22). Thus, in this research, the isolation of bovine LF by SSBB cation exchange chromatography was developed with a good potential for an industrial scale up.

At bovine colostrum whey pH 7.5 and 50 mM Na_2HPO_4, the major whey proteins were charged negatively based on the principle shown in Figure 2-13. At that pH, the LF was charged positively since the pI of LF is 8.5, whereas other whey proteins having pI in the range of 4.2 to 7.3 were charged negatively. As the whey proteins passed through the SSBB matrix, the negatively charged whey proteins passed through the column without binding to negatively charged groups on the matrix. However, the positively charged LF molecules were strongly bound by electrostatic forces. Bound LF was eluted from the ion exchange matrix by increasing the concentration of ionic strength to 1 M NaCl in the eluting buffer.

A profile of bovine LF isolated by SSBB cation exchange chromatography is shown in Figure 3-3, and a stepwise gradient of NaCl developed for isolation of bovine LF is given in Table 3-1. Under established conditions of separation, whey proteins such as α-LA and β-LG were washed out from the SSBB column by an equilibrating buffer during the first 25 minutes at flow rate of 1ml/min. During next 15 minutes, the weakly bound whey proteins with retention time of about 30 minutes were washed out at 0.3 M NaCl in an equilibrating buffer. Strongly bound LF eluted at 50 minutes and at 1 M NaCl in the same buffer. Many preliminary experiments were carried out in order to establish a gradient

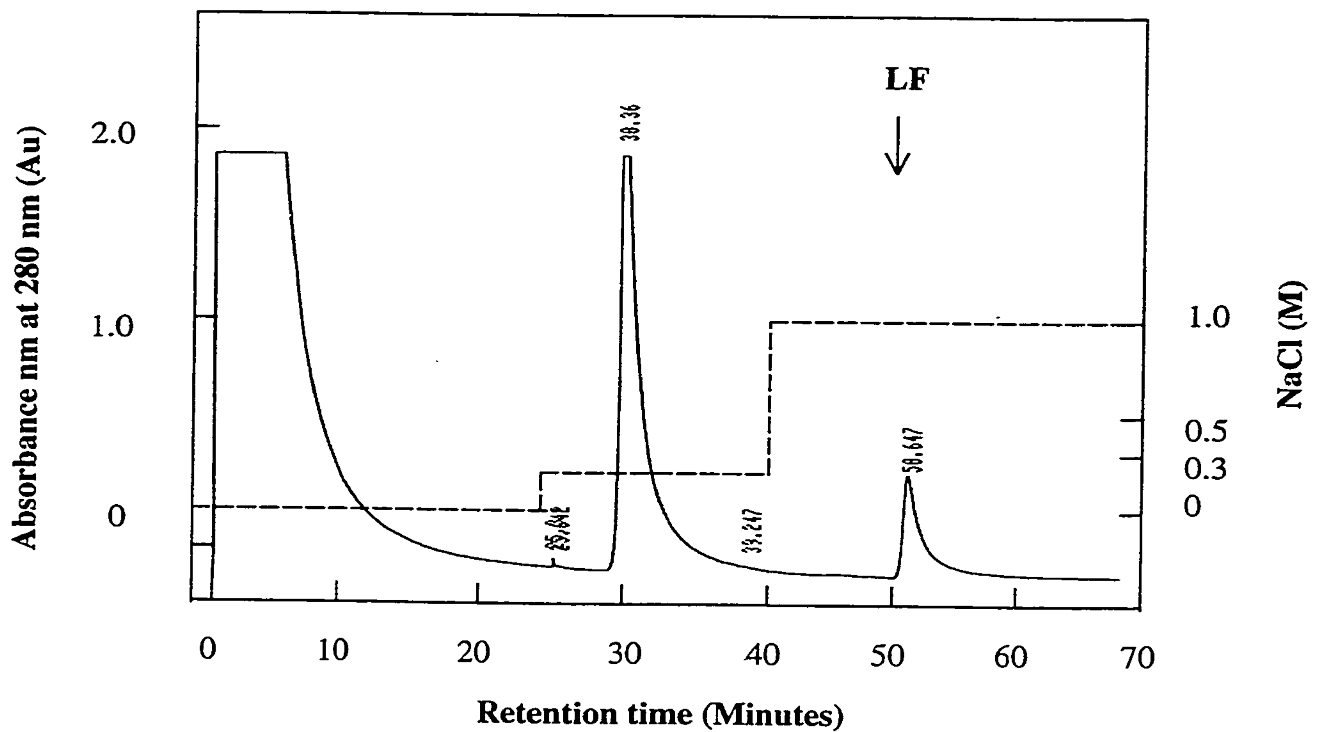

Figure 3-3. Chromatogram of bovine LF isolation on sp-sepharose big beads (SSBB) matrix. Buffer A: 50 mM Na_2HPO_4, pH 7.5; Buffer B: 1 M NaCl in buffer A.

profile separating other proteins from LF. Developed parameters allow for the separation of whey proteins into three main species: 1) LF-depleted whey proteins (fractions eluted between 1-25 minutes); b) fraction eluted at 30 minutes, and 3) LF. As is shown in Figure 3-3, the separation of LF from colostrum whey by the SSBB matrix resulted in very good resolution, allowing for much easy scale-up purification compared to that reported in separation protocols.

3.3.4 SP-SEPHAROSE BIG BEADS (SSBB) CATION EXCHANGER MATRIX CAPACITY AND LF RECOVERY

The yield and recovery parameters are critical in industrial application of separation techniques, industrial membrane processing techniques, and chromatographic techniques.

The recoveries of standard and bovine whey LF from SSBB matrix were evaluated (Table 3–3). 40 mL of 0.5 mg Lf/mL of standard and 13 mL whey (0.45 mg Lf/mL) were applied to 1 mL SSBB gel. About 80% of standard LF was recovered whereas 45% was recovered from colostrum whey. When LF isolated from colostrum was applied to the column (data not included), the recovery was similar to that of the standard. However, from the practical point of view involving the potential application of this process, the recovery of LF from colostrum whey containing a mixture of different proteins was also important. For this reason, colostrum whey with a known LF concentration (0.45 mg Lf/mL) was used to determine the recovery under proteins mixture conditions. The lower recovery of LF from colostrum whey can be attributed to the fact that a portion of LF may interact with other proteins. For example, LF may associate with caseins, α-LA and β-LG and lysozyme (1), and it can be speculated that under current separation conditions, a portion of LF was lost with other protein complexes.

The capacity of the SSBB matrix to bind standard LF was about 39 mg Lf/mL gel (Figure 3-4), and the capacity to bind LF from whey (0.45 mg Lf /mL) was about 10 mg Lf/mL gel (Figure 3-5). The lower capacity of the SSBB matrix for LF in colostrum might be due to the reasons discussed above.

The effect of column washing by buffer (0.3 M NaCl in 50 mM Na_2HPO_4; pH 7.5) before applying elution gradient on LF purity was also investigated, and the results are

Table 3-3. Recovery of LF from SSBB matrix

	Standard	Whey*
Feeding LF (mg)	20	5.85
Recovered LF*(mg)	16	2.63
Recovery (%)	80	45

* Concentration of LF in whey was 0.45 mg Lf/mL.

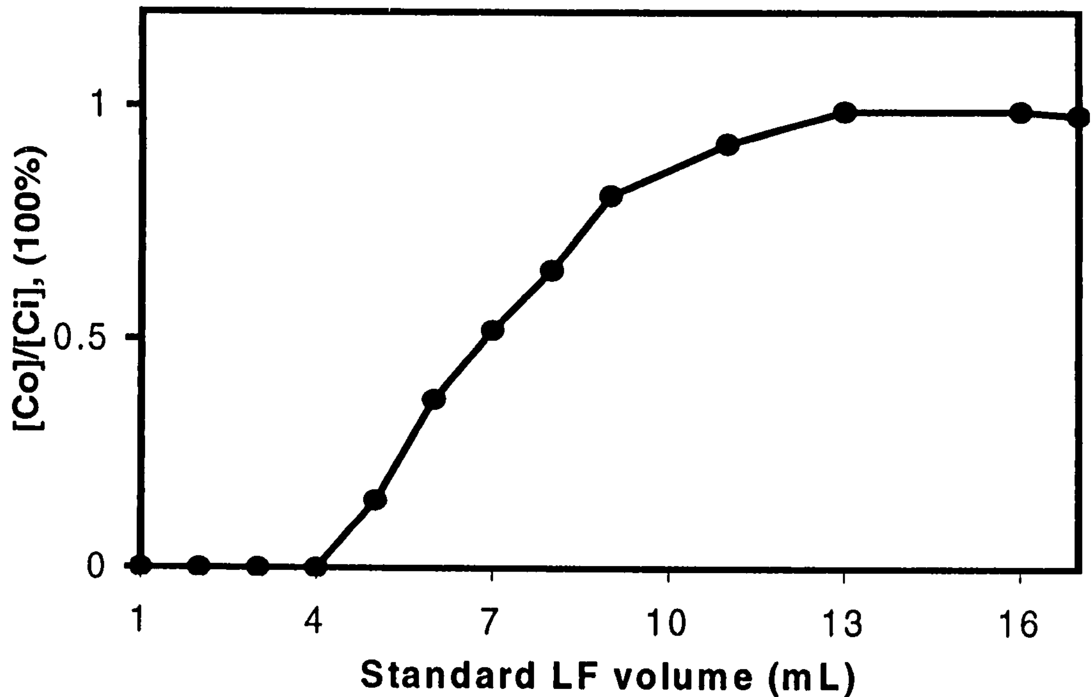

Figure 3-4. Binding capacity of sp-sepharose big beads (SSBB) matrix (1mL), where, [C_i] is LF concentration at inlet to column, [C_o] is LF concentration at outlet to column. The concentration of standard LF is 3 mg/mL.

