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AFFINITY PURIFICATION OF BOVINE LACTOFERRIN AND BOVINE
TRANSFERRIN FROM WHEY USING IMMOBILIZED GANGLIOSIDES

by

Seung-Hee Nam

A thesis submitted in partial fulfillment
of the requirements for the degree

of

MASTER OF SCIENCE

in

Nutrition and Food Sciences

UTAH STATE UNIVERSITY
Logan, Utah

2000

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ABSTRACT

Affinity Purification of Bovine Lactoferrin and Bovine Transferrin
from Using Immobilized Gangliosides

by

Seung-Hee Nam, Master of Science

Utah State University, 2000

Major Professor: Dr. Marie K. Walsh
Department: Nutrition and Food Sciences

Bovine lactoferrin (BLF) and bovine transferrin (BTF) are major-iron transport and regulation proteins found in bovine whey. BLF and BTF must interact with the eukaryotic cell surface to mediate their biological function of iron delivery and cellular functions of inflammatory and immunological modulation. As common components of the eukaryotic cell surface, gangliosides were used for affinity purification of BLF and BTF.

Bovine gangliosides were isolated from fresh buttermilk and covalently immobilized onto controlled-pore glass beads (66 $\mu\text{g/g}$ beads). After the matrix was loaded with whey protein (WPI or WPC), lactoferrin was eluted with 1 M NaCl and identified by N-terminal protein sequencing. Pretreated whey isolate (1% wt/vol) showed the highest lactoferrin purity with 40% among protein sources, and whey protein isolate (10% wt/vol) showed the highest recovery with 105%.

Bovine transferrin was eluted with sodium phosphate buffers at pH 7 after the immobilized matrix was loaded with a 2% (wt/vol) whey solution. The ganglioside

column resulted in a 74.2% recovery of BTF from whey, and the BTF was enriched to 61% purity after Mono-Q chromatography. Bovine transferrin was identified by SDS-PAGE analysis, Western analysis, and isoelectrofocusing. In conclusion, immobilized gangliosides can be used to purify BLF and BTF from bovine whey.

(81 pages)

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Seung-Hee Nam

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LIST OF SYMBOLS, NOTATION, DEFINITIONS**Abbreviation Key**

α -LA=alpha lactalbumin

β -LG=beta lactoglobulin

BLF=bovine lactoferrin

BSA=bovine serum albumin

BTF=bovine transferrin

Con-A=concanavalin-A

CPG=controlled pore glass

EDC=carbodiimide

HPLC=high performance liquid chromatography

IEF=isoelectrofocusing

LF=lactoferrin

MFGM=milk fat globule membrane

OPA=O-phthaldialdehyde

PI=isoelectric point

PP3=proteose peptone

PVDF=polyvinylidene difluoride

SDS-PAGE=sodium dodecyl sulfate-polyacrylamide gel electrophoresis

TF=transferrin

WPC=whey protein concentrate

WPI=whey protein isolate

CHAPTER I

INTRODUCTION

Besides nutritional roles as amino acids, whey proteins in milk have functional roles such as iron-binding proteins, antibacterial agents, immunoglobulins, growth factors, vitamin-binding, and enzymes. Major whey proteins include immunoglobulins, α -lactalbumin, β -lactoglobulin, and bovine serum albumin. The minor proteins include lactoferrin, lactoperoxidase, transferrin, proteose-peptones, prosaposin, growth factors, vitamin-binding proteins, and enzymes. During recent decades, the identification of minor whey components for functional and nutritional applications has greatly increased.

Among the minor components of whey, bovine lactoferrin (BLF) and bovine transferrin (BTF) are well-known bioactive proteins, which are found at concentrations of 20 to 200 $\mu\text{g/ml}$ in bovine milk. Each of the two proteins has a major role in iron metabolism. Bovine lactoferrin is an iron-sequestration protein and bovine transferrin is an iron-transport protein (43). Bovine lactoferrin and bovine transferrin transport iron from the biological fluids into the cytoplasm via plasma membrane by receptor-mediated endocytosis (44). Due to ubiquitous iron requirements, the cell receptors for both proteins have been observed in a wide range of eukaryotic species and cell types. Besides its role as an iron transporter, BLF shows multifunctional roles such as antimicrobial activity, immunomodulatory activity, regulation of myelopoiesis, cellular growth promotion, and antioxidant effects (55).

In order for BLF and BTF to mediate the biological function of iron delivery and cellular functions such as inflammatory and immunological reactions, they must interact with the eukaryotic cell surface. Gangliosides are common components of eukaryotic cell surfaces.

Current purification procedures for BLF and BTF produce low recovery due to the co-purification of other proteins. Some methods lead to high purification efficiency,

but there are multiple steps. Bovine lactoferrin and bovine transferrin have been purified from complex sources such as milk, serum, and plasma. This research studied the development of an affinity chromatography technique using immobilized gangliosides, common components of eukaryotic cell surface, for the purification of BLF and BTF. Commercially available bovine whey protein concentrate (WPC) and whey protein isolate (WPI) were used for this study.

This affinity method presents several economic advantages in comparison with others. This procedure provides efficient isolation and enrichment of highly purified BLF and BTF. The inclusion of pH and ionic strength differences in the chromatography buffers removes some of the nonspecifically absorbed whey proteins before elution of BLF and BTF. Gangliosides coupled to CPG beads have good mechanical properties which are stable to organic solvents, acidic solutions, and drying. The immobilized ganglioside column has been used repeatedly without apparent decrease in binding capacities of BLF and BTF for 6 to 12 months. The support can be cleaned in ethanol, urea, or high salt buffers.

Chapter III outlines the affinity purification of BLF using the immobilized ganglioside matrix from various whey sources. Chapter IV describes the fractionation of BTF using the immobilized ganglioside matrix from WPI. Bovine transferrin was further purified by Concanavalin-A sepharose and Mono-Q anion exchange chromatography. In the Appendix, the details of the purification of bovine prosaposin from whey are described.

CHAPTER II

LITERATURE REVIEW

BIOACTIVE PROTEINS IN BOVINE WHEY

Whey proteins in milk have nonnutritional roles such as iron-binding, antibacterial agents, immunoglobulins, growth factors, vitamin binding, and enzymes. Major components include immunoglobulins, α -lactalbumin, β -lactoglobulin, and bovine serum albumin. Minor components include lactoferrin, lactoperoxidase, transferrin, proteose-peptones, prosaposin, growth factors, vitamin-binding proteins, and enzymes (8, 33). Table 1 shows the composition and biological functions of the identified bioactive proteins in bovine whey.

Among the minor components of whey, bovine lactoferrin (BLF) and bovine transferrin (BTF) are well-known bioactive proteins, which are found at concentrations of 20 to 200 $\mu\text{g/ml}$ in bovine milk. Each of the two proteins plays a major role in iron metabolism. Bovine lactoferrin is an iron-sequestration protein and bovine transferrin is an iron-transport protein (43). Bovine lactoferrin and bovine transferrin are 80 kDa glycoproteins, consisting of a single chain of about 700 amino acids and organized with two terminal lobes with a high degree of similarity (44). Although BLF displays sequence and structural homology with BTF, BLF is unique in terms of its various locations and multifunctional roles in the body. Bovine lactoferrin is known to interact with the cell surface of various eukaryotic and bacterial cells, mucosal components, many biopolymers, and other small molecules. Besides its role as an iron sequester, BLF shows multifunctional roles such as antimicrobial activity, immunomodulatory activity, regulation of myelopoiesis, cellular growth promotion, and antioxidant effects (55).

TABLE 1. Composition and biological functions of some bioactive proteins in bovine whey.

Whey protein	Weight contribution (g/L of milk)	Biological function
Major ¹		
β-lactoglobulin	3.2	(Pro) vitamin A transfer
α-lactalbumin	1.2	Lactose synthesis, Ca ²⁺ carrier
Bovine serum albumin	0.4	Fatty acid transfer, anticancer
Immunoglobulins	0.8	Immune protection, anticancer
Minor ^{1,2}		
Lactoferrin	0.2	Bacteriocidal, iron absorption
Transferrin	0.2	Iron absorption and transport
Lactoperoxidase	0.03	Antibacterial agent
Enzymes (> 50)	0.03	Health indicators
Protease-peptones	≥ 1	Opioid activity
Prosaposin	Trace	Neurotrophic factor
Growth factors stimulation	Trace	Mammalian cell growth
Vitamin-binding proteins	Trace	Vitamin bioavailability improvement

¹ De-Wit (8).

² Adapted from Lonnerdal et al. (33).

Bovine lactoferrin and bovine transferrin transport iron from the biological fluids into the cytoplasm via plasma membrane receptor-mediated endocytosis (44). Bovine lactoferrin and bovine transferrin interact with specific receptors present in variable amounts on target cells. Important target cells include the liver, bone marrow, and muscle. Due to ubiquitous iron requirements, the cell receptors for both proteins have been observed in a wide range of eukaryotic species and cell types. These receptors have been found on the surfaces of various eukaryotic cells such as erythroid, reticulocyte, hepatocyte, placental trophoblast, and macrophage cells (41).

In order for BLF and BTF to mediate the biological function of iron delivery and cellular functions such as inflammatory and immunological reactions, they must interact with the eukaryotic cell surface. Gangliosides are common components of eukaryotic cell surfaces. The surface of mammalian cells contains many types of glycoconjugates, which include proteins, glycosaminoglycans (heparin, heparin sulfate, and N-acetylneuraminic acid), and glycolipids (gangliosides and cerebroside). Their biologic function is thought to be involved in cell growth, differentiation, cell-to-cell interactions, receptor mediated membrane modulation, and malignancy (3). Glycoconjugates commonly act as receptors for a variety of compounds including interferon, serotonin, a variety of pathogenic organisms, and bacterial toxins (3, 28).

Recently, prosaposin has been discussed as another minor bioactive component in whey. Prosaposin is the precursor of the lysosomal saposin proteins, which are required for hydrolysis of glycosphingolipids, but is also found in its unprocessed form in milks. This protein plays a broad role in the development, maintenance, and repair of the nervous system (21). Hiraiwa et al. (23) have demonstrated that prosaposin displayed a strong affinity to gangliosides at pH 4.0 with great reduction at neutral pH value.

Bovine lactoferrin, bovine transferrin, and prosaposin as bioactive proteins are of commercial interest due to medical and nutritional benefits. They could be used in infant

formulas to strengthen the immune system, specialty dietary formulations to assist iron absorption, pharmaceuticals to treat harmful bacteria, and personal health items such as antibacterial toothpaste or anti-microbial cosmetics. For commercial uses, a simple inexpensive purification of BLF, BTF, and prosaposin is needed.

GANGLIOSIDES

Structure, Distribution, and Function

Gangliosides are glycosphingolipids containing one or more N-acetyl or N-glycosy-neuraminic acid (sialic acid) residues as part of the carbohydrate chain. They are abundant in the central nervous system, especially in grey matter, but smaller amounts are also widely distributed in the plasma membranes of a variety of tissue cells.

Gangliosides in bovine milk are derived from the apical membrane of the bovine mammary secretory cell, which is the source of the milk fat globule membrane (MFGM). This membrane contains 10 to 25% of the total cellular gangliosides of these cells. The MFGM has the same ganglioside profile as the mammary gland. The MFGM contains 90% of the gangliosides in milk, mainly GM2, GM3 (20% of the total gangliosides), and GD3 (50% of the total gangliosides). Minor gangliosides in milk include GM1, GD1b, and GD2 (26) (Table 2).

TABLE 2. Gangliosides in bovine milk.

Ganglioside ¹	Bovine milk
GM1	1.2 µg/l
GM2	700 µg/l
GM3	300 µg/l
GD3	8.8 mg/l
GD1b	1.2 mg/l
GD2	Trace

¹Adapted from Jensen (26).

Total quantities of gangliosides are similar in bovine and human milks but human MFGM contains more GM1 and GM3 but less GM2 and GD3 than does bovine milk (Table 2).