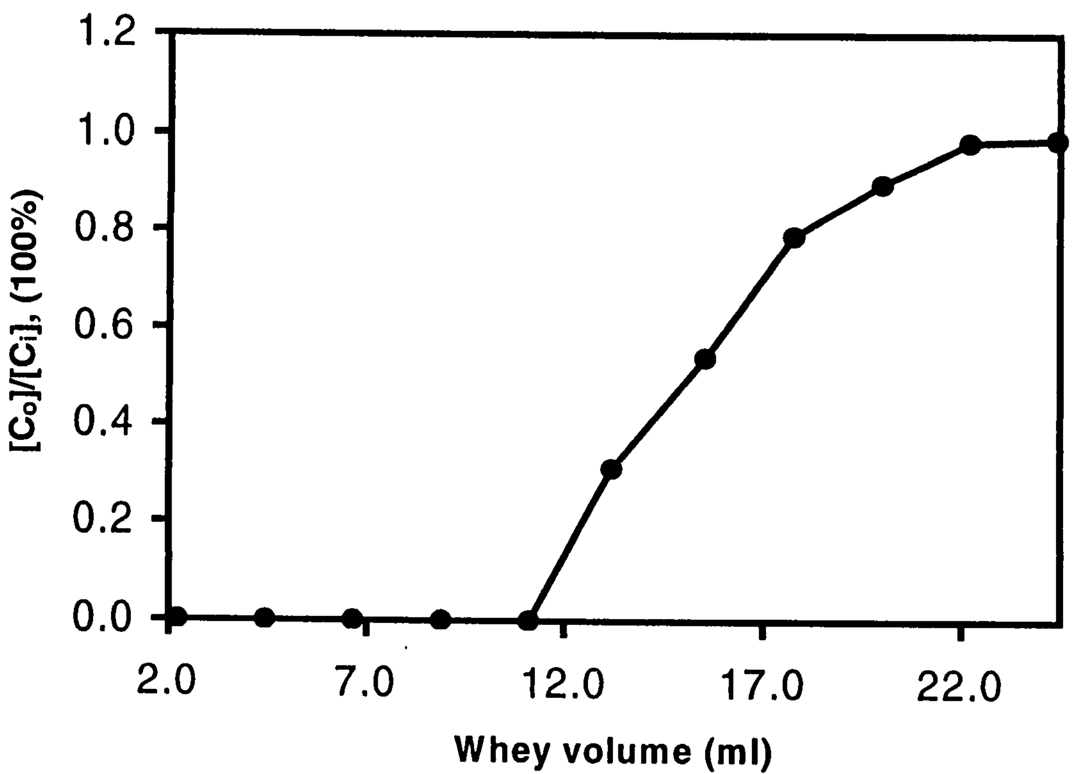

Figure 3-5. Binding capacity of sp-sepharose big beads (SSBB) matrix (1mL) to LF in colostrum whey (0.45 mg Lf/mL). [C_i] is LF concentration at inlet to column, [C_o] is LF concentration at outlet to column.

shown in Figure 3-6. As the volume of washing buffer increased from 0 to 100 × bed volume, the purity of isolated LF was significantly improved. Isolated LF after 20 bed volume washing had a good purity; however, if higher purity is needed, then 80 to 100 × bed volume washing is recommended as the purity was demonstrated by narrower and sharper LF bands. Some loss of LF may occur during higher bed volume washings. It appears that 40 to 60 × bed volume of washing with 0.3 M NaCl in 50 mM Na_2HPO_4; pH 7.5 leads to a reasonable yield and purity of isolated LF.

3.3.5 IDENTIFICATION OF ISOLATED LF FROM COLOSTRUM WHEY BY REVERSR PHASE AND SIZE EXCLUSION CHROMATOGRAPHY, MALDI-MS, SDS-PAGE AND AMINO ACID COMPOSITION ANALYSIS

Isolated LF from colostrum whey was further analyzed and compared to the standard by reverse phase HPLC. The profile is shown in Figure 3-7. Both isolated bovine LF and the standard eluted out at about 20 minutes, and their patterns are very similar. The reverse phase HPLC was used in order to identify isolated LF. The profile can not be compared to literature data as separation conditions chosen were not similar, reverse phase HPLC was not used for isolation but as a analytical tool in quality control.

Size-exclusion chromatography was carried out at pH 6.8, and the chromatograms are shown in Figure 3-8. Both isolated LF and the standard eluted at 10 minutes, and obtained results indicate that they have similar MW size. Bovine LF molecular weight varies from 76 Kdal to 85 Kdal (14,15,16,17), and this variation might be due to different analytical methods used or analysis accuracy. In this research, matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS) was also used to determine the molecular weight of isolated LF. Two LF peaks were found by MALDI-MS (Figure 3-9). The first one with molecular weight of 77.9 Kdal refers to the LF monomer molecule, and the second peak with molecular weight of 152.0 Kdal seems to be a LF dimer molecule, based on the fact that the MW of the second peak is about 2 fold bigger. The molecular weights of isolated and standard LF were 78 Kdal and 83 Kdal, respectively (Figure 3-10). The difference in molecular weight (5 Kdal) may be due to: 1) different sources of

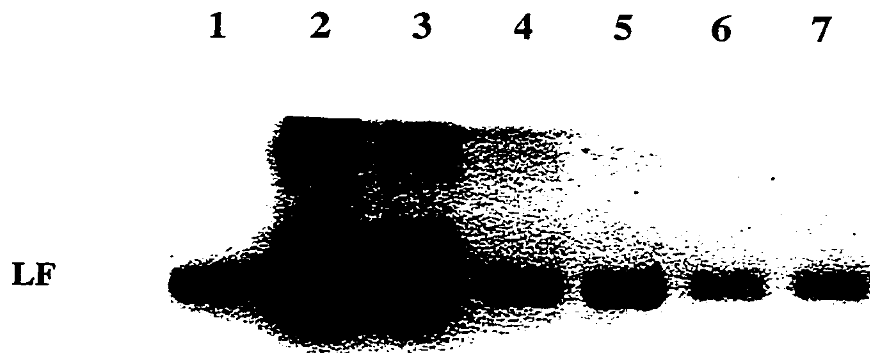

Figure 3-6. The effect of variable bed volume washing (0.3 M NaCl in 50 mM Na_2HPO_4, pH 7.5) on LF purity as measured by SDS-PAGE. Lane 1: Standard LF, lane 2 to lane 7 are isolated LF with 0 ×, 20 ×, 40 ×, 60 ×, 80 ×, 100 × bed volume washing, respectively.

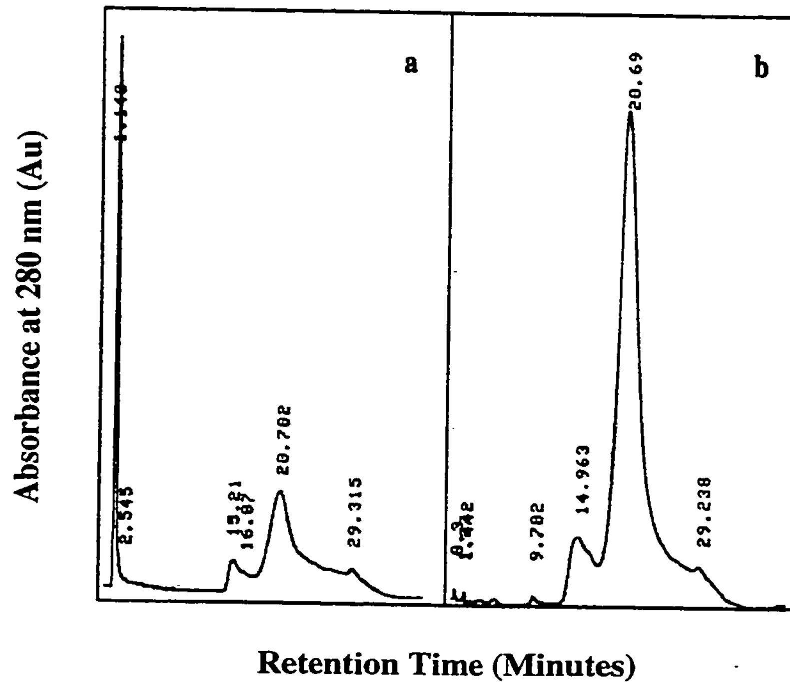

Figure 3-7. Reverse phase chromatography of isolated and standard lactoferrin. a) isolated lactoferrin from bovine colostrum, b) standard lactoferrin

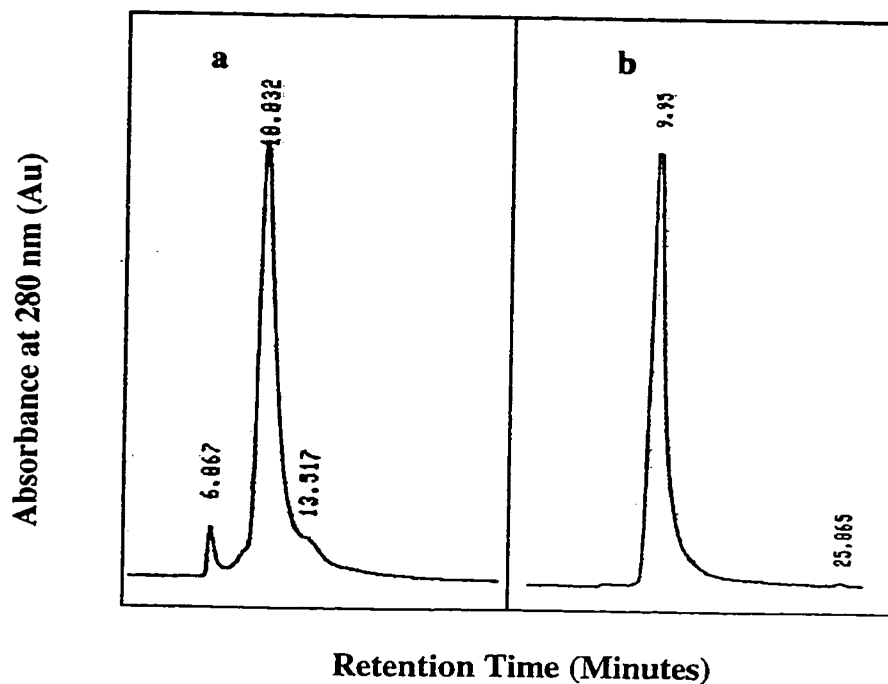

Figure 3-8. Size exclusion chromatography of isolated LF and standard. a) isolated LF from colostrum whey, b) standard

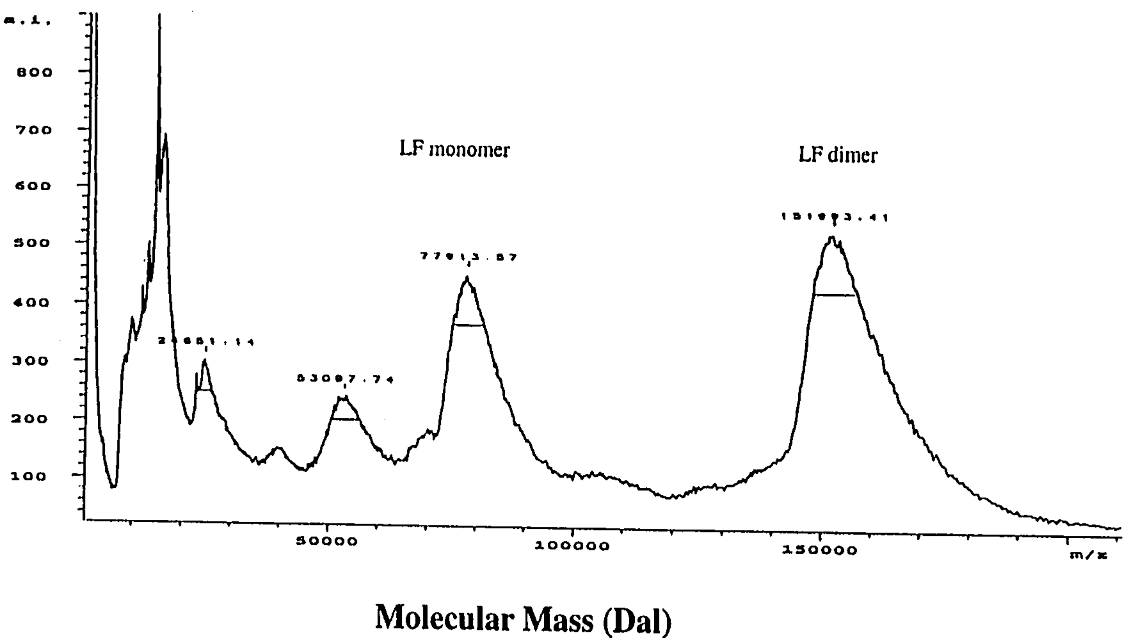

Figure 3-9. Matrix assisted laser desorption/ionization mass spectrometry (MALDI-MS) of isolated LF. The matrix was sanipinic acid.

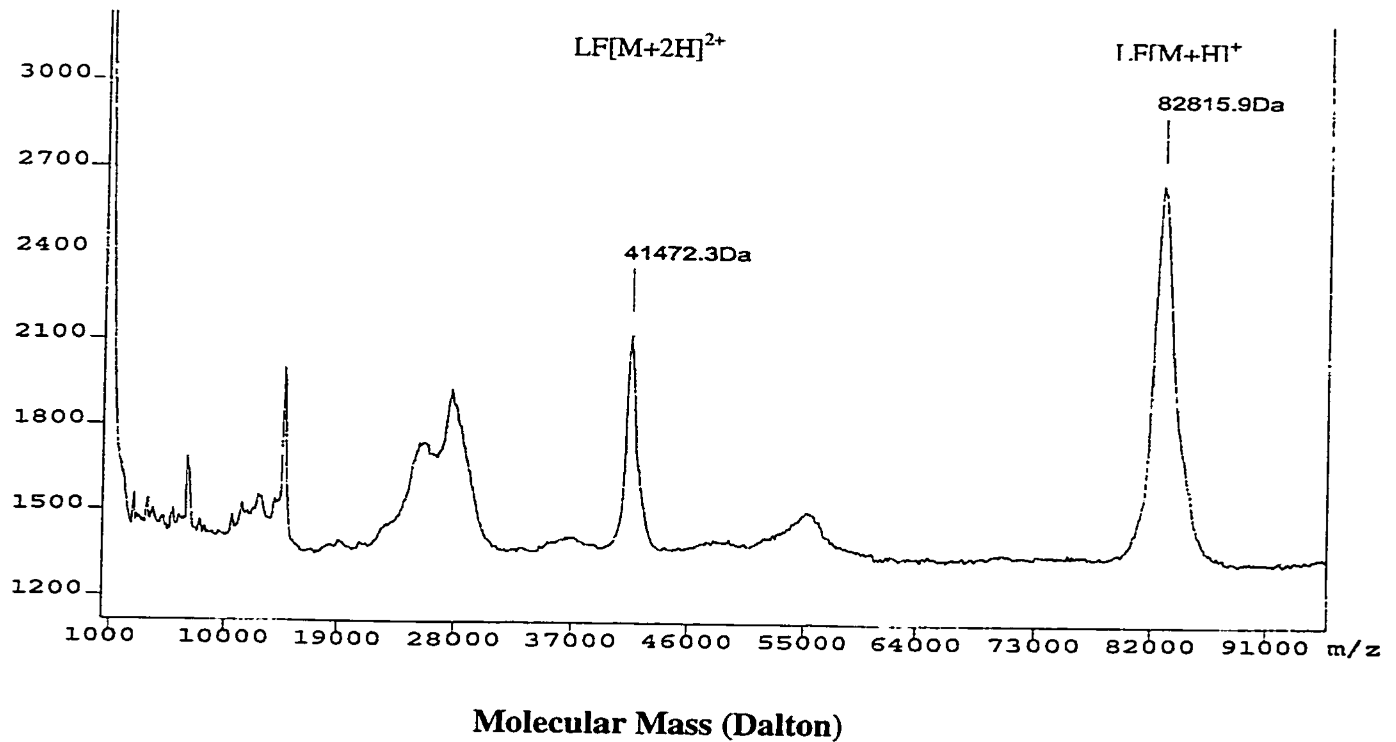

Figure 3-10. Matrix assisted laser desorption/ ionization mass spectrometry of standard lactoferrin. The matrix was sanipinic acid.

LF (colostrum *vs* whey); 2) different degree of iron-saturation; 3) difference in carbohydrate content between these LF molecules. The molecular weight of LF isolated from colostrum (78 Kdal) reported here is comparable to the SDS-PAGE result reported by Hutchens (4).

Electrophoretic analysis of LF isolated from colotrum whey and the standard on the SDS-PAGE gel are illustrated in Figure 3-11. Both proteins showed similar average mobility on a 12.5% polyacrylamide SDS-gel. The molecular weight of isolated LF was within the range of 67-94 Kdal, and it was estimated to be appropriately 80 Kdal molecular weight is comparable to the molecular weight of 78 Kdal, as determined by MALDI-MS, and the purity of isolated LF was also comparable to that of standard one (Figure 3-10).

The amino acid composition of LF isolated from colostrum whey is shown in Table 3-4, and similar data was reported by Wang (24). Methionine, cysteine and tryptophan were not determined. These three amino acids are decomposed by 5.7 N HCl during sample treatment. In order to detect these three amino acids, performic acid oxidation for methionine and cysteine should be performed first, then followed by HCl treatment. The amino acid composition of isolated LF was also comparable to theoretical values; therefore, the isolated LF was additionally identified by amino acid composition as well.

3.3.6 SCALE-UP ISOLATION OF LF FROM CLOSTRUM WHEY BY SSBB MATRIX

Cation exchange chromatography (11,14) and affinity chromatography (4, 25) were used in LF isolation from mammalian milk whey. In this research, a one-step LF isolation method was developed using cation exchange chromatography on a SSBB matrix. The experimental data were obtained by using a 1 mL matrix.

In order to obtain experimental data for the scale-up of this purification procedure, the matrix volume was increased from 1 mL to 17 mL (1700% increase). 12 × bed volume of colostrum whey (0.45 mg Lf /mL) was loaded to a column containing 17 mL matrix. The effect of NaCl concentration in a washing buffer in the range from 0.2 to 0.6 M on LF purity and the yield is shown in Figure 3-12. The buffer volume was constant and equal

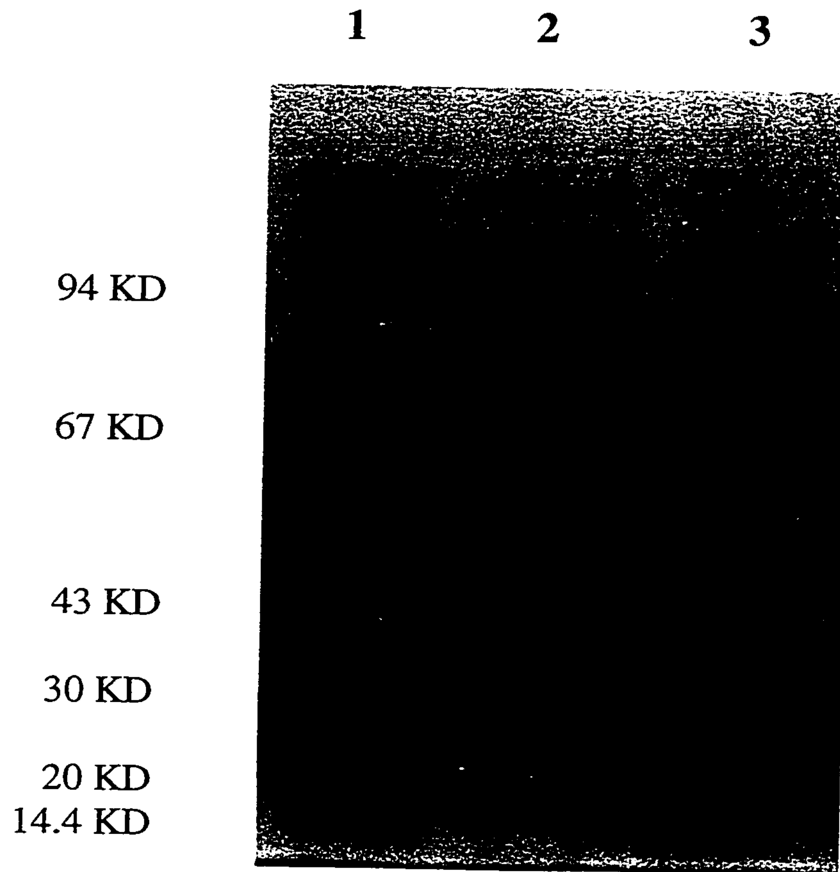

Figure 3-11. The SDS-Gel Electrophoresis (12.5% Polyacrylamide) of isolated bovine LF by SSBB Cation exchange chromatography. Lane 1: Bio-Rad MW standard; Lane 2: isolated bovine LF; Lane 3: standard LF.