Huang (24) found a very high concentration of gangliosides in commercially available buttermilk. When expressed in terms of the weight of gangliosides per gram of total lipid, the ganglioside concentration in buttermilk exceeded even that in the grey matter of the brain (10 to 20 mg/L vs. 0.5 mg/kg). The GD3 ganglioside in buttermilk comprises 85% of total gangliosides and consists of two species, one having mainly C16 and C18 sphingosine and the other having C22 to C24 fatty acids with C16 and C18 sphingosine (18). Takamizawa et al. (58) reported that buttermilk contains 0.92 μ M of lipid bound sialic acid per gram of dry weight (72 mg sialic acid/kg), 80% of which is in the form of GM3, GD3, and GT3.

Gangliosides consist of a lipid moiety (ceramide) to which is attached an oligosaccharide chain containing at least one sialic acid residue. Gangliosides are synthesized on the Golgi apparatus and are predominantly located in the plasma membrane with their oligosaccharide chain exposed on the cell surface. As the ceramide portion is too short to span the bilayer, gangliosides appear to be confined to the outer half of the lipid bilayer. Because of their orientation on the cell surface, gangliosides have been implicated in various recognition phenomena (12). As ubiquitous compounds in all mammalian cell membranes, gangliosides are known to play important biological roles. Gangliosides act as receptors for viruses, bacteria, toxins, and other ligands. Gangliosides are also involved in immunomodulation and cell to cell recognition. Their significance in neurobiology has been reported because of the importance of glycosphingolipids as tumor-associated antigens (19). Possible roles of gangliosides in cellular activity have been investigated. GM3, GQ1b, and neolacto series gangliosides

showed stimulation of cell proliferation and differentiation in growth factor receptors, leukemic cell line, and neuronal cells, respectively (20).

Purification

The classical method used for the isolation of gangliosides involves a chloroform:methanol extraction, partitioning of the gangliosides into an aqueous upper phase, and dialysis to remove salts and other water-soluble, low molecular weight molecules. Another approach to ganglioside isolation is the use of a total lipid extraction and column chromatography such as DEAE-sephadex, anion exchanger Q-sepharose, TMAE (trimethylaminoethyl) Fractogel, and Sep-Pak C18 cartridges (19, 38, 64). The structural analysis of gangliosides is performed by periodate oxidation and gas chromatography (24).

GM1, GM2, GM3, GM4, GD3, and 9-O-acetyl GD3 gangliosides were purified from milk, brain, meconium, and malignant melanoma in human (27, 32, 59). Mouse brain and hepatocytes were used to isolate GM1, GM4, GD1a, GD1b, GD3, GT1b, and GQ1b gangliosides (36, 38). As an excellent source, bovine buttermilk has been used extensively to obtain gangliosides. GD3a and GD3b gangliosides, representing 85% of total lipid-bound sialic acid, have been isolated using various chromatography methods. A unique ganglioside, 9-O-acetyl GD3, was isolated in large quantities by a combination of ion exchange and silica gel column chromatography methods. O-acetylated gangliosides, GD3, and GT3 were purified using chloroform/methanol extraction and chromatography on DEAE-sephadex A-25 and Iatrobeads columns (2, 18, 49).

LACTOFERRIN

Structure and Function

Lactoferrin (LF), an 80 kDa iron-binding glycoprotein, is present in all exocrine fluid such as milk, seminal fluid, tears, sweat, and nasal secretion (47, 63). It is found in milk at concentrations of 20-200 mg/L and in whey at 0.3 to 3% of total protein (55). Lactoferrin can bind two Fe^{3+} ions with high affinity, with both apo and saturated forms present in milk (46). Lactoferrin has various functions, which include blocking the growth of various bacteria, fungi, and protozoa. The ability to chelate iron relates to its antibacterial property, but other functions are the result of the specific interactions with target cells including monocytes and macrophages (39). Lactoferrin binds surface structures found on bacteria, and has been shown to interact with eukaryotic cells (10). Specifically, lactoferrin has been shown to bind glycosaminoglycans, fibrinogen, collagen type I, collagen type IV, and laminin. Lactoferrin binding to eukaryotic cells is independent on its degree on iron saturation, and the interaction is thought to be electrostatic involving basic amino acids at its NH_2 terminus and sulfated glycosaminoglycans (63). Lactoferrin also binds specific cell receptor proteins on both eukaryotic and prokaryotic cells (47). Lactoferrin has roles in cellular immunity including regulation of antibody and cytokine production (54). Lactoferrin binds to various types of cells such as monocytes/macrophages, lymphocytes, intestinal brush border membranes, neutrophils, and platelets (34, 52).

Purification

Lactoferrin contains a strongly basic region close to the N-terminus that contributes to the interaction of LF with a variety of anionic biological molecules (63). The N-terminal stretch of LF contains four consecutive arginine residues which have a decisive role in the binding of LF to physiologically relevant ligands such as heparin

(55), bacterial lipopolysaccharide, human lysozyme, and DNA (63). The binding of LF to immobilized heparin (55) was not influenced by pH or 8 M urea, but did dissociate in the presence of 1.0 M NaCl. The authors concluded the main binding force was electrostatic interactions between the negatively charged sulfate and carboxyl groups of heparin and positively charged groups in LF.

The interactions between LF and various anionic biological molecules have been applied for affinity chromatography or ion exchange chromatography. Bovine lactoferrin was easily purified using 5'-p-aminophenylphosphoryl, uridine 2'-3'-phosphate agarose (57), or aminohexyl-divinylsulfonyl agarose as affinity chromatographic methods (25). However, these affinity chromatographic methods needed further purification steps to separate BLF from co-purifying proteins. Purification methods using ion-exchange chromatography have been used to purify BLF and BLF peptides (48). Hydrophobic interaction chromatography followed by carboxymethyl ion-exchange chromatography was used to purify BLF and bovine lactoperoxidase from acid whey. This purification scheme resulted in 80-88 mg/L of BLF protein (66). To increase the BLF purification yield, acid whey was pretreated with ammonium sulfate and sodium sulfate to remove major whey fractions and to concentrate the BLF in whey before being applied to chromatographic methods (65). Ion-exchange chromatographic methods lead to high purification efficiency but still have the drawback of requiring multiple steps.

Recently, a one-step ion exchange chromatography procedure has been demonstrated for bovine lactoperoxidase and BLF using a cation exchange membrane containing immobilized sulfonic acid moieties (6). Using elution buffers of different ionic strengths, the purification efficiency was 50% for BLF and 73% for bovine lactoperoxidase. Other purification methods using cationic exchange membranes have been patented (4, 62).

TRANSFERRIN

Structure and Function

Transferrin (TF) is a monomeric glycoprotein of 679 amino acids and has a relative molecular weight of approximately 80 kDa (15). Transferrin exists mainly in the serum and interstitial compartments of vertebrates and some invertebrates (1, 50). Transferrin is found at much lower concentration in human milk ($< 50 \mu\text{g/ml}$) in comparison to bovine milk (20 to 200 $\mu\text{g/ml}$) (53). The principal physiological function of TF in mammals is to transport ferric iron from sites of absorption to sites of utilization. Transferrin transports iron from the biological fluids into the cytoplasm via the plasma membrane by receptor-mediated endocytosis (44). Transferrin interacts with specific receptors present in variable amounts on target cells. Important target cells include the liver, bone marrow, and muscle. Due to ubiquitous iron requirements, TF receptors on the cells have been observed in a wide range of eukaryotic species and cell types. Transferrin receptors have been found on the surfaces of various eukaryotic cells such as erythroid, reticulocyte, hepatocyte, placental trophoblast, and macrophage (41). Necker and Cossman (40) demonstrated that TF receptors are induced in human lymphocytes by interleukin. Melanoma plasma membrane-associated glycoprotein (p97) from chondrocytes has been demonstrated to have sequence homology with TF (29). Transferrin acts as a growth factor *in vitro* and as an essential component in defined culture media (51). Transferrin has profound stimulatory effects on the cell proliferation such as hematopoiesis and metanephric differentiation (11, 56).

Purification

Structurally, TF consists of two homologous lobes. Each lobe is made up of two domains containing one specific iron-binding site. These sites require synergistic anion binding for effective iron binding (42). Anions, physiologically carbonate or bicarbonate,

bind to cationic side chains in the protein in the immediate vicinity of the metal-binding site and result in metal-anionic molecule-protein ternary complex (5, 17, 42).

The interaction between TF and TF receptors is influenced by pH (9). TF receptors, at physiologic pH, prefer to bind ferric-TF, resulting in a ferric-TF complex. The ferric-TF complex is transported to the cell cytoplasm by an undiscovered mechanism and becomes exposed to the acidic pH of the cell cytoplasm, causing rapid dissociation of iron. The resulting apo-TF complex is recycled to the neutral pH compartment, including the cell exterior, and apo-TF on the complex is dissociated from TF receptors (60, 61).

The interactions between TF and various anionic biological molecules have been used for affinity chromatography or ion exchange chromatography. Previously, several methods for TF separation from possum milk (14), porcine serum (13), sheep serum (37), human serum (67), and bovine serum and plasma (35) have been published. Most separation methods include several steps, including precipitation with ammonium sulfate, gel filtration, DEAE ion exchange, Mono-Q anion exchange, Blue sepharose affinity, and Concanavalin-A affinity separation. These methods produced low recovery and less purity of TF since there was co-purification of other proteins. Milk, serum, and plasma as TF sources were expensive and inconvenient to handle. Recently, a single step method purified human TF with 75% yield by DEAE sphadex from a by-product of the chromatographic fraction of plasma (51).

PROSAPOSIN

Prosaposin from humans is a 70 kDa glycoprotein and is the precursor for saposins (spingolipid activator proteins). Prosaposin is proteolytically processed into mature saposins within lysosomes. Prosaposin exists unprocessed as an integral

membrane protein, and also as a secreted protein in biological fluids such as seminal plasma, milk, and cerebrospinal fluids (22, 30). In humans, prosaposin exists in intracellular form of 66 kDa and extracellular form of 73 kDa due to varying levels of glycosylation since both forms can be converted to a 50 kDa form by deglycosylation (7, 16). Human prosaposin consists of 525 amino-acid residues and approximately 20% carbohydrate.

Prosaposin binds various gangliosides tightly and facilitates their transfer from micelles to membranes (23). Gangliosides, sialic acid containing glycosphingolipid, are enriched in nerve tissue and components of plasma membranes. Human prosaposin was shown to bind 13 different gangliosides with high affinities in the micromolar range (23). Prosaposin binds to GM1, GD1a, GT1b, and GQ1b with the highest affinities. The binding of prosaposin to gangliosides was pH dependent (optimal pH 4) and occurred over a wide temperature range (4 °C to 80 °C) and was completed within 3 min of incubation at room temperature.

Gangliosides have a unique ability to enhance neuronal cell proliferation and increase number and total length of neurites in neuroblastoma cell lines. Therefore, prosaposin may play an important role in ganglioside transport during neuronal differentiation and neuritogenesis (23).

The presence of prosaposin was first investigated in human milk by Western analysis according to Hineno et al. (21). Kondoh et al. (31) and Hiraiwa et al. (22) isolated prosaposin using several steps such as ion exchange, size exclusion, Con-A sepharose, and immunoaffinity chromatography from human milk. Only 16.8% of total prosaposin in human milk was recovered with these multiple methods.

Recently, prosaposin was also isolated from bovine milk and WPC by Patton et al. (45). Fresh and commercial whey were rich sources of prosaposin. Prosaposin did not associate with casein micelles, fat globules, membrane fragments, or somatic cells.