Table 3-4. Amino acid composition of isolated bovine LF from colostrum whey

	Theoretical value	I-LF (SSDA)	I-LF (SSBB)
		Mol %	
Lysine	7.8	9.0	8.6
Histidine	1.4	1.6	1.6
Arginine	5.8	5.7	5.4
Aspartic acid	9.7	10.6	10.3
Threonine	5.2	4.9	5.1
Serine	5.0	5.4	6.3
Glutamic acid	9.8	11.2	11.2
Proline	4.5	5.6	5.9
Glycine	7.4	7.7	8.3
Alanine	9.8	10.2	9.4
Cysteine	3.8	ND	ND
Valine	6.6	7.2	7.6
Methionine	0.4	ND	ND
Isoleucine	2.6	2.7	2.6
Leucine	10.6	10.4	10.1
Tyrosine	3.5	3.3	3.4
Phenylalanine	4.3	4.3	4.1
Tryptophan	1.7	ND	ND

Theoretical value is adapted from Wang et al. (1984).
ND: not determined

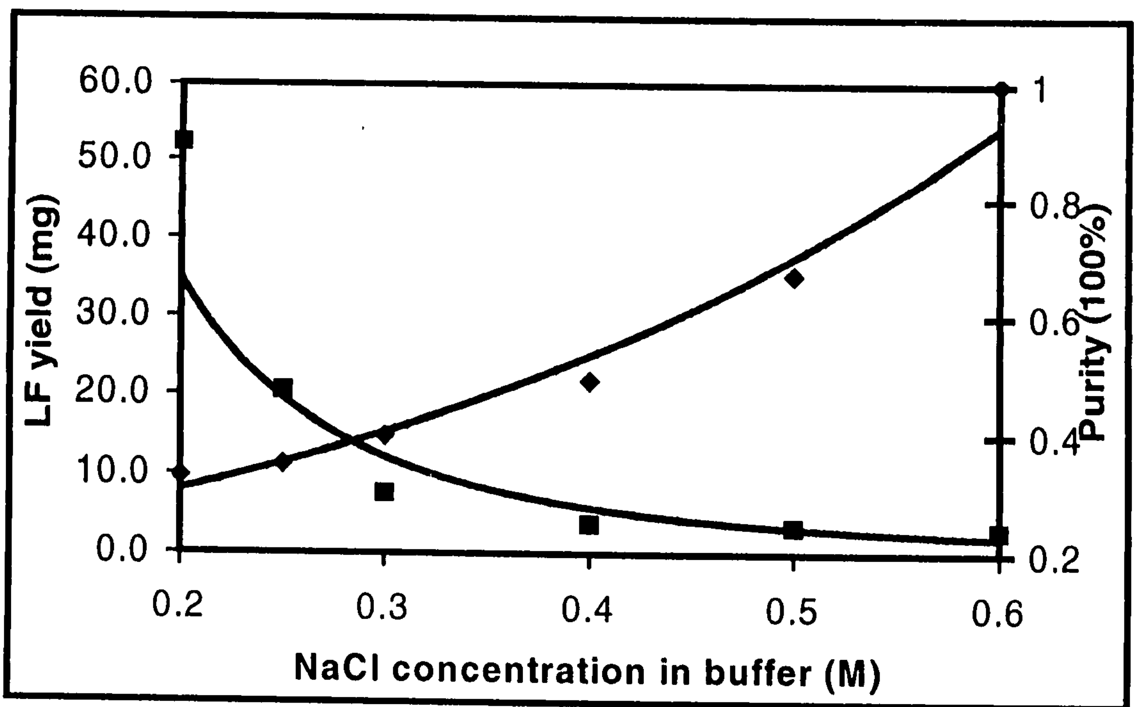

Figure 3-12. The effects of NaCl concentration in washing buffer on isolated LF purity and yield. Washing volume was constant at 3 × bed volume. The concentrations of NaCl were: 0.2, 0.3, 0.4, 0.5 and 0.6 M in 50 mM Na_2HPO_4, pH 7.5, respectively. After washing the LF was eluted with 1 M NaCl in the same buffer.

to 3 × bed volume. After washing, the LF was eluted from the column with the buffer at 1 M NaCl concentration. We found that the LF purity increased from 30% to 92% as the concentration of salt in the washing buffer increased from 0.2 M to 0.6 M. However, at the same time, the LF yield decreased from 35 mg to 3 mg (Figure 3-12). At the intersection point of the LF yield curve and LF purity curve, the LF purity was 40% and yield 18%, while the salt concentration was 0.3 M. This experiment revealed that washing the column with 3 × bed volume of buffer (0.3 M NaCl in 50 mM Na_2HPO_4; pH 7.5) before elution of bound LF results in LF with 40% purity without losing much of the LF yield. However, if higher purity of LF is needed, then LF yield has to be sacrificed to less than 18%.

The effect of the loading rate (mL/min) of bovine colostrum whey on the yield of isolated LF was looked at, and results are shown in Figure 3-13. The relative yield of LF decreases from 100% to 40% when the loading rate increases 3 fold (Figure 3-13). This result indicate that at a faster loading rate, LF molecules may not have enough time to reach available binding sites on the matrix for efficient binding; therefore, some LF molecules pass through the SSBB matrix without any specific binding. Thus, the whey loading rate should be considered in setting up separation protocols. From an industrial and practical point of view, a shorter processing time is still a key factor that will determine large-scale LF isolation.

3.4 CONCLUSIONS

A simple bovine LF isolation method was established using sp-sepharose big beads (SSBB) cation-exchange chromatographic techniques. The bovine LF isolated by this method had reasonable purity and yield. The molecular weight of isolated LF was determined as about 78 Kdal by MALDI-MS and SDS-PAGE. The binding capacities of LF to the SSBB matrix were on average 39mg Lf/mL gel (standard) and 10 mg Lf/mL gel (colostrum whey), respectively. The recovery of standard LF was 80%, and that of LF from whey (0.45 mg Lf/mL) was 45%. The purity of LF separated by SSBB cation exchange chromatography was on average 93%. Based on the amino acid composition of the isolated LF, it was also identical compared to the standard. Further work needs to be

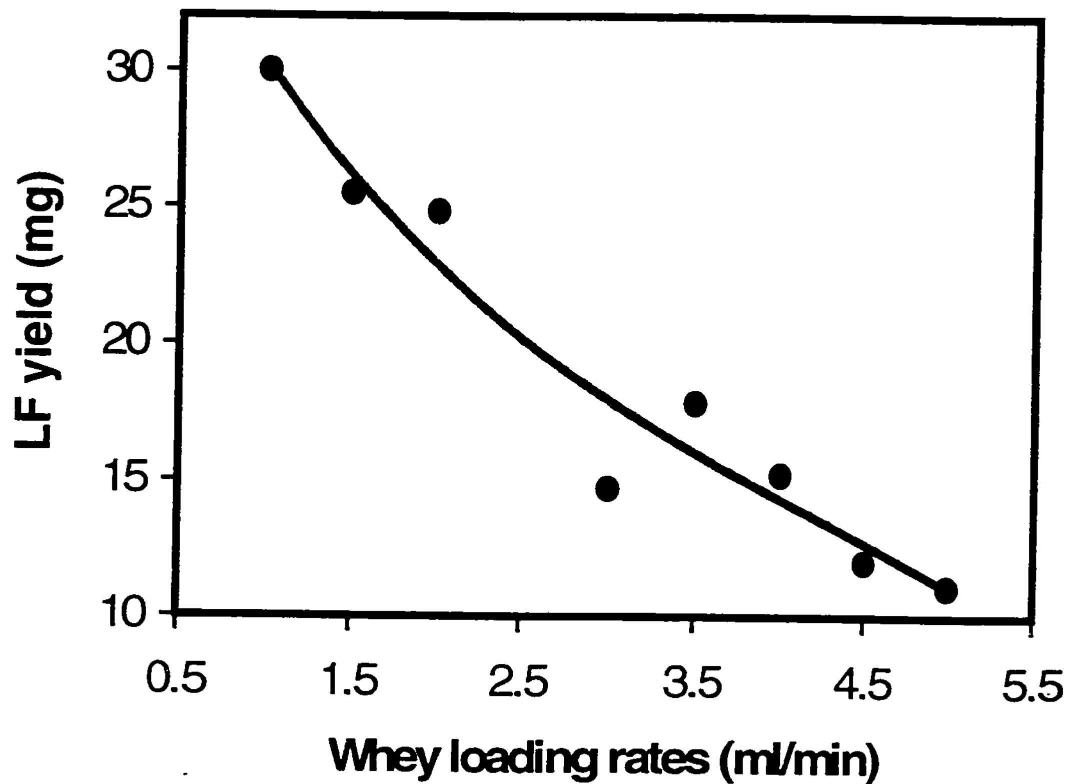

Figure 3-13. The effect of colostrum whey loading rate on yield of isolated LF. Column: 17 mL bed volume of SSBB matrix.

done in order to improve LF purity and yield and to optimize the technical parameters of LF separation at the industry scale.

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CHAPTER 4

ISOLATION OF LACTOFERRIN FROM BOVINE COLOSTRUM BY AFFINITY CHROMATOGRAPHY

4.1 INTRODUCTION

Lactoferrin (LF), an iron-binding glycoprotein synthesized by neutrophils and glandular epitheial cells (21), is a major whey protein in human milk with concentration range from 1 to 2 mg/ml, and a minor constituent of cow's milk with concentration from 0.01 to 0.1 mg/ml (22). LF concentration in colostrum or milk varies both among and within species (Figure 2-2). LF concentration changes during lactation and is highest during the first week (colostrum) and increases again during the dry period (Figure 2-3). LF has been shown to inhibit the growth of several microbes, including *Escherichia coli*, *Salmonella typhimurium*, *Shigella dysenteria*, *Listeria monocytogenes*, *Bacillus stearothermophilus* and *Bacillus Subtilis* (23, 24, 25, 26, 27). It has been also reported that LF has antiviral effects against herpes simples virus type-1 (HSV-1), human immunodeficiency virus-1 (HIV-1) and human cytomegalovirus *in vitro* (28, 29). Studies have demonstrated that about 2-6% of ingested LF survives the GI tract, a result suggesting that intact LF has a important role to play in the antimicrobial defense of the neonatal GI tract. If 94-98% of LF is actually broken down in the GI tract to lactoferricin (LFC), peptide released by pepsin hydrolysis of LF molecule may be a more important factor rather than native LF in antimicrobial defense mechanism (30,31). In this case, LF may serve as a precursor of LFC. Some data indicate that LF stimulates cell growth and acts as a growth factor or iron carrier molecule (32, 33, 34). The evidence has shown that LF could be a possible growth factor for the intestinal mucosa cells, and the role for LF in cellular proliferation has been recognized. Such evidence include 1) better gastrointestinal development in newborn animals fed maternal milk in comparison to newborns fed commercial formulas, 2) increased thymidine incorporation with LF supplementation of

milk formulas, and 3) in vitro augmentation of thymidine incorporation into rat crypt cell DNA by LF. Recent studies showed that LF could bind DNA and activate transcription, a finding that might explain the molecular basis of growth regulation (4).

Literature indicates that LF intends to become tightly associated with other macromolecules including DNA, and has been isolated successfully from human milk and urine of human-milk-fed infants by a single-strand DNA-agarose (SSDA) matrix (1,2, 3,4,5). Bovine LF can be recovered from skim milk or cheese whey on an industrial scale and made available as a functional ingredient. Using LF as the starting material, the large-scale production of LF hydrolysates and LFC-B is achievable. These new materials derived from bovine LF have potentially broad-spectrum antimicrobial properties, and considerable potential appears to exist for their widespread commercial use. Such material could be useful as a 'natural' preservative agent in foods or cosmetics, and as a functional component in new clinical foods and hygienic products for prevention or treatment of microbial infections and disease. Until now, no research had been reported on LF isolation from bovine colostrum whey by SSDA affinity chromatography. The objective of this research was to develop a LF isolation protocol based on SSDA affinity chromatography.

4.2 MATERIALS AND METHODS

4.2.1 BOVINE COLOSTRUM WHEY TRAETMENT

Colostrum was collected from the Dairy Technology Center at University of Alberta Research Station and stored at –20°C until use. Frozen colostrum was thawed at ambient temperature, and fat was removed by centrifugation at 15,000 × g at 4°C for 35 minutes. Thereafter, the temperature of colostrum was raised to 50 °C, and casein was precipitated by adjusting pH to 4.6, using 1 M HCl. Casein curd was removed by secondary centrifugation at 25,000 × g at 4°C for 30 minutes. The retained whey was re-adjusted to pH 8.0 with 1 M NaOH. Solid urea (Sigma Chemical Co., St. Luious, USA) was added to colostrum whey up to the concentration of 6 M, followed by filtration through a Millipore Millex-GS 0.22 μm membrane (Millipore Corporation, USA) before colostrum whey was applied to a SSDA affinity column.

4.2.2 AFFINITY GEL, COLUMN PREPARATION AND ISOLATION PARAMETERS

Single strand DNA-agarose (SSDA) was purchased from Pharmacia Biotech Inc.(Uppsala, Sweden). The standard LF (purity of 93%) was a commercial product from DMV International Inc.(DMV International Inc., Netherland). Other chemicals were purchased from Sigma Chemical Co.(St. Louise, USA), with the exception of acetonitrile and formic acid, which were purchased from Fisher Scientific Inc. (Hampton, USA).

The SSDA matrix was packed into a Pharmacia HR 5/5 column (i.d. 0.5 × 5cm) to 1 ml bed volume (1-3 mg DNA/mL gel). Then the column was washed with 20 × bed volumes of HPLC grade H_2O and regenerated with 5 × bed volumes of 2M NaCl in 20mM HEPES buffer; pH 8.0. The column was then washed sequentially with several bed volumes of HPLC grade H_2O, 8M guanidine-HCl in 20 mM HEPES; pH 8.0, and HPLC grade H_2O.

The apparatus that was used in the experiment consisted of the following Shimadzu LC-6A HPLC components: LC-6A binary pump system, CTO-6A column oven, SCL-6A system controller, SIL-6A auto-sample injector, C-R6A integrator and SPD-10A UV spectrophotometric detector from Shimadzu Inc. (Kyoto, Japan) and Fractomtte® α-200 fraction collector from Buchler Intruments.(NJ, USA).

4.2.3 ISOLATION OF LF FROM BOVINE COLOSTRUM WHEY

The self-packed SSDA column (i.d. 0.5 × 5 cm) was washed with degassed Milli-Q water and was equilibrated with 6 M urea in 20 mM HEPES; a pH 8.0 buffer (Buffer C) at a flow rate of 0.2 ml/min was used. The separation procedure was performed at room temperature (20-22°C). Colostrum whey, to which urea was added up to the concentration of 6 M, was applied to the SSDA affinity column. The column was washed with 12 bed volumes of buffer C. Before gradient elution, the urea was washed out by several column volumes of buffer A (20mM HEPES; pH 8.0). LF was eluted by applying linear gradient of NaCl (0 to 1M) using buffer B (1M NaCl in 20 mM HEPES; pH 8.0). The eluates were monitored at 280 nm for protein content. After each purification run, the SSDA column was washed extensively, first with 2 M NaCl in buffer A, then with 8 M guanidine-HCl in

buffer A, and finally with Milli-Q H_2O. Fractions of LF were pooled, dialyzed against Mill-Q H_2O, and then lyophilized at –52°C.

4.2.4 SINGLE STRAND DNA AGAROSE (SSDA) MATRIX CAPACITY

SSDA matrix capacity was investigated by loading known amounts of standard LF and colostrum whey into the SSDA column. The bound LF was calculated by following equation:

$$\text{Capacity (mg/mL)} = Lf_{max}/\text{mL Gel}$$

Lf_{max} - maximum amount of LF bound to SSDA column (mg)

4.2.5 PURITY OF ISOLATED LF FROM COLOSTRUM WHEY

The purity of isolated LF from colostrum whey by affinity chromatography was evaluated using C_{18} reverse phase chromatography and SDS-PAGE. The purity of LF was calculated by following equation:

$$\text{Purity, (\%)} = W_{lf} / W_{fraction} \times 100\%$$

$W_{fraction}$- Fraction dried weight (mg), W_{lf}- LF dried weight (mg), $Purity_{lf}$ - Purity of isolated LF.

4.2.6 REVERSE PHASE CHROMATOGRAPHY OF LF ISOLATED FROM COLOSTRUM WHEY

An aliquots (10 µL) of purified LF or 10 µL of LF standard (2.0 mg/ml) was applied separately to a Pharmacia PepRPC C_{18} RPC column (Pharmacia Biotech Inc, Sweden). The buffer system was established as follows: Buffer A: 10% (v/v) mixture (10% (w/v) NaCl in 1% (v/v) CH_2O_2), 10% (v/v) acetonitrile, 80% (v/v) H_2O; Buffer B: 10% (v/v) mixture (10% (w/v) NaCl in 1% (v/v) CH_2O_2), 80% (v/v) acetonitrile, 10% (v/v) H_2O. The gradient used in this analysis is same as that in 3.2.6. The flow rate was 1ml/min throughout the analysis procedure, and eluted protein was monitored by UV absorbance at 280 nm.

4.2.7 CATION EXCHANGE CHROMATOGRAPHY OF LF ISOLATED FROM COLOSTRUM WHEY

An aliquots of purified LF recovered from the SSDA column was applied to a Pharmacia HR 5/5 Mono-S cation-exchange column (i.d. 0.5 × 5cm, Pharmacia Biotech Inc., Sweden). The equilibrating buffer consisted of 50 mM Na_2HPO_4; pH 7.5. LF was eluted by a stepwise NaCl gradient (0 - 0.3 M - 1M NaCl) in an equilibrating buffer for about 31 minutes of running time. The flow rate was constant at 1 ml/min through all procedures, and the eluent was monitored for protein content by absorbance measurement at 280 nm.

4.2.8 SIZE EXCLUSION CHROMATOGRAPHY OF LF ISOLATED FROM COLOSTRUM WHEY

An aliquots of purified LF eluted from the SSDA column was applied to a TosoHAAS TSK-Gel 3000SW column (i.d. 0.78 × 30cm, TosoHAAS Inc., Japan). The buffer consisted of 20 mM sodium phosphate and 0.4 M KCl; pH 6.8. Samples were eluted at a flow rate of 1 ml/min. Protein content were monitored by measuring absorbance at 280 nm.

4.2.9 ELECTROPHORETIC ANALYSIS OF LF ISOLATED FROM COLOSTRUM WHEY

An aliquots of isolated LF was mixed with an equal volume of solubilization buffer (containing 2% sodium dodecyl sulfate, 3% mercaptoethanol), heated in a boiling water bath for 90 seconds, and electrophoresed on a 12.5% polyacrylamide gel with stacking gel essentially according to Laemmli (6). After electrophoresis, the gel was fixed in a 10% acetic acid solution with 50% methanol. The gel was stained with 0.1% Coomassie blue containing 10% acetic acid, according to Laemmli (6).

4.2.10 MATRIX ASSISTED LASER DESORPTION/IONIZATION MASS SPECTROMETRY (MALDI-MS) OF ISOLATED LF

The MALDI–MS system was calibrated with a BSA dimer (MW_1 =66.4 Kdal, MW_2 =132.9Kdal). Isolated LF from colostrum whey or standard was dissolved in 0.1% TFA to obtain the concentration of 1.5 mg Lf /mL. Sinapinic acid (MW =224.21 dal) was chosen to be the matrix in analysis. Saturated solutions of matrix were prepared by dissolving sinapinic acid in 70% (v/v) trifluoroacetic acid / 30% (v/v) acetonitrile solution at room temperature. Samples for MALDI-MS were prepared by mixing 10 μL of the LF stock solution with 10 μL of the saturated sinapinic acid matrix solution and applying 2 μl mixture to a stainless steel insertion probe tip. Prepared samples were allowed to air-dry before insertion into the vacuum system of the mass spectrometer. Mass spectrometry was performed on a Hewlett Packard MALDI linear time-of-flight mass spectrometer (Hewlett Packard Inc., USA) operating in the positive-ion mode of detection. All MALDI mass spectra were recorded with a Hewlett Packard Data Analysis System (Hewlett Packard Inc., USA). Ionization was achieved using a conventional nitrogen laser (337 nm beam, 3 ns pulse width, 5 Hz) set at attenuation between 15 and 20. In the reflection mode, LF was accelerated at 16 kV and reflected at 20 kV.

4.2.11 AMINO ACID COMPOSITION ANALYSIS OF ISOLATED LF

The amino acid composition of isolated LF by SSDA affinity chromatography was determined with a Beckman System 6300 amino acid analyzer, system gold version 6.01. The sample was hydrolyzed in 5.7 N HCl for 24 hours at 110°C before analysis. However, methionine, cysteine and tryptophan were not determined by this method.