The heat-stable property of prosaposin was used for purification. A large majority of the heat-labile whey proteins were precipitated after boiling a solution of WPC. The heat-stable proteins (α -lactalbumin, BSA, and prosaposin) were subjected to multiple chromatographic steps as described by Hiraiwa et al. (22). Ion-exchange chromatography reduced the number and amounts of whey proteins, but also resulted in a 50% reduction of prosaposin. Low yields of prosaposin were obtained by immunoaffinity chromatography (45). Non-reducing SDS-PAGE analysis was used to separate two glycoproteins, BSA and prosaposin, since each has the same size (66 kDa). The authors found that the concentration of prosaposin in bovine milk was about 5-10 mg/L, which is similar to that found in human milk. Prosaposin exists in bovine milk as well as in milk of other species (human, chimpanzee, rhesus, goat, and rat).

HYPOTHESIS

Bovine lactoferrin and bovine transferrin are major-iron transport and regulation proteins in vertebrates and in some invertebrates. Bovine lactoferrin and bovine transferrin must interact with the eukaryotic cell surface to mediate their biological function of iron delivery and cellular functions of inflammatory and immunological reactions. Gangliosides are common components of eukaryotic cell surfaces. Therefore, immobilized gangliosides can be used to purify lactoferrin and transferrin from whey.

OBJECTIVES

1. To develop an immobilized ganglioside matrix for the affinity purification of BLF and BTF. Gangliosides were extracted from buttermilk and covalently linked to controlled pore glass (CPG) beads.

2. To purify BLF using the immobilized ganglioside matrix and study the purification efficiency of BLF from various whey sources.
3. To fractionate BTF using the immobilized ganglioside matrix from WPI and assess the purity of BTF using Con-A sepharose and Mono Q anion exchange chromatography.

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CHAPTER III

AFFINITY PURIFICATION OF BOVINE LACTOFERRIN FROM WHEY

ABSTRACT

Bovine lactoferrin was purified by affinity chromatography using immobilized gangliosides. Bovine gangliosides were isolated from fresh buttermilk using a combination of ultrafiltration and organic extraction. Isolated gangliosides were covalently immobilized onto controlled-pore glass beads. The immobilized matrix contained 66 micrograms of gangliosides per gram beads. After loading the matrix with whey protein isolate or whey protein concentrate (WPI or WPC), it was washed with sodium phosphate buffer (pH 7) followed by sodium acetate buffer (pH 4) before elution of lactoferrin with 1 M NaCl in sodium acetate buffer. Lactoferrin was identified by N-terminal protein sequencing. From the intensities of the protein bands in SDS-PAGE, lactoferrin constituted a minimum of 40% of the total protein in the 1 M NaCl eluted sample. Pretreated WPI (1% wt/vol) showed the highest lactoferrin purity among protein sources and WPI (10% wt/vol) showed the highest recovery. These results show that immobilized gangliosides can be used to purify lactoferrin from whey. This affinity purification procedure is suitable to obtain bovine lactoferrin in large quantities.

INTRODUCTION

Bovine lactoferrin, an 80 kDa iron-binding glycoprotein, is present in all bovine exocrine fluid such as milk, seminal fluid, tears, sweat, and nasal secretion (1). It is found in milk at concentrations of 20-200 mg/L and in whey at 0.3 to 3% of total protein. Bovine lactoferrin can bind two Fe^{3+} ions with high affinity with both apo and saturated forms present in milk (20). Lactoferrin (LF) has various functions, which include blocking the growth of various bacteria, fungi, and protozoa. The ability to chelate iron

relates to its antibacterial property, while other functions are the result of the specific interactions with target cells, including monocytes and macrophages (13). Lactoferrin binds surface structures found on bacteria, and has been shown to interact with eukaryotic cells (5). Specifically, LF has been shown to bind glycosaminoglycans, fibrinogen, collagen type I, collagen type IV, and laminin. Lactoferrin binding to eukaryotic cells is independent of its degree of iron saturation. The interaction of LF with eukaryotic cells is thought to be electrostatic involving basic amino acids at its NH₂ terminus and sulfated glycosaminoglycans on cell surfaces (24). Lactoferrin also binds specific cell receptor proteins on both eukaryotic and prokaryotic cells (15). Lactoferrin has roles in cellular immunity, including regulation of antibody and cytokine production (19). Lactoferrin binds to various types of cells such as monocytes/macrophages, lymphocytes, intestinal cells, neutrophils, and platelets (12, 18).

In order for LF to mediate cellular functions, such as inflammatory reactions, it must interact with the eukaryotic cell surface. Gangliosides are common components of eukaryotic cell surfaces. The surface of mammalian cells contains many types of glycoconjugates, which include proteins, glycosaminoglycans (heparin, heparin sulfate, and N-acetylneuraminic acid), and glycolipids (gangliosides and cerebroside). Their biologic function is involved in cell growth, differentiation, cell-to-cell interactions, receptor-mediated membrane modulation, and malignancy (2). Glycoconjugates commonly act as receptors for a variety of compounds, including interferon, serotonin, a variety of pathogenic organisms, and bacterial toxins (2, 10).

Bovine lactoferrin denatures at temperatures greater than 90 °C, and is stable to pasteurization temperatures. The iron-saturated form is more thermostable than the apo form. Paulsson et al. (14) demonstrated that both unheated and pasteurized BLF showed similar antibacterial properties. Bovine lactoferrin is of commercial interest because of its medical and nutritional benefits.

In this study we have purified bovine gangliosides from buttermilk and covalently linked them to a solid support for the affinity purification of BLF. The BLF recovery from various whey sources is reported.

MATERIALS AND METHODS

Ganglioside Purification

Gangliosides were purified from fresh bovine buttermilk (30% solids, Breaden Butter, Logan, UT). Buttermilk was diluted 1:10 with water and ultrafiltrated using a prep/scale-TFF 2.5 ft² cartridge membrane with 10 kDa (Millipore, Bedford, MA) to remove lactose. The efficiency of lactose removal was confirmed with a lactose enzymatic bioanalysis kit (Boehringer Mannheim, Indianapolis, IN). Moisture, protein, minerals, and lipid content of lactose-free buttermilk were determined by oven, Kjeldahl, ash, and Majonnier, respectively. Lactose-free buttermilk was freeze-dried and gangliosides were extracted using 20 vol of organic mixture of chloroform:methanol:water (40:80:30 vol) per gram dry solids (6). The sample was centrifuged at 11,000 x g, and the supernatant was collected and evaporated under nitrogen gas. The total amount of gangliosides purified was determined by the periodate-resorcinol method using sialic acid as standard according to Jourdian et al. (9).

Ganglioside Immobilization

Controlled pore glass (CPG) beads (2000 Å, 120-200 mesh) (Sigma, St. Louis, MO) were derivatized with 3-aminopropyltriethoxy silane and succinylated with succinic anhydride according to Walsh and Swaisgood (25). Acetic anhydride (10% by vol) in acetone was used to cap excess amino groups. Gangliosides were saponified to produce the lyso-derivative containing a free amino group by the reflux boiling method (22). The OPA method (O-phthaldialdehyde) as described by Weimer and Oberg (26) was used to

confirm saponification of gangliosides. The carboxyl matrix was reacted with Sulfo-NHS (Pierce Chemical Co., Rockford, IL) in 0.01 M MES (pH 6) containing water-soluble carbodiimide (EDC). The lyso-gangliosides, dissolved in MES buffer (pH 6), were circulated through the Sulfo-NHS matrix to couple via amide bond formation for 12 h at 4 °C using a peristaltic pump. Hydroxylamine (10 mM) was added to quench the reaction by hydrolyzing any unreacted NHS present. The efficiency of gangliosides immobilized was determined by measuring the amount of sialic acid on the matrix and the soluble gangliosides in the immobilization solution as described above.

Whey Protein Preparation

Bovine whey protein isolate (WPI) was obtained from Avenmore West (Twinfalls, ID), and whey protein concentrate was obtained from Cache Valley Dairy (Logan, UT). Crude whey protein isolate (WPI) and whey protein concentrate (WPC) were dissolved in 0.05 M sodium acetate buffer, pH 4, yielding various concentrations such as 1%, 5% and 10% wt/vol. As a pretreated sample, 1% WPI solution (1.5 g) was heated in boiling water for 5 min followed by centrifugation to precipitate denatured proteins. The remaining heat stable proteins were concentrated using an Amicon concentrator (Amicon, Beverly, MA) with a 50 kDa membrane and adjusted to pH 4, with 1 M sodium acetate (pH 4).

Ganglioside Affinity Chromatography

Immobilized gangliosides (5 ml) were packed into stop-flow columns (Pierce, Rockford, IL) and equilibrated with 0.05 M sodium acetate buffer (pH 4). Crude and pretreated whey samples were applied to the column and circulated for 5 min at room temperature. Lactoferrin was eluted by batch mode using four kinds of 0.05 M buffers in

order: A= sodium phosphate, pH 7; B= sodium acetate, pH 4; C= sodium acetate, pH 4 containing 0.5 M NaCl; and D= sodium acetate, pH 4 containing 1.0 M NaCl.

Protein Concentration

The protein concentration of samples eluting from the ganglioside column was determined by Bradford assay (Bio-Rad, Hercules, CA) with BSA as the standard.

SDS-PAGE Analysis and Densitometry

Protein samples were analyzed by SDS-PAGE under nonreducing conditions with 10% resolving and 5% stacking discontinuous gel conditions according to a modified method of Laemmli (11). Gels were stained for 30 min in Coomassie brilliant R-250 then destained in 25% methanol, 5% acetic acid (vol/vol). Dried gels were analyzed by densitometry to measure the intensities of the protein bands. Densitometry was performed on photographic images of dried gels using a scanning laser densitometer and Imager Quant software.

Determination of the N-Terminal Amino Acid Sequence

After SDS-PAGE analysis of samples, proteins were electro-transferred onto polyvinylidene difluoride (PVDF) membrane followed by staining with Coomassie blue R-250. The protein band of interest was cut out of the PVDF membrane and subjected to amino acid sequence analysis by the Edman degradation procedure using an Applied Biosystem 477 protein sequencer.

RESULTS

Ganglioside Extraction

The efficiency of ganglioside purification from fresh buttermilk is shown in Table 3. Lactose and proteins were removed from buttermilk to prevent Maillard browning during saponification. The lactose content was reduced approximately 100-fold after ultrafiltration (10 kDa membrane) and 1000-fold after both ultrafiltration and organic extraction. The molecular weight of bovine gangliosides ranges from 1 to 3 kDa, although they form micelles in aqueous solutions in the range of 200 to 250 kDa. Bovine gangliosides in fresh buttermilk are integrally associated with the milk fat globule membrane (MFGM) which are typically greater than 0.2 μ M in length (8). Gangliosides were purified from freeze-dried lactose-free buttermilk powder using a mixture consisting of chloroform, methanol, and water (6).

TABLE 3. Extraction of gangliosides from fresh buttermilk.

Treatment	Ganglioside content ² (g/L)	Lactose content ³ (mg/L)	Protein content (g/L)
Fresh buttermilk ¹	1600.0	6700.0	87000.0
Ultrafiltration	1400.0	72.0	4171.0
Ultrafiltration and organic extraction	400.0	6.6	1.0

¹Control as fresh buttermilk contained 87.9% moisture, 8.7% crude protein, 2.7% lipid, and 0.365% minerals.

²Gangliosides content was determined by periodate-resorcinol method.

³Lactose content was determined by enzymatic bioanalysis kit.

The gangliosides were soluble in the organic phase, and the proteins and residual lactose were removed with the water phase. The organic extraction of buttermilk decreased the gangliosides content by 75%, but also decreased the protein level to 1 mg/L and the lactose to 6.6 mg/L.

The method used to measure the gangliosides content of buttermilk (9) actually measures sialic acid, which is part of the sugar moiety of gangliosides. Most of the MFGM-carbohydrates are covalently bound to both lipids and proteins (8). During the organic extraction step, the gangliosides content was reduced, but this may result in a loss of glycosylated proteins. However, Table 3 demonstrates that both ultrafiltration and organic extraction efficiently removed the lactose and protein from buttermilk, while concentrating the gangliosides fraction.

Ganglioside Immobilization

Gangliosides were saponified to release the fatty acid resulting in a free amino group. The extent of saponification was monitored using OPA. There was a 74% increase in amino groups after saponification. The Sulfo-NHS was used to stabilize the amine-reactive intermediate that was formed between EDC and the carboxyl groups of the support matrix. The immobilized ganglioside beads were housed in a column and washed with 100 bead volumes of 0.05 M sodium phosphate buffer in pH 7, followed by 1 L of the same buffer containing 1 M NaCl to remove noncovalently bound gangliosides.

Hydroxylamine was added to quench the reaction, which also served to cap the unreacted carboxyl group to prevent nonspecific protein binding. Determination of CPG-bound sialic acid with the resorcinol reaction showed that less than 5% of the lyso-gangliosides were present in the washing solutions. This result indicates that

immobilization efficiency is almost 95%. Covalent immobilization yielded 66 micrograms gangliosides per gram bead.

Affinity Purification of Lactoferrin

Whey protein isolate and WPC were used as sources of BLF since they are essentially fat-free, devoid of caseins, and commercially available. Pretreated WPI, WPI, and WPC were applied to the ganglioside column, and proteins eluting with the different buffers (A = sodium phosphate, pH 7; B = sodium acetate, pH 4; C = sodium acetate, pH 4 containing 0.5 M NaCl; D = sodium acetate, pH 4 containing 1.0 M NaCl) were collected. Figures 1, 2, and 3 show the SDS-PAGE analysis under nonreducing conditions of proteins eluting from the column using the three whey protein sources. Sodium phosphate, pH 7, and sodium acetate, pH 4, without salts were used as washing steps to remove nonspecifically bound proteins (Figures 1, 2, and 3, lanes A and B). Sodium acetate, pH 4, containing 1 M NaCl, was applied to elute BLF (Figures 1, 2, and 3, lanes C and D). A protein of 80 kDa molecular weight appears in samples eluted with salt (Figures 1, 2, and 3, lanes C and D) from the ganglioside column. N-terminal sequence analysis revealed that the 80 kDa protein was identical with BLF in the 12 N-terminal amino acids sequenced.

The bovine lactoferrin protein band is not easily visible in the starting sample (Figures 1, 2, and 3, lanes 1 and 2) or in the washing solutions (Figures 1, 2, and 3, lanes A and B). Buffers at pH 7 and pH 4 without salts removed some of the nonspecifically absorbed BSA (66 kDa), β -LG (18 kDa), and α -LA (14 kDa). Increasing salt concentrations to 0.5 and 1.0 M NaCl were then used to specifically elute BLF. There was approximately the same amount of BLF in samples C and D (Table 4), but, in general, the BLF in sample D contained fewer co-eluting whey proteins.

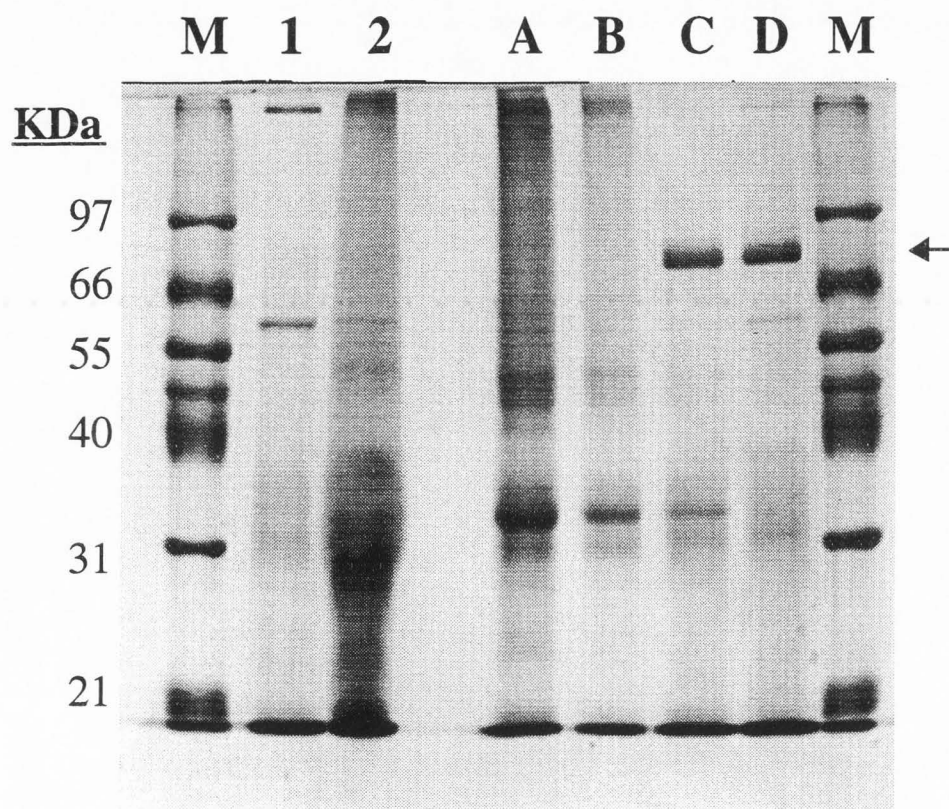


Figure 1. Nonreducing SDS-PAGE analysis of heat-stable protein from 1% whey protein isolate solution. Arrow designates BLF in whey samples. Whey samples were collected from the ganglioside column using various buffers. Approximately 20 μg of protein was loaded for each sample. Lane 1, 1% whey protein; lane 2, pretreated 1% WPI; lane A, elutant at pH 7; lane B, elutant at pH 4; lane C, elutant at pH 4 (0.5 M NaCl); lane D, elutant at pH 4 (1.0 M NaCl); lanes M, protein standards with molecular mass $\times 10^{-3}$ indicated.

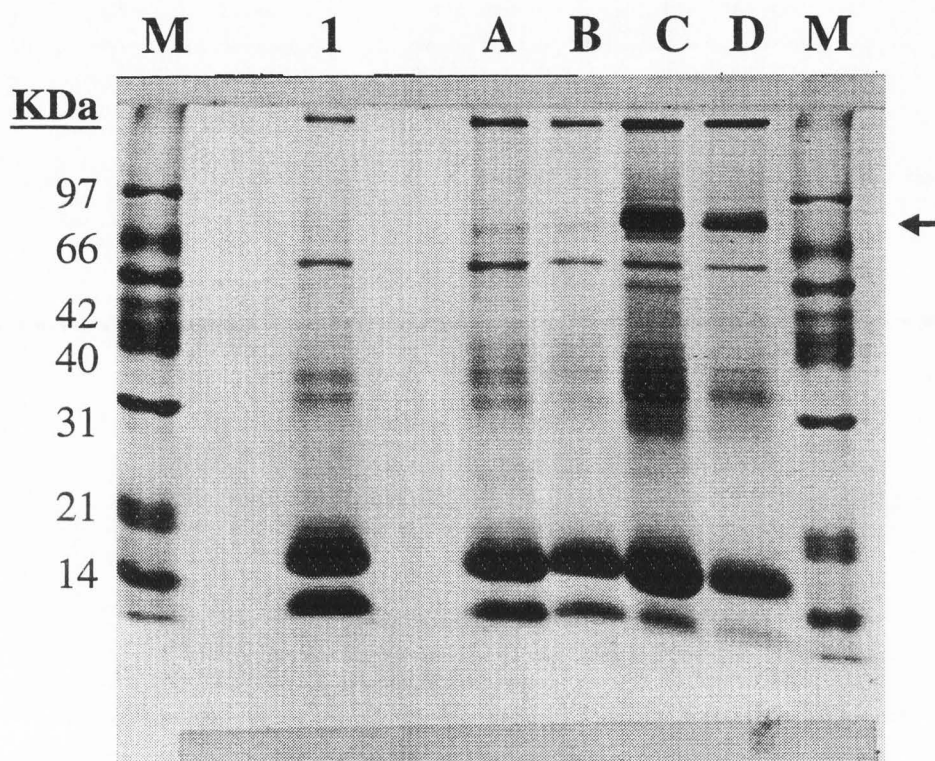


Figure 2. Nonreducing SDS-PAGE analysis of 1% whey protein isolate solution. Arrow designates BLF in whey samples. Whey samples were collected from the ganglioside column using various buffers. Approximately 20 μg of protein was loaded for each sample. Lane 1, 1% whey protein; lane A, elutant at pH 7; lane B, elutant at pH 4; lane C, elutant at pH 4 (0.5 M NaCl); lane D, elutant at pH 4 (1.0 M NaCl); lanes M, protein standards with molecular mass $\times 10^{-3}$ indicated.

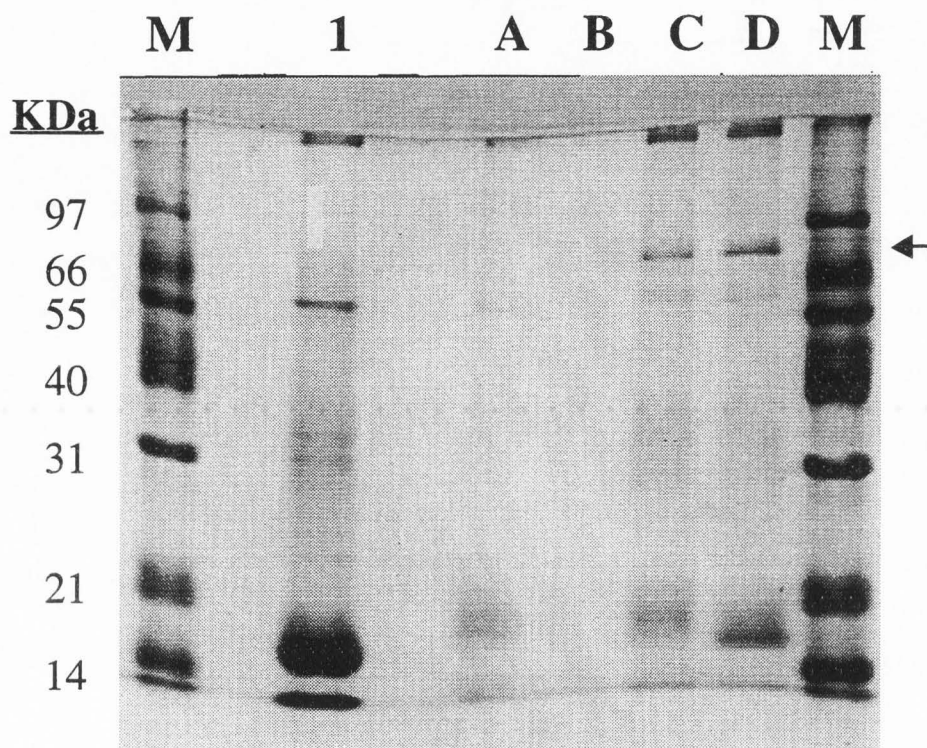


Figure 3. Nonreducing SDS-PAGE analysis of 1% whey protein concentrate solution. Arrow designates BLF in whey samples. Whey samples were collected from the ganglioside column using various buffers. Approximately 20 μg of protein was loaded for each sample. Lane 1, 1% whey protein; lane A, elutant at pH 7; lane B, elutant at pH 4; lane C, elutant at pH 4 (0.5 M NaCl); lane D, elutant at pH 4 (1.0 M NaCl); lanes M, protein standards with molecular mass $\times 10^{-3}$ indicated.

TABLE 4. Estimated recovery of bovine lactoferrin from various whey samples using immobilized gangliosides.

Sources	Starting samples ¹ (mg)	BLF in C ² (%)	BLF in D ³ (%)	Total BLF ⁴ (mg)	Estimated recovery ⁵ (%)
Pretreated WPI (1%)	1500.0	36.1	40.3	4.7	7.0
WPI (1%)	135.0	14.2	22.4	1.53	38.0
WPI (5%)	135.0	15.7	8.0	3.10	76.5
WPI (10%)	135.0	28.3	38.0	4.29	105.0
WPC (1%)	16.5	18.5	22.8	0.33	67.3
WPC (5%)	16.5	24.5	33.3	0.24	48.5
WPC (10%)	16.5	20.0	30.0	0.10	20.2

¹Samples were quantitated by Bradford protein assay (Bio-Rad, Hercules, CA).