4.3 RESULTS AND DISCUSSIONS

4.3.1 COLOSTRUM WHEY TREATMENT

Bovine colostrum is a mammary gland secretion produced by cows during the first 24-48 hours after calf birth. Colostrum is not only a source of nutrients such as proteins, carbohydrates, fat, vitamins and minerals, but also contains several biologically active

molecules essential for specific biological functions. The concentration of biological components such as LF in bovine colostrum is significantly higher than in milk (Figure 2-3), but the colostrum is not yet used as a source of these components on the industrial scale. The development of new functional ingredients and nutraceuticals derived from milk requires development of technological processes for their isolation and purification. The colostrum whey was harvested by acidic precipitation of caseins from colostrum at pH 4.6. The pH of colostrum whey was readjusted to 8.0 and 20 mM HEPES to optimize binding conditions between LF and DNA based matrix. Solid urea was added (up to 6 M) to colostrum whey in order to prevent LF interaction with acidic whey proteins such as caseins, α-LA, β-LG and BSA (20). However, urea may not prevent LF association with basic proteins such as secretory IgA and lysozyme. These interactions may be important in intestinal maturation by promoting the binding of LF to the intestinal musoca, where LF can exert its function as a microbial inhibitor or growth stimulator (20). In our case, urea was added to mobile phase as a modifier and to promote selective adsorption or to eliminate the interaction of other proteins with immobilized DNA.

4.3.2 ISOLATION OF LF FROM BOVINE COLOSTRUM WHEY

The elution profile of bovine LF isolated from bovine colostrum whey by a SSDA matrix is shown in Figure 4-1. During the first 60 minutes, unbound proteins, which were mainly β-LG, α-LA, BSA and other minor enzymes, were washed out of the column by 27mL of 20 mM HEPES (6M Urea; pH 8.0) buffer followed by 3mL of 20 mM HEPES (pH 8.0). Then a linear gradient of NaCl from 0 to 1 M in 20mM HEPES (pH 8.0) was used during the next 40 minutes. LF bound tightly to the DNA matrix eluted only at 1 M NaCl in 20mM HEPES buffer under these conditions. LF eluted at a retention time of 113 minutes. LF appears to be a very unique protein in colostrum whey and binds tightly to immobilized DNA on the SSDA matrix under described experimental conditions. The reasons for strong LF binding to DNA could be 1) urea was used as a mobile phase modifier to promote selective adsorption or to eliminate the interaction of other whey proteins with immobilized DNA on SSDA matrix (5), and therefore, the possibility of

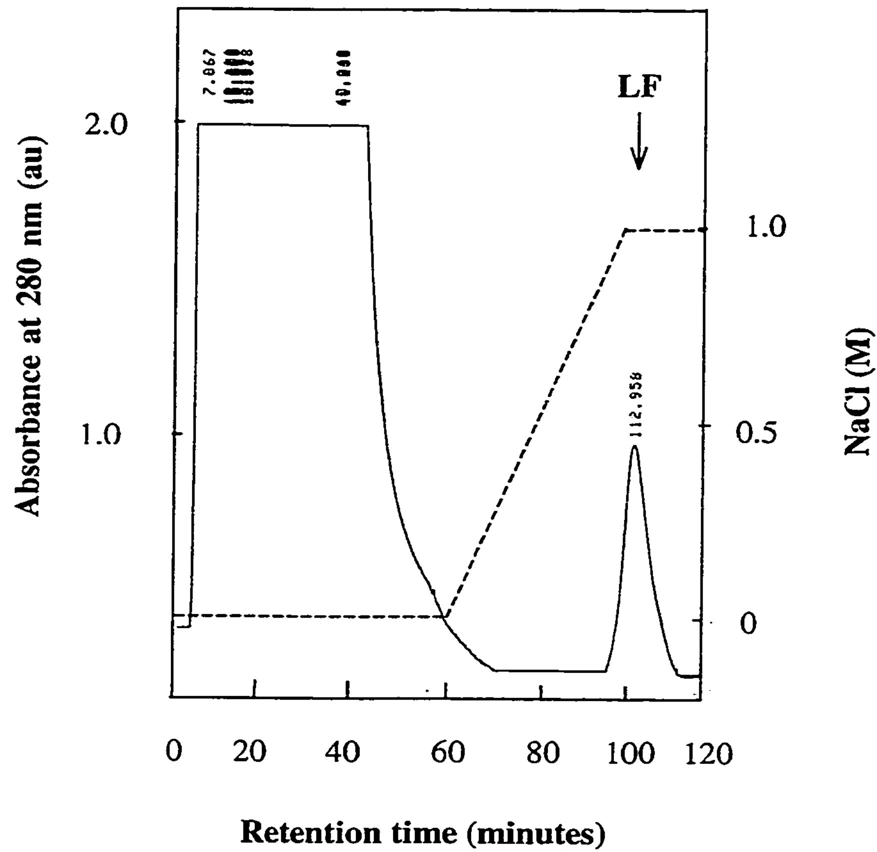

Figure 4-1. Chromatogram of bovine LF isolation on SSDA affinity matrix. Buffer A: 20 mM HEPES, pH 8.0; Buffer B: 1 M NaCl in buffer A.

DNA binding to other whey proteins was minimized, and 2) LF might bind to DNA more strongly than other whey proteins in colostrum under experimental conditions.

Calf single-strand DNA-agarose (SSDA) was used to purify LF from human milk and infant urine by Hutchens *et al* (1,2,3,5), and metal-chelate affinity chromatography was used by Lonnerdal (8, 14, 15) to purify LF from human milk and cheese whey. The other reported affinity methods used in LF isolation are antibody affinity chromatography (16), heparin-sepharose affinity chromatography (17,18), Cibacron-blue F3G-A affinity chromatography (19), and immobilized β-lactoglobulin affinity chromatography (20).

4.3.4 BINDING CAPACITY OF SSDA MATRIX, PURITY AND YIELD OF ISOLATED LF FROM COLOSTRUM WHEY

The binding capacity of SSDA matrix to standard and isolated LF in colostrum whey was determined, and the results are shown in Figure 4-2 and Figure 4-3, respectively. The binding capacity was defined as the maximum amount of LF bound to the SSDA matrix per mL of gel. The binding capacity of the SSDA matrix was 12 mg Lf/mL gel and 1.0 mg Lf/mL for standard and LF in colostrum whey, respectively. These differences in the binding capacity of the SSDA matrix to standard and LF in colostrum whey indicate that other proteins present in whey interfere with LF binding to the DNA matrix.

The purity and yield of LF isolated from colostrum whey by SSDA affinity chromatography were also investigated. The SDS-PAGE gel of LF isolated by affinity chromatography showed similar mobility and band to those of the standard, and the molecular weights were also similar (Figure 4-7). The resolution and purity of isolated LF were comparable to those of the standard.

The yield of isolated LF by SSDA affinity chromatography was not comparable to that purified by SSBB cation-exchange chromatography. The amounts of isolated LF by SSDA affinity chromatography were variable within a large range, even though the same amount of colostrum whey (0.05mg Lf/mL) was applied to the SSDA matrix. The experimental data indicate that SSDA affinity chromatography might not be the method of choice for large-scale production of LF from colostrum or cheese whey because of this

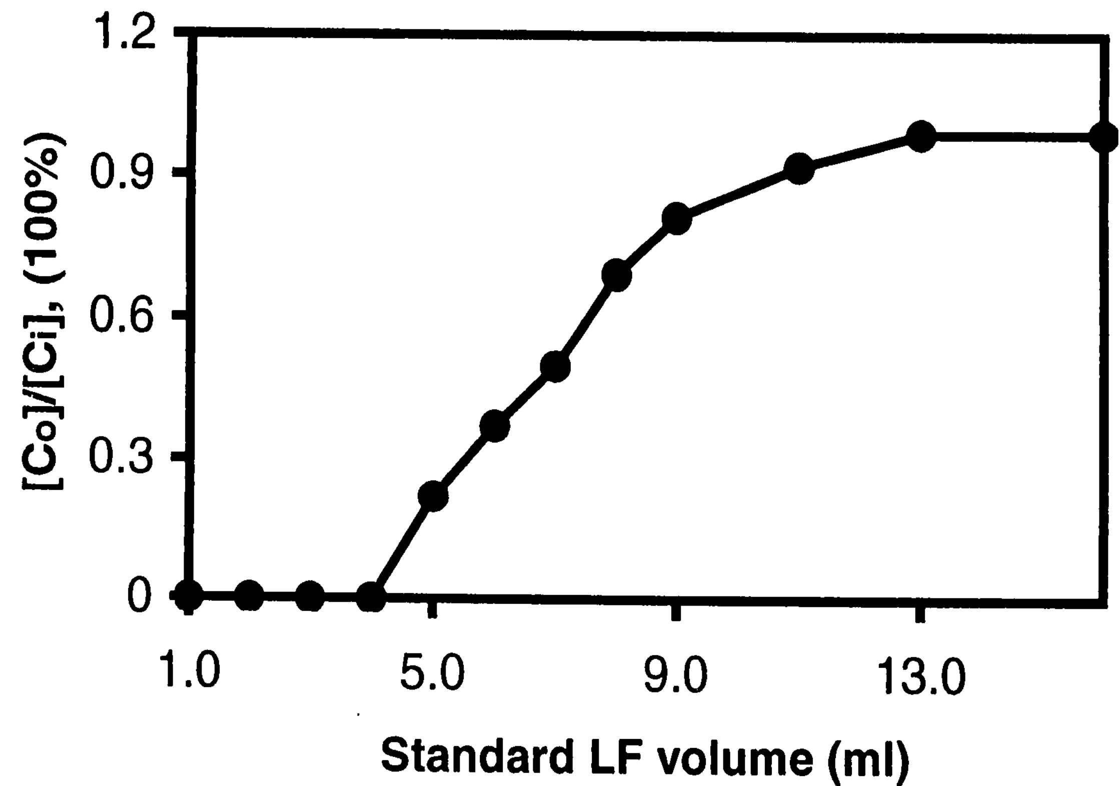

Figure 4-2. Binding capacity of SSDA matrix (1mL) to standard LF (1mg Lf/mL). [C_i] is LF concentration at inlet to column, [C_o] is LF concentration at outlet to column.