²Lactoferrin (%) present in sample C.

³Lactoferrin (%) present in sample D.

⁴Total amount of lactoferrin was calculated on the basis of % of total protein eluted.

⁵Calculation based on a BLF concentration of 3% in whey since bovine milk (1L) contains 2 mg of BLF among 6 mg of whey protein (20).

Protein fractions from the ganglioside column were analyzed by nonreducing SDS-PAGE to clearly distinguish BLF (80 kDa) from other whey proteins with a similar molecular weight that may have been co-purified (28). Lactoperoxidase (78 kDa) and transferrin (72-76kDa) have similar molecular weights but differ in the number of disulfide bonds. Therefore, we used nonreducing SDS-PAGE. No lactoperoxidase or transferrin was detected in purified samples as determined by N-terminal protein sequencing. Lactoperoxidase is also very heat labile and, therefore, may not have been in the starting whey protein material.

Whey protein isolate solution (1%) was pretreated by heat-denaturation, ultrafiltration, and pH adjustment to remove the major heat-labile whey proteins such as α -lactalbumin, β -lactoglobulin, BSA, and immunoglobulins. This resulted in a BLF

purity of 40% after affinity purification. Although BLF is thermostable under acidic conditions, particularly at pH 4.0 (17), pretreatment may have reduced the amount of BLF in this sample because the BLF recovery was only 7% (Table 4).

Since approximately the same amount of BLF was eluted at salt concentrations of 0.5 to 1 M NaCl, the interaction between BLF and gangliosides appears to be influenced by ionic strength. The use of 0.5 M NaCl was effective in removing BLF and other bound whey proteins. There was BLF remaining on the column after this wash, which was eluted with 1 M NaCl. This is clearly shown in lanes D of Figures 1, 2, and 3.

Estimated Recovery

The estimated recovery of BLF among WPI, WPC, and pretreated WPI was compared in Table 4. BLF was recovered with varying efficiency from 105% to 7% depending on the whey protein source and concentration. The WPI contained 90% protein, and the WPC contained 11% protein on a gram basis. Each WPI concentration tested contained 135 mg protein and each WPC concentration tested contained 16.5 mg protein. Pretreatment of a 1% WPI solution resulted in a 90% loss in protein content from 1500 mg to 150 mg.

Bovine lactoferrin is found at a concentration of 0.3% to 3% in whey protein, and the estimated recovery listed in Table 4 was calculated based on 3% BLF in the whey protein. Whey protein isolate, 10% and 5%, showed the highest recovery among whey samples, and pretreated WPI showed the lowest. Whey protein concentrate also proved to be a valuable source of BLF with recovery ranging from 20% to 67%.

The ganglioside column was packed with 2g glass beads containing 132 μg immobilized gangliosides. The column capacity was calculated using Avogadro's number (6.023×10^{23} molecules), using an average ganglioside molecular weight of 1500 g/1 mole and a molecular weight (80,000g/1 mole) for BLF in equations (1) and (2).

$$132\mu\text{g} \times \frac{1 \text{ mole}}{1500 \text{ g}} \times \frac{6.023 \times 10^{23} \text{ molecules}}{1 \text{ mole}} = 5.3 \times 10^{16} \text{ molecules} \quad (1)$$

$$5.3 \times 10^{16} \text{ molecules} \times \frac{1 \text{ mole}}{6.023 \times 10^{23} \text{ molecules}} \times \frac{80,000 \text{ g}}{1 \text{ mol}} = 7.0 \times 10^{-3} \text{ g} \quad (2)$$

In this study, we applied 150 mg whey containing 4.5 mg BLF (3% of whey) to the ganglioside column, which shows maximum 7 mg or 7.0×10^{-3} g BLF binding capacity. This calculation indicates that the applied sample was not limiting.

DISCUSSION

Bovine lactoferrin contains a strongly basic region close to the N-terminus that contributes to the interaction of BLF with a variety of anionic biological molecules (24). The N-terminal stretch of BLF contains four consecutive arginine residues, which have a decisive role in the binding of BLF to physiologically relevant ligands such as heparin (20), bacterial lipopolysaccharide, human lysozyme, and DNA (24). The binding of BLF to immobilized heparin (20) was not influenced by pH or 8 M urea, but did dissociate in the presence of 1.0 M NaCl. The authors concluded the main binding force was electrostatic interactions between the negatively charged sulfate and carboxyl groups of heparin and positively charged groups in BLF.

We have shown that BLF interacts with immobilized gangliosides. The interaction is stable at acidic and neutral pH values, but is influenced by salt concentration. BLF was eluted from the ganglioside column with both 0.5 and 1 M NaCl. This interaction is electrostatic and may involve the negatively charged N-acetylneuraminic acid groups in gangliosides. This interaction may also be biospecific, since BLF is known to interact with eukaryotic membranes.

The interactions between BLF and various anionic biological molecules have been applied for affinity chromatography or ion-exchange chromatography. BLF was easily extracted using 5'-p-aminophenylphosphoryl, uridine 2'-3'-phosphate agarose (21), or aminohexyl-divinylsulfonyl agarose as affinity chromatographic methods (7). However, these affinity chromatographic methods needed further purification steps to separate BLF from co-purifying proteins.

Purification methods using ion-exchange chromatography have been used to purify BLF and BLF peptides (16). Hydrophobic interaction chromatography followed by carboxymethyl ion-exchange chromatography was used to purify BLF and bovine lactoperoxidase from acid whey. This purification scheme resulted in 80-88 mg/L of BLF (28). To increase the BLF purification yield, acid whey was pretreated with ammonium sulfate and sodium sulfate to remove major whey fractions and to concentrate the BLF in whey before applying to chromatographic methods (27). Ion-exchange chromatographic methods lead to high purification efficiency but still have drawbacks in requiring multiple steps.

Recently, a one-step ion-exchange chromatography procedure has been demonstrated for bovine lactoperoxidase and BLF using a cation-exchange membrane containing immobilized sulfonic acid moieties (4). Using elution buffers of different ionic strengths, the purification efficiency was 50% for BLF and 73% for bovine lactoperoxidase. Other purification methods using cationic exchange membranes have been patented (3, 23).

Our affinity column using gangliosides presents several economic advantages in comparison with the methods used previously. This affinity chromatography procedure provides efficient isolation of enriched BLF without the need to pretreat whey. The inclusion of pH difference in the chromatography buffers removes some of the nonspecifically absorbed whey proteins before elution of BLF. Gangliosides coupled to

CPG beads have good mechanical properties and are stable to organic solvents, acidic solutions, and drying. The immobilized ganglioside column has been used repeatedly without apparent decrease in BLF binding capacity for 6 to 12 mo. The support can be cleaned in ethanol, urea, or high salt buffers.

CONCLUSIONS

Bovine lactoferrin was purified by affinity chromatography using immobilized gangliosides. Our newly developed immobilized gangliosides can be used repeatedly for a maximum 12 mo with resistance to organic solvents, acidic conditions, and mechanical pressure. Bovine lactoferrin was efficiently purified using immobilized gangliosides with 0.5-1 M NaCl. Pretreated WPI (1% wt/vol) showed 40% BLF purity among protein sources, while WPI (10% wt/vol) showed the 105% BLF recovery. This affinity purification procedure is suitable to obtain BLF in large quantities using inexpensive commercial whey.

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CHAPTER IV

RAPID FRACTIONATION OF BOVINE TRANSFERRIN USING IMMOBILIZED GANGLIOSIDES

ABSTRACT

Bovine transferrin (BTF) was fractionated from bovine whey using ganglioside affinity chromatography. After loading the immobilized matrix with a 2% whey solution, the matrix was washed with sodium acetate buffer at pH 4 containing 1 M NaCl before elution of BTF with sodium phosphate buffers at pH 7. Concanavalin-A affinity and Mono-Q anion exchange chromatography were used for further purification. The ganglioside column showed a 74.2% BTF recovery from whey, and BTF was enriched to 61% purity with Mono-Q chromatography. Bovine transferrin was identified by SDS-PAGE analysis, Western analysis, and isoelectrofocusing. The Concanavalin-A affinity and Mono-Q anion exchange chromatography steps enriched BTF in the samples and removed other whey proteins from ganglioside purified fractions. These results indicate that immobilized ganglioside can be used to fractionate BTF from bovine whey. Our novel ganglioside affinity is rapid and efficient for BTF fractionation from whey.

INTRODUCTION

Transferrin (TF) is a monomeric glycoprotein of 679 amino acids, with a relative molecular weight of approximately 80 kDa (11). Transferrin exists mainly in the serum and interstitial compartments of vertebrates and some invertebrates (2, 23). Transferrin is found at a much lower concentration in human milk (< 50 $\mu\text{g}/\text{ml}$) in comparison with bovine milk (20 to 200 $\mu\text{g}/\text{ml}$) (25). The principal physiological function of TF in mammals is to transport ferric irons from sites of absorption to sites of utilization.

Transferrin transports iron from the biological fluids into the cytoplasm via plasma membrane by receptor-mediated endocytosis (22). Transferrin interacts with specific receptors present in variable amounts on target cells. Important target cells include the liver, bone marrow, and muscle. Due to ubiquitous iron requirements, TF receptors on the cells have been observed in a wide range of eukaryotic species and cell types.

Transferrin receptors have been found on the surfaces of various eukaryotic cells such as erythroid, reticulocyte, hepatocyte, placental trophoblast, and macrophage (20). Neckers and Cossman (19) demonstrated that TF receptors are induced in human lymphocytes by interleukin. Melanoma plasma membrane-associated glycoprotein (p97) from chondrocytes has been demonstrated to have sequence homology with TF (16).

Transferrin acts as a growth factor *in vitro* and an essential component in defined culture media (24). Transferrin has profound stimulatory effects on the cell proliferation such as hematopoiesis and metanephric differentiation (6, 26).

In order for BTF to mediate the biological function of iron delivery and stimulatory effects on cell proliferation and differentiation, it must interact with the eukaryotic cell surface. Gangliosides are common components of eukaryotic cell surfaces. Gangliosides have been implicated in various recognition phenomena (7). Gangliosides act as receptors for viruses, bacteria, toxins and other ligands. Gangliosides also involve in immunomodulation and cell to cell recognition. Gangliosides showed stimulations of cell proliferation and differentiation in growth factor receptors, leukemic cell lines, and neuronal cells, respectively (14).

In the present study, we developed a novel ganglioside affinity chromatography procedure for the fractionation of BTF from whey protein isolate (WPI). The purity of BTF was increased by Con-A and Mono-Q chromatography, and BTF was identified by SDS-PAGE analysis, Western analysis, and isoelectrofocusing.

MATERIALS AND METHODS

Starting Material

Bovine whey protein isolate (WPI) (Avenmore West, Twinfalls, ID) was used as the source of TF. Whey solution (2% WPI) dissolved in distilled water, was vacuum filtered through a 0.2 μ M membrane filter (Gelman Science, Ann Arbor, MI), and stored at 4 °C. The whey solution was adjusted to pH 4.0 by addition of 1.0 M sodium acetate at pH 4, just before use.

Ganglioside Extraction

Gangliosides were extracted from fresh bovine buttermilk (Breaden Butter, Logan, UT). Buttermilk was diluted 1:10 with distilled water and ultrafiltrated using a pre/scale-TFF 2.5 ft² cartridge membrane with 10 kDa (Millipore, Bedford, MA) to remove lactose. The efficiency of lactose removal was confirmed with a lactose enzymatic bioanalysis kit (Boehringer Mannheim, Indianapolis, IN). Lactose-free buttermilk was freeze-dried and gangliosides were extracted using 20 vol of organic mixture of chloroform:methanol:water (40:80:30 vol) per gram dry solids according to Heitmann et al. (13). The total amount of gangliosides extracted was determined by the periodate-resorcinol method using sialic acid as standard (15).

Ganglioside Immobilization

Controlled pore glass (CPG) beads (2000 °A, 120-200 mesh) (Sigma, St.Louis, MO) were derivatized with 3-aminopropyltriethoxy silane and succinylated with succinic anhydride according to Walsh and Swaisgood (32). Acetic anhydride (10% by vol) in acetone was used to cap excess amino groups. Gangliosides were saponified to produce the lyso-derivative containing a free amino group by the reflux boiling method (27). The

carboxyl matrix was reacted with Sulfo-NHS (Pierce, Rockford, IL) in 0.01 M MES (pH 6) containing water-soluble carbodiimide (EDC). The lyso-gangliosides, dissolved in MES buffer (pH 6), were circulated over the Sulfo-NHS matrix to couple via amide bond for 12 h at 4 °C using a peristaltic pump. Hydroxylamine (10 mM) was added to quench the reaction by hydrolyzing any unreacted NHS present. The extent of gangliosides immobilized was determined by measuring the amount of sialic acid in the supernatant and washing buffers after immobilization (15).

Ganglioside Affinity Chromatography

Immobilized gangliosides were packed into stop-flow columns (Pierce, Rockford, IL) and equilibrated with 0.05 M sodium acetate buffer (pH 4). Whey sample (2% WPI) was applied to column and circulated for 5 min at room temperature. Bovine transferrin was purified by batch mode using four kinds of 0.05 M buffers; A = sodium acetate (pH 4), 1 M NaCl; B = sodium acetate (pH 4); C = sodium phosphate (pH 7); D = sodium phosphate (pH 7), 1 M NaCl. For the complete removal of lactoferrin and bovine serum albumin (BSA), the whey sample was applied successively to the ganglioside column and followed by washing with sodium acetate buffer at pH 4 (buffers A and B) for several times. Finally, BTF was eluted with sodium phosphate buffer at pH 7 (buffers C and D) and analyzed by SDS-PAGE under nonreducing conditions.

Con-A Affinity Chromatography

Concanavalin-A sepharose (Con-A) beads (Pharmacia, Uppsala, Sweden) were packed into disposable polypropylene column (Pierce, Rockford, IL) and equilibrated with 3 column vol of 10 mM sodium phosphate (pH 7). The column was loaded with samples from the ganglioside column after being ultrafiltrated through the 50 kDa membrane (Amicon, Beverly, MA). The column was washed with 2 column vol of 10

mM sodium phosphate (pH 7) containing 1.5 M NaCl to remove nonspecifically bound proteins and glycoproteins were eluted with 1 column vol of 10 mM sodium phosphate (pH 7) containing 0.75 M methylmannoside.

Anion Exchange Chromatography

The liquid chromatography system (System Gold Nouveau, Beckman, Fullerton, CA) consisted of two 125 pumps, a 125 pump-direct controller, a 502 autosampler, and a 168 detector operated at a wavelength of 280 nm. The anion exchange column, Mono-Q HR 5/5, 5.0 x 50 mm (Pharmacia, Piscataway, NJ), was used with 0.02 M Tris-Cl, pH 7 (buffer A), and the same buffer with 1 M NaCl (buffer B). A stepwise gradient of buffer B with the following steps was used for BTF separation: 1) 5 ml of 0-10%; 2) 25 ml of 11-35%; 3) 2 ml of 36-100%; 4) 2 ml of 100-0%; and 5) 10 ml of 0%. Prior to chromatography, the Con-A purified whey fractions were filtered through a 0.2- μ m membrane filter (Gelman Science, Ann Arbor, MI). Buffers were vacuum-filtered through a 0.2- μ m membrane filter (Gelman Science, Ann Arbor, MI) and degassed before use.

SDS-PAGE and Western Analysis

To identify BTF among whey proteins in gels, whey fractions were resolved by nonreducing or reducing SDS-PAGE with 10% polyacrylamide according to Laemmli (17) with modification. One gel was stained with Coomassie blue and destained in 25% methanol, 5% acetic acid (vol/vol). The dried gel was measured by densitometry to measure the concentration of BTF in samples. The other gel was electroblotted onto nitrocellulose membrane as described by Towbin et al. (31). The membrane was blocked with superbloc blocking buffer in TBS (Pierce, Rockford, IL) with gentle agitation for 2 h at room temperature. The membrane was incubated overnight at 4 °C with sheep anti-

BTF antibody conjugated alkaline phosphatase (Bethyl Lab., Montgomery, TX) in 20 ml of TTBS, diluted 1: 500. Finally, after washing four times with TBS, the blot was developed by incubation with 10 ml of substrate solution, 1-step NBT/BCIP (Pierce, Rockford, IL).

Isoelectrofocusing

Isoelectrofocusing (IEF) electrophoresis was carried out vertically at room temperature using the Mini-protein II electrophoresis cell (Bio-Rad, Richmond, CA). The anode and the cathode solutions were 7 mM phosphoric acid and 20 mM lysine containing 20 mM arginin. Proteins were focused at 250 V for 1 hour using a precasted IEF gel (pH 5/8) (Bio-Rad, Hercules, CA). The gel was stained with IEF staining solution (Bio-Rad, Hercules, CA) and destained in 40% methanol, 10% acetic acid mixture with multiple changes. As standards, BTF, BSA purchased from Sigma (St.louis, MO), and IEF standard (PI 3.5/9.3) (Pharmacia, Piscataway, NJ) were used.

RESULTS

Immobilized Gangliosides

Gangliosides were saponified to release the fatty acid resulting in a free amino group. These lyso-gangliosides were covalently coupled to succinylated controlled pore glass (CPG) beads via an amide bond using carbodiimide (EDC) and Sulfo-NHS. The Sulfo-NHS was used to stabilize the amine-reactive intermediate that was formed between EDC and the carboxyl group of support matrix. The immobilized gangliosides beads were housed in a column and washed with 100 bead volumes of 0.05 M sodium phosphate buffer in pH 7, followed by 1 L of the same buffer containing 1 M NaCl to remove noncovalently bound gangliosides. Hydroxylamine served to quench the reaction and cap the unreacted carboxyl group to prevent nonspecific protein binding.

Determination of sialic acid with the resorcinol reaction showed that less than 5% of the lyso-gangliosides were present in the washing solutions. This result indicates that immobilization efficiency is more than 95%. Covalent immobilization yielded 66 micrograms gangliosides per gram bead.

Transferrin Purification

After 2% whey (5 g WPI) in sodium acetate was applied to the ganglioside column, two protein bands (approximately 76 kDa, 72 kDa) appeared in whey fractions eluted at pH 7. These protein bands appeared between lactoferrin (80 kDa) and BSA (66 kDa) and had the expected location of BTF (30). These bands were not visible in the starting sample (Figure 4, lane W). Buffer at pH 4 containing salt (buffer A) removed lactoferrin and BSA (Figure 4, lanes A). This washing step was carried out after every application of the same whey sample four times to remove whey proteins. Buffers at pH 7 with (buffer D) and without salt (buffer C) were then applied to specifically elute bound proteins (Figure 4, lanes C and D). Collected whey fractions were analyzed by nonreducing SDS-PAGE to clearly distinguish BTF from both lactoferrin and BSA, which have similar molecular weights within the 60 to 80 kDa range (arrows in Figure 4). Bovine transferrin was eluted with buffers at pH 7 (Figure 4, lanes C and D). After applying buffer A, the column was equilibrated with buffer B and the eluting sample was reapplied (Figure 4, lanes A and B). The interaction between BTF and gangliosides was influenced by pH rather than salt concentration in that BTF was eluted with buffers at pH 7. Prior to eluting BTF from the ganglioside column, multiple washing steps with buffer A were efficient at removing other whey proteins, especially lactoferrin and BSA. However, the eluted BTF fraction (Figure 4, lanes C and D) still contained other proteins including lactoferrin and BSA.

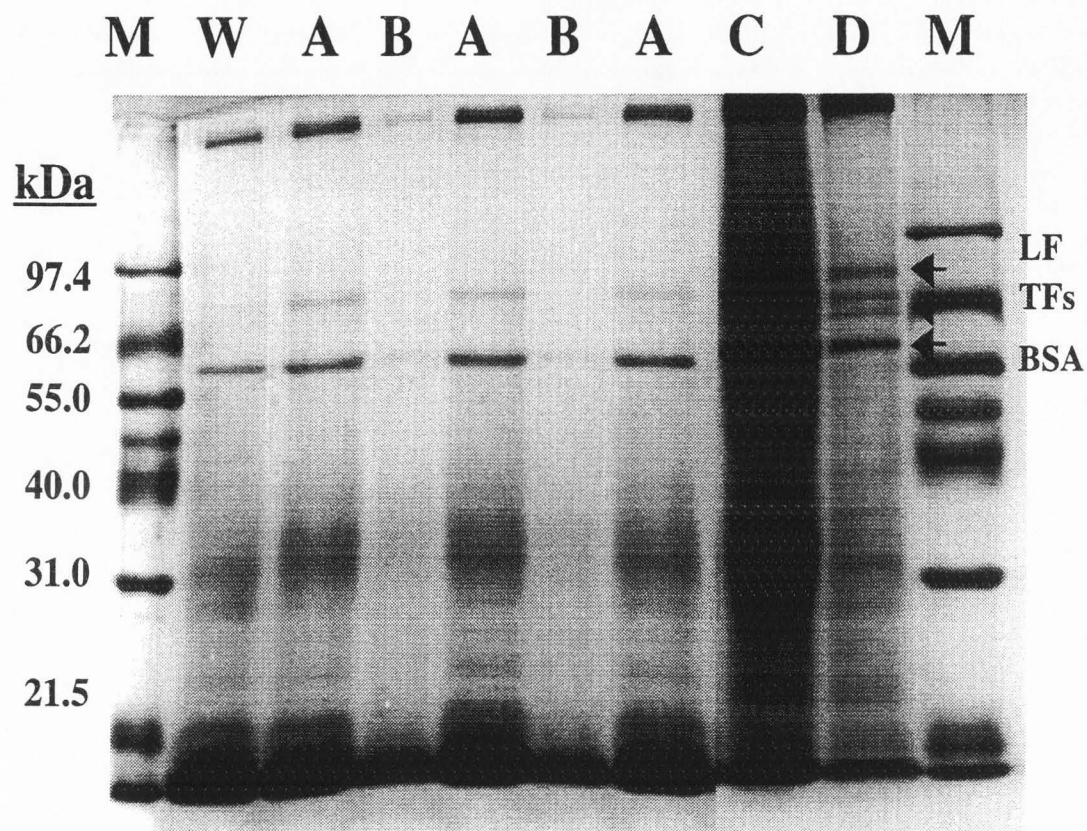


Figure 4. Nonreducing SDS-PAGE analysis of whey samples from ganglioside column. Arrows designate BSA (bovine serum albumin), TFs (bovine transferrin), and LF (bovine lactoferrin). Lanes M, protein molecular weight markers; lane W, WPI; lanes A, successive elution of proteins at pH 4, 1 M NaCl; lanes B, successive elution of proteins at pH 4; lane C, elution of proteins at pH 7; lane D, elution of proteins at pH 7, 1 M NaCl.