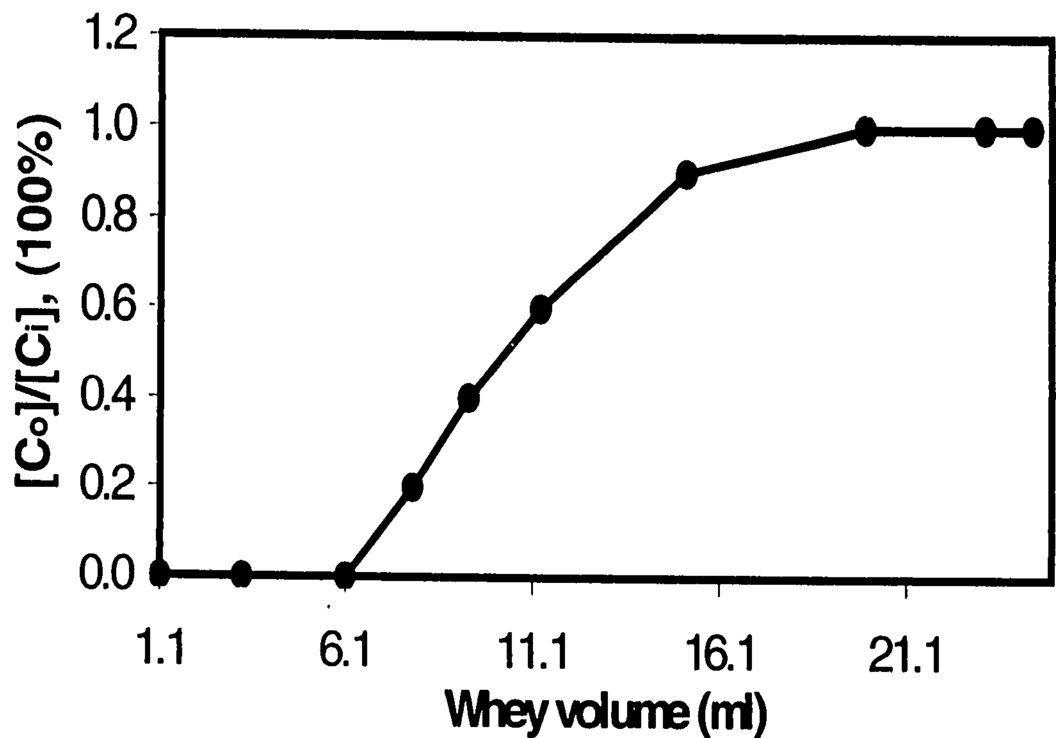

Figure 4-3. Binding capacity of SSDA matrix (1mL) to LF in colostrum whey (0.05 mg Lf/mL). [C_o] is LF concentration at inlet to column, [C_i] is LF concentration at outlet to column.

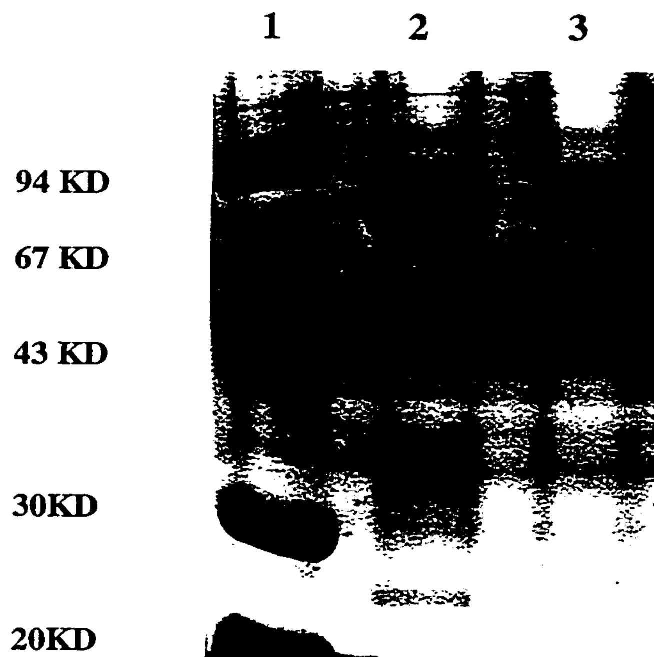

Figure 4-7. The SDS-PAGE gel of isolated bovine LF by SSDA affinity chromatography. Lane 1: Low range MW; Lane 2: standard LF; lane 3: Isolated bovine LF.

chromatography's small binding capacity, low yield, non-food gradient buffer system, potential DNA leakage from matrix, and the high coast of SSDA matrix.

4.3.5 IDENTIFICATION OF LF ISOLATED FROM BOVINE COLOSTRUM WHEY BY REVERSE PHASE, SIZE EXCLUSION AND CATION EXCHANGE CHROMATOGRAPHY, AND SDS-PAGE, MALDI-MS AND AMINO ACID COMPOSITION

Reverse phase chromatography of LF isolated from colostrum by affinity chromatography was used to identify isolated LF. The reverse phase chromatography profile (Figure 4-4) showed that retention time (15 minutes) and purity of LF isolated by affinity chromatography appeared similar to those of the standard, and results were consistent with SDS-PAGE data.

Cation exchange chromatography of isolated LF by affinity chromatography performed on Mono-S analytical column also confirmed that both standard and isolated LF eluted at similar a retention time (25 minutes) and their chromatography profiles were comparable (Figure 4-5).

Size-exclusion chromatography (SEC) of isolated LF by affinity chromatography and standard LF were performed on a TosoHAAS TSK-Gel 3000SW size-exclusion column (Figure 4-6). The SEC elution profiles were similar for standard and isolated LF; however, isolated LF retention time was longer by 2 minutes. This result may indicate a slightly higher molecular weight of the isolated LF as compared to that of the standard. The reason for the higher molecular weight might be due to 1) different degrees of iron-saturation between standard and isolated LF (11,12); 2) different portions of carbohydrate between these two LFs; and 3) LF may have variable forms with different molecular sizes. The literature reports the range in LF molecular weight from 76 to 85 Kdal (35, 36, 37, 38).

Isolated LF was further analyzed by matrix-assisted laser desorption/ionization mass spectrometry. Its MALDI-MS spectrum is shown in Figure 4-8 and that of the standard in Figure 3-12. The MALDI-MS data revealed that isolated LF molecules mostly appeared

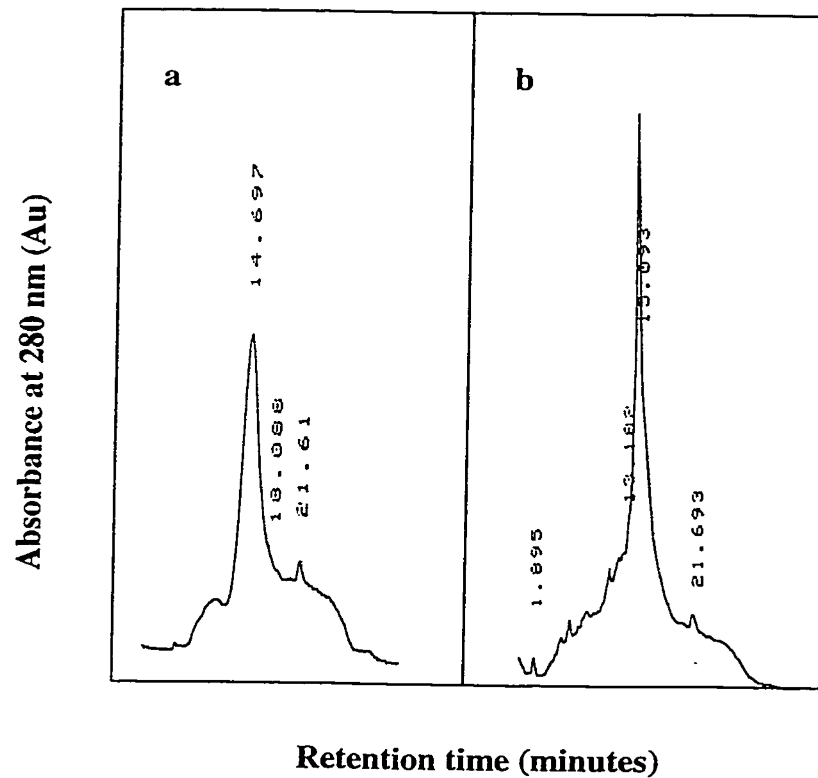

Figure 4-4. Reverse phase chromatography of isolated LF by affinity chromatography and standard, a) standard, b) isolated LF.

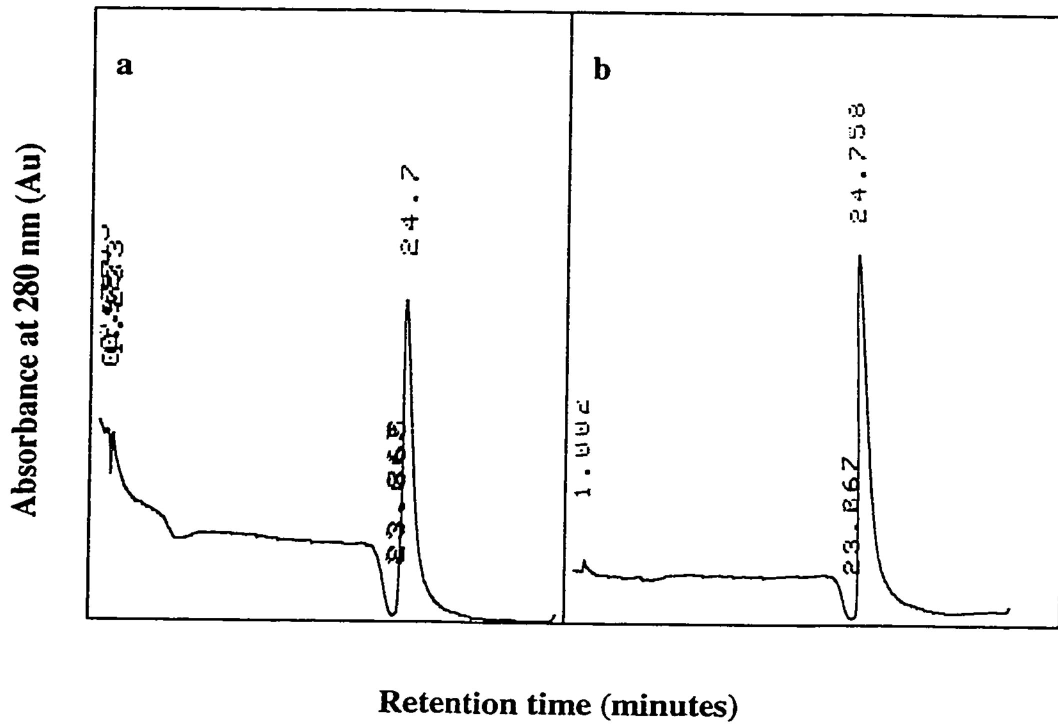

Figure 4-5. Cation exchange chromatography of isolated LF by affinity chromatography and standard. a) isolated bovine LF, b) standard.