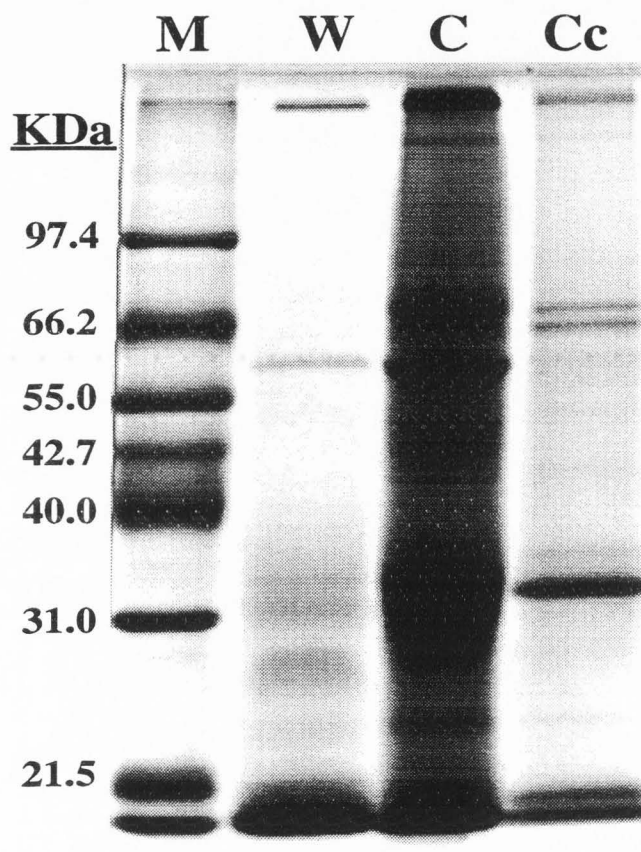


Figure 5. Nonreducing SDS-PAGE of WPI fraction at pH 7 after Con-A chromatography. Lane M, protein molecular weight marker; lane W, WPI; lane C, ganglioside purified WPI fraction at pH 7; lane Cc, sample C after Con-A purification.

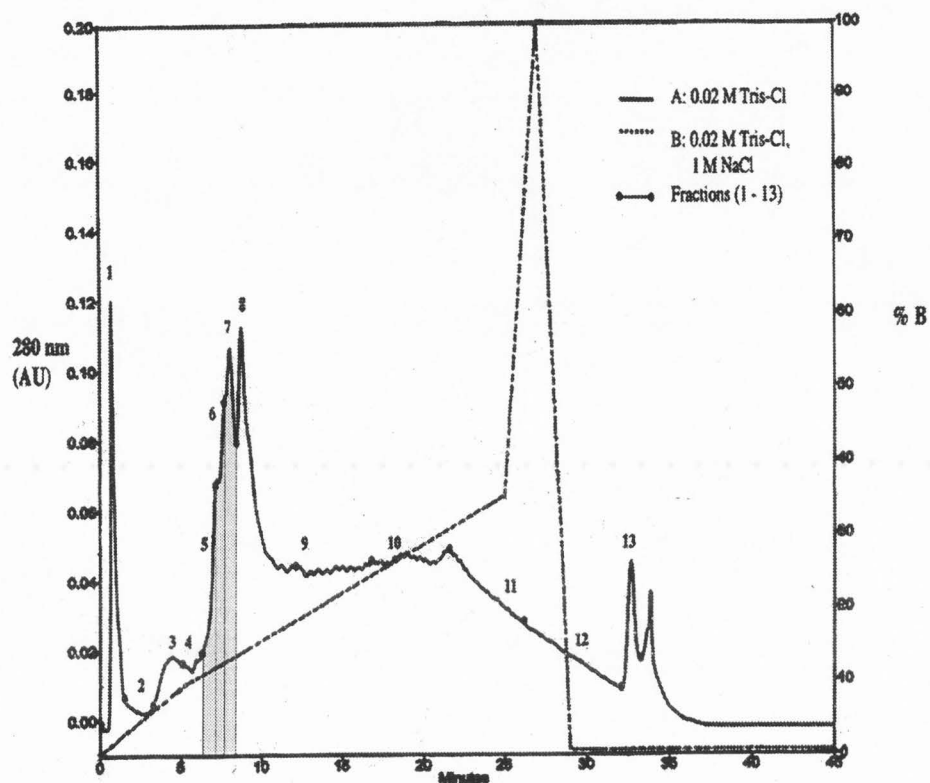


Figure 6. Mono Q anion exchange chromatography of Con-A purified WPI fraction at pH 7 containing 1 M NaCl. Grey painted sections contain BTF enriched samples. A 100 μ l sample (10 mg/ml) in 20 mM Tris-Cl buffer (pH 7) was applied to column and eluted with the same buffer containing 1 M NaCl, at a flow rate of 1 ml/min.

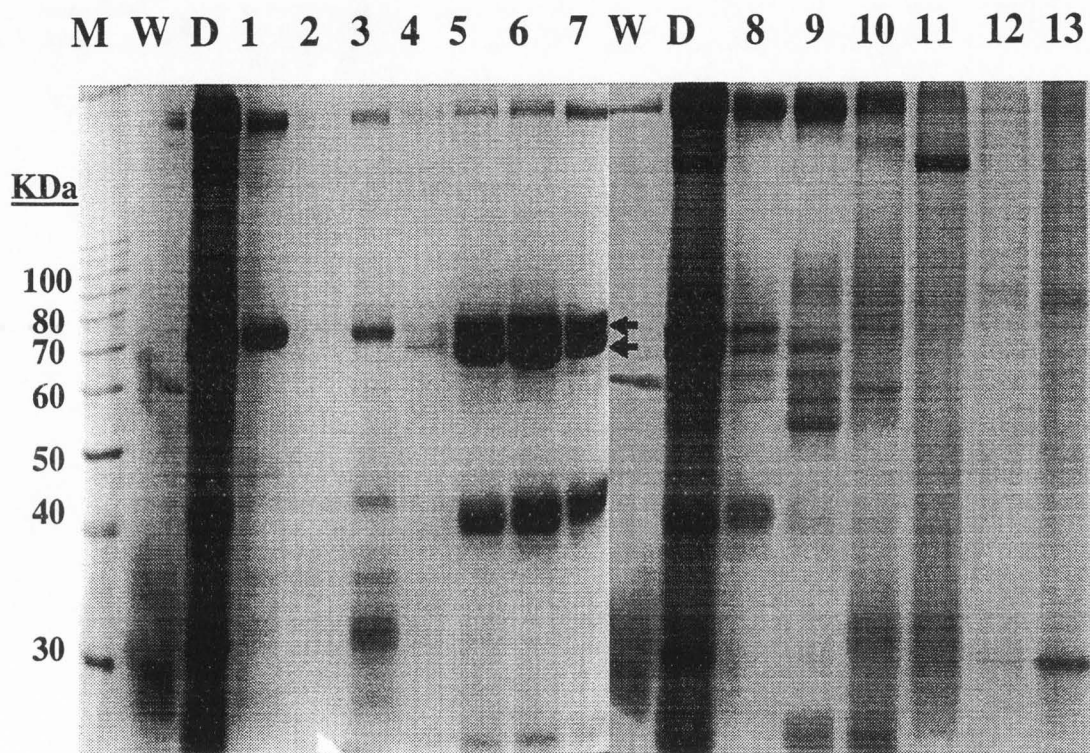


Figure 7. Nonreducing SDS-PAGE analysis of HPLC fractions. Arrows designate enriched BTF in fractions. Lane M, 10 kDa protein ladder; lanes W, WPI; lane D, gangliosides fraction at pH 7, 1 M NaCl; lanes 1-13, fractions collected from Figure 6.

Con-A Affinity Chromatography

An example of Con-A purification on WPI fraction C is shown in Figure 5. Con-A purification was used to purify the glycoproteins present in lane C of Figure 4. Figure 5 shows that nonglycosylated whey proteins were efficiently removed by Con-A (Figure 5, lane Cc). There are two prominent protein bands at approximately 66 kDa, one band at 31 kDa and two bands less than 21 kDa. After Con-A purification, the BTF purity in samples was increased from 13.9% to 24%.

Mono Q Anion Exchange Chromatography

Con-A purified WPI fraction D was applied to the Mono-Q column and its elution profile is shown in Figure 6. To assess the purity, the fractions obtained from HPLC were collected and analyzed by SDS-PAGE under nonreducing conditions (Figure 7). The major protein of fraction 1 has the expected location as lactoperoxidase since it is known to have faster movement than lactoferrin in SDS-PAGE (33). Lactoperoxidase is known to have a physiologically basic PI 9.2 to 9.9 (4) and to be released from positive column matrix without electrostatic interaction.

Fractions 5, 6, and 7 eluted within 0.1 to 0.15 M NaCl salt have 76 kDa and 72 kDa molecular weights, which correspond to BTF. These protein bands constitute about 64%, 62%, and 57%, respectively, with an average of 61% of the total protein in SDS-PAGE gel. Each of these three fractions also contains a 31 kDa molecular weight band, expected to be PP3. This band appeared in the ganglioside column fractions (Figure 4, lanes C and D), Con-A fraction (Figure 5, lanes C and Cc), and Mono-Q fractions (Figure 7, lanes 5, 6, and 7). PP3 is lipid-binding glycoprotein present in bovine whey (9).

Interestingly, fraction 9 eluted within 0.15 M to 0.2 M NaCl gradient contained several whey proteins with molecular weights between 50 kDa and 90 kDa. Anion

exchange chromatography efficiently separated Con-A purified whey fraction and increased the BTF purity from 24% to 61%.

Western Analysis

Whey protein isolate fractions 5, 6, and 7 from Mono-Q chromatography were analyzed by SDS-PAGE under reducing conditions with BSA, BTF, and lactoferrin as standards (Figure 8). The location of BTF in the Mono-Q fractions was the same as that of commercial BTF and one of the two BTF bands showed similar mobility to BSA under reducing SDS-PAGE conditions. Native-glycosylated and deglycosylated (by N-glycosidase F) samples of the three HPLC fractions were analyzed by Western analysis using alkaline phosphatase conjugated sheep anti-BTF antibody (Figure 9). On the Western blot, each fraction (Figure 7, lanes 5, 6, and 7) with and without N-glycosidase F treatment had two bands recognized by the anti-BTF antibody. These BTF bands were decreased 2 to 3 kDa molecular mass after deglycosylation (Figure 9, lanes 5N, 6N, and 7N).

Isoelectrofocusing

Isoelectrofocusing (IEF) (Figure 10) showed that only fraction 5 contained pure BTF bands. The PIs for BTF is 5.8 and 6.0, corresponding to the value obtained for the standard BTF in Figure 10. Fraction 6 contained two other bands with PIs of 5.5 and 5.7 in addition to the two BTF bands, and fraction 7 contained three other bands with PIs of 5.4, 5.5, and 5.6 as well as one band of BTF. No fraction had BSA, lactoferrin, or lactoperoxidase.

BTF Purification

Bovine transferrin purification was shown in Table 5. The ganglioside column was used to fractionate BTF from whey and the Con-A and Mono Q columns were used

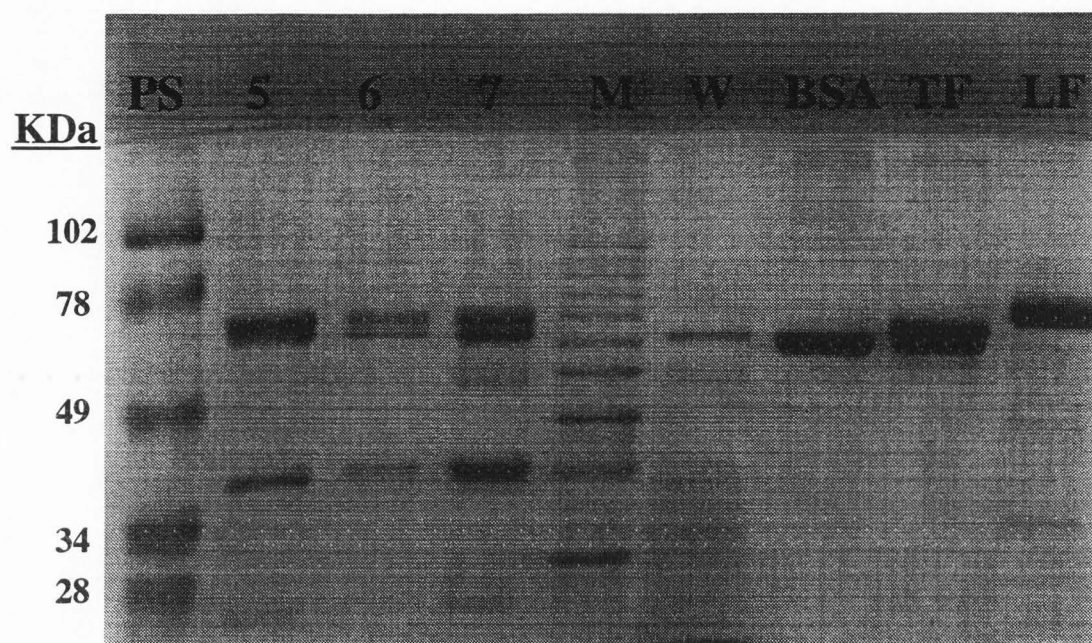


Figure 8. SDS-PAGE analysis of WPI Mono Q fractions and commercial standards. Lane PS, prestained protein standard; lane M, 10 kDa protein ladder; lanes 5, 6 and 7, Mono Q fractions; lane W, WPI; lane BSA, commercial bovine serum albumin; lane TF, commercial bovine transferrin; lane LF, commercial bovine lactoferrin.