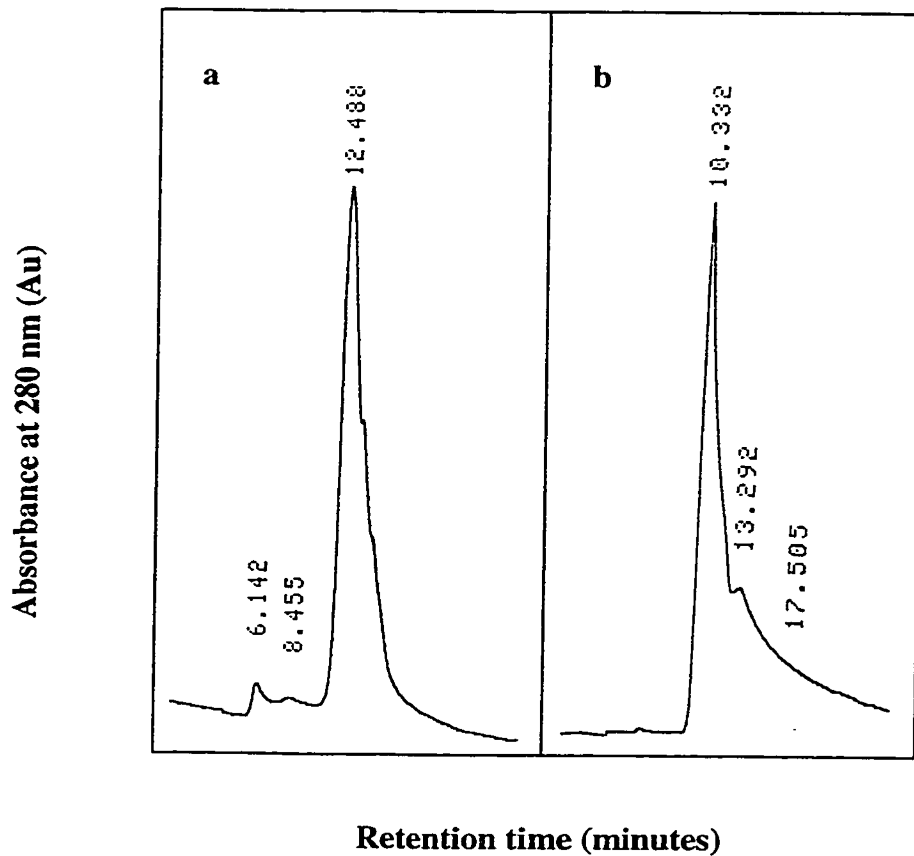

Figure 4-6. Size exclusion chromatography of isolated LF by affinity chromatography and standard, a) isolated LF, b) standard.

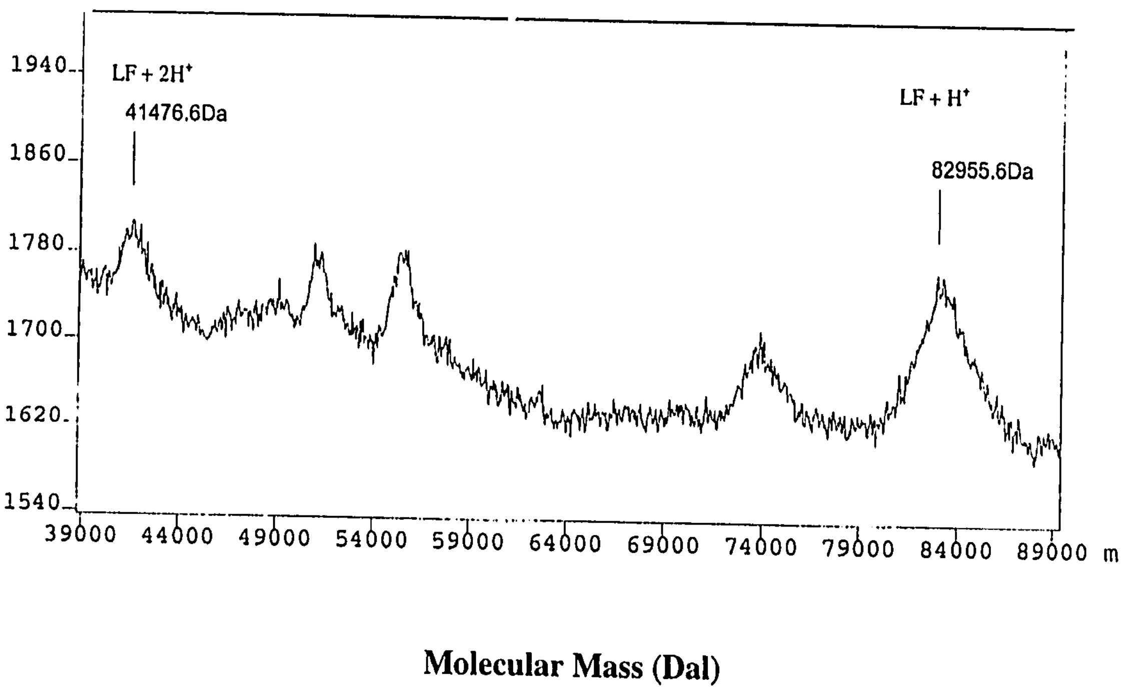

Figure 4-8. The matrix assisted laser desorptio/ionization mass spectrometry (MALDI-MS) of isolated LF by affinity chromatography. The matrix was sinipanic acid.

as monomer with a single positive charge and a molecular weight of about 83 Kdal. The molecular weight of isolated LF was also confirmed by both SEC and SDA-PAGE data.

The amino acid composition of LF isolated from colostrum whey is shown in Table 3-4 and is nearly identical to the data reported by Wang (24). Methionine, cysteine and tryptophan were not determined. These three amino acids are decomposed by 5.7 N HCl during sample treatment. The amino acid composition of isolated LF was comparable to the theoretical values; therefore, the isolated LF was additionally identified by amino acid composition as well.

4.4 CONCLUSIONS

Lactoferrin was isolated from colostrum whey by SSDA affinity chromatography under defined experimental conditions. The binding capacity of a SSDA matrix to LF is very low compared to that of a cation exchange matrix. The isolated LF was further identified by C_{18} reverse phase chromatography, SDS-polyacralamide electrophoresis, cation exchange chromatography, MALDI-MS and amino acid composition. All applied methods confirmed that LF was isolated from colostrum whey by SSDA affinity chromatography. The molecular weight of isolated LF by SSNA affinity chromatography was 83 Kdal, and its purity was similar to that of the standard. However, SSDA affinity chromatography protocol under established experimental conditions may not be a suitable method for large-scale production and for food use because of low yield, the high cost of the matrix, the potential leakage of NDA from matrix, and the buffer system needed for elution.

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CHAPTER 5

CONCLUDING CHAPTER

5.1 SUMMARY OF RESEARCH FINDINGS

Protocols of lactoferrin isolation from bovine colostrum whey by cation exchange chromatography on SSBB matrix and affinity chromatography on SSDA matrix were established. The recovery of LF in colostrum whey was about 45%, whereas the recovery of standard LF was about 80 %. The capacity of SSBB matrix was 39 mg Lf/mL gel when standard LF was used and 4.5 mg Lf /mL gel when colostrum whey was applied. The yield of isolated LF decreased when LF purity increased by modifying ion strength (NaCl concentration) in a washing buffer system. A 0.3 M NaCl concentration in a washing buffer might be considered as optimum for both the yield and purity of isolated LF. The molecular weight of isolated LF by SSBB cation exchange chromatography was about 78 KD, as shown by MALDI-MS analysis.

Bovine LF was also isolated from colostrum whey by SSDA chromatography. LF binds tightly to DNA in the presence of urea as compared to other whey proteins. The molecular weight of LF isolated by affinity chromatography was 83 kdal. The capacity of SSDA matrix was about 12 mg Lf/mL gel when pure LF was applied and was about 1.0 mg Lf/mL gel when colostrum whey was applied. Isolated LF had similar purity compared to that of standard LF.

In conclusion, as compared to the affinity chromatography, the isolation of bovine lactoferrin by cation exchange chromatography on a SSBB matrix is a method of choice for industrial scale-up.

5.2 RECOMMENDATIONS FOR FUTURE RESEARCH

There is a tremendous potential for the use of LF as functional ingredients and nutraceuticals, because of its biological functions and physiological benefits to human health. More research is needed in the area of biological functionality, the mechanism of bactericidal effects of lactoferrin and lactoferricins, and immunological functions. The

improvement of the LF isolation technique by SSBB cation exchange chromatography is also required in order to meet the criteria for scale-up to industrial production.

5.2.1 BIOLOGICAL ROLE OF LF

The potential of LF as an antimicrobial agent has been widely considered; however, the antimicrobial mechanism is not clear as yet. Lactoferrin antimicrobial activity was linked to its iron-binding properties or to a direct affinity to cell-wall and to release of LPS (12, 16). Clinical and scientific evidence is needed to prove whether LF might be active in the human gastrointestinal tract, and to identify if LF or its peptides are absorbed in the human body in order to enhance human immuno-system or to be a cell-growth factor (17, 18).

5.2.2 IMPROVEMENT OF LF ISOLATION

The SSBB cation exchange chromatography method can be scaled up to large-scale production; however, more research is needed in order to optimize separation parameters. In order to facilitate study on the biological role of LF, the purity of the final product should be improved and following steps might be considered: 1) using a high concentration of NaCl in the buffer to wash away impurity before eluting LF; doing so might lead to lower LF yield, but purity can be increased; and 2) combining ion exchange chromatography with a membrane processing technique (1, 2, 3, 4). All approaches to improving LF purity should have the following advantages: 1) the ion-exchanger can be maintained easily, with long-term usage; 2) repeatability is good; and 3) the purification protocol should be simple and applicable for commercial production.

5.2.3 SOURCES OF LF AND ITS BIOLOGICAL FUNCTIONAL PEPTIDES

The isolation protocol established for LF isolation from colostrum whey can be applied to cheese whey as well. A huge amount of whey is produced from cheese production; therefore, cheese whey might be good starting material for industrial-scale LF purification.

Peptides derived from the hydrolyses of bovine LF are called lactoferricins (LFc-B) and posses bactericidal activity against a wide range of organisms such as Gram-negative and Gram-positive bacteria as well as yeast (12). Current LFc-B research is focused mainly on its bacterostatic or bactericidal properties (13, 14, 15). Industrial production and isolation of LFc-B need to be conducted in order to meet the potential for pharmaceutical or nutraceutical applications.

In this thesis, research results documented the potential for the commercial production of LF. This production might provide the traditional dairy industry with new opportunities to invest in new business in the area of functional ingredients and nutraceuticals.

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