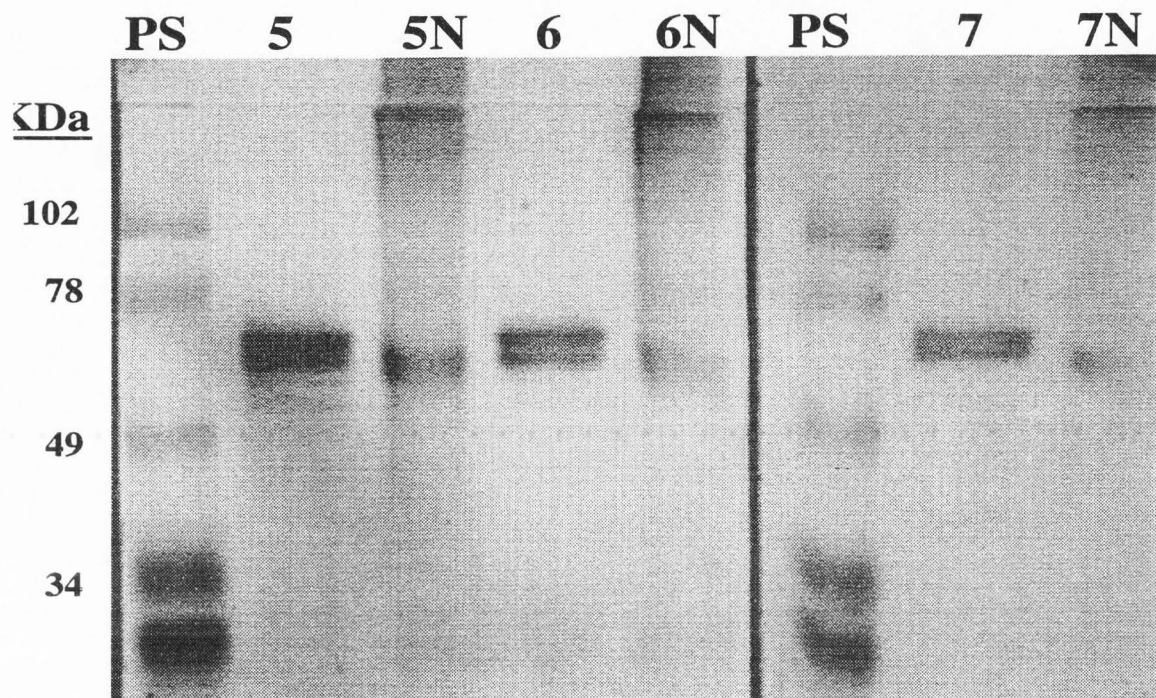


Figure 9. Western analysis of Mono Q fractions for BTF detection. Lane PS, prestained protein standard; lanes 5, 6 and 7, Mono Q fractions; lanes 5N, 6N and 7N, deglycosylated Mono Q fractions.

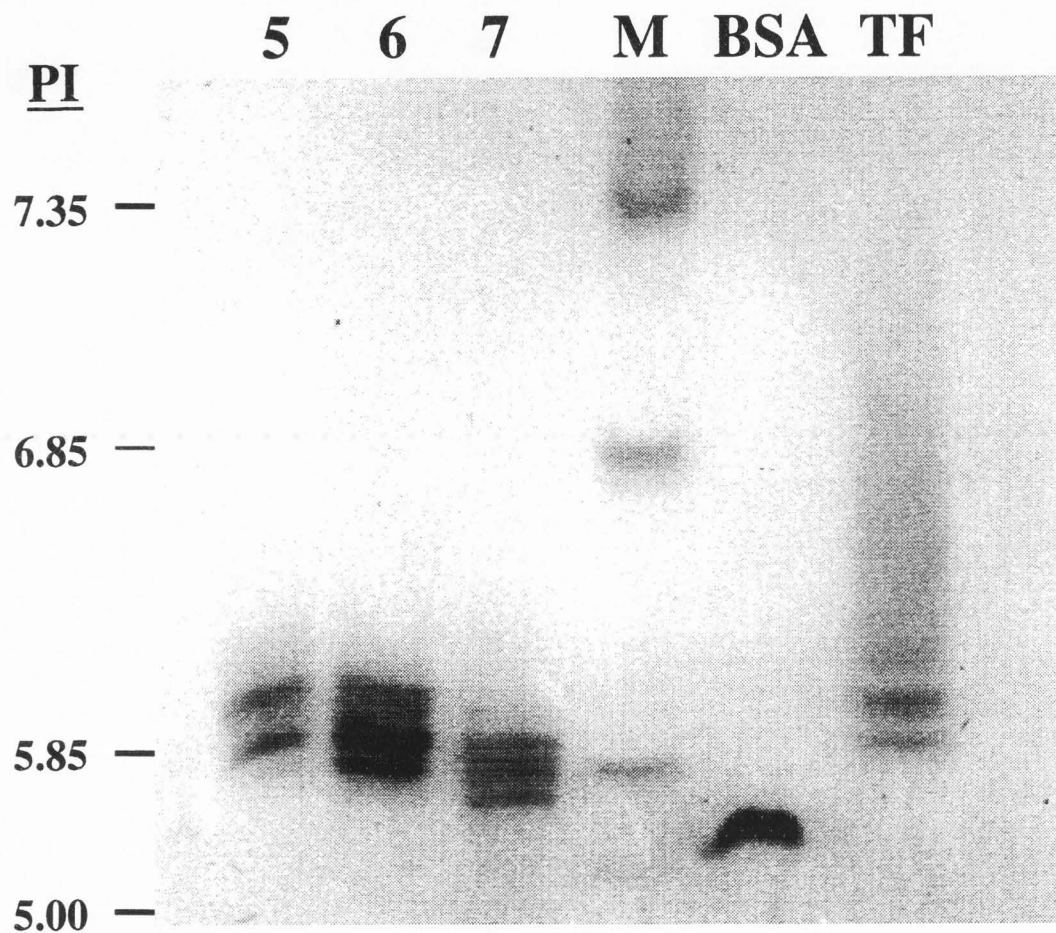


Figure 10. Isoelectrofocusing analysis of Mono Q fractions at pH 5/8. Lane M, Isoelectrofocusing standard marker with PI 3.6/9.3; lanes 5, 6 and 7, Mono Q fractions; lane BSA, commercial bovine serum albumin; lane BTF, commercial bovine transferrin.

TABLE 5. Bovine transferrin purification.

Source	Total protein ¹ (mg)	Total BTF ² (mg)	BTF purity ³ (%)	Estimated recovery ⁴ (%)
2% WPI	5000	5000.0	-	100.0
Ganglioside column ⁵	801	111.3	13.9	74.2
Con-A column ⁵	53	12.6	24.0	-
Mono Q column ⁶	20	12.2	61.0	-

¹Total protein were quantitated by Bradford protein assay (Bio-Rad, Hercules, CA).

²Total amount of transferrin was calculated on the basis of % of total protein eluted.

³Densitometry detected transferrin (%) of total protein present in effluent.

⁴Transferrin is found at a concentration of 3% of total whey protein.

⁵WPI fractions at pH 7 with (D) and without salt (C).

⁶Fractions 5, 6, and 7 from Con-A purified WPI fraction D.

for further BTF purification. Bovine transferrin is found at concentrations of 3% in whey protein and the BTF purification listed in Table 5 were calculated based on 3% BTF in the whey protein (25). The ganglioside column showed 74.2% BTF recovery with 13.9% purity. The Con-A column and Mono-Q column increased the BTF purity to 24%, 61% respectively. The ganglioside column was effective for BTF fractionation from 2% whey solution (5 g WPI). The Con-A column and Mono-Q column steps enriched BTF in the samples and removed other whey proteins from gangliosides purified fractions.

DISCUSSION

Structurally, TF consists of two homologous lobes. Each lobe is made up of two domains containing one specific iron-binding site. These sites require synergistic anion-binding for effective iron-binding (21). Anions, physiologically carbonate or bicarbonate

bind to cationic side chains in the protein in the immediate vicinity of the metal-binding site and result in metal-anionic molecule-protein (TF) ternary complex (3, 12, 21).

The interaction between TF and TF receptors is known to be influenced by pH (5). TF receptors, at physiologic pH, bind ferric-TF, resulting in a ferric-TF complex. The ferric-TF complex is transported to the cell cytoplasm by an undiscovered mechanism and becomes exposed to the acidic pH of the cell cytoplasm. This results in a rapid dissociation of iron. The resulting apo-TF complex is recycled to the neutral pH compartment, including the cell exterior, and apo-TF on the complex is dissociated from TF receptor (28, 29).

The interactions between TF and various anionic biological molecules have been used for affinity chromatography or ion exchange chromatography. Previously, several methods for TF separation from possum milk (10), porcine serum (8), sheep serum (18), human serum (34), and bovine serum and plasma (1) have been published. Most of separation methods involved several steps, including precipitation with ammonium sulfate, gel filtration, DEAE ion exchange, Mono-Q anion exchange, Blue sepharose affinity, and Concanavalin-A affinity separation. These methods produced low recoveries of TF since there was co-purification of other proteins. Transferrin sources used are expensive and inconvenient to handle, such as milk, serum, and plasma. Recently, a single-step method has been demonstrated whereby human TF was purified with a 75% yield by DEAE spherodex from plasma (24).

In this study, BTF eluting from the gangliosides affinity column contained a molecular weight heterogeneity of 76 kDa and 72 kDa as shown by SDS-PAGE analysis and showed two bands with decreased molecular mass after deglycosylation. Transferrin heterogeneity has been observed only in bovine, but not in human or in rat. This is due to

the different number of sialyl residues attached to each TF molecule and variant in cattle (30).

Gangliosides coupled to CPG beads have good mechanical properties resistant for high pressure, organic solvents, and acidic solutions. The immobilized gangliosides beads could be repeatedly used for 6 to 12 mo without apparent decrease in BTF binding capacity if the support is washed with high salt or urea and the immobilized beads are kept at 4 °C in 0.05% sodium azide.

Transferrin, the iron-binding glycoprotein, is found at much lower concentrations in human milk (< 50 µg/ml) in comparison to bovine milk (20 to 200 µg/ml) (25). In this paper, BTF was fractionated with a high recovery by gangliosides affinity chromatography using commercial whey.

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CHAPTER V

SUMMARY

Bovine lactoferrin (BLF) and bovine transferrin (BTF) are major-iron transport and regulatory proteins found in bovine whey. BLF and BTF must interact with the eukaryotic cell surface to mediate their biological function of iron delivery and cellular functions of inflammatory and immunological reactions. As common components of eukaryotic cell surface, gangliosides were used for affinity purification of BLF and BTF. Bovine gangliosides were isolated from fresh buttermilk using a combination of ultrafiltration and organic extraction. Isolated gangliosides were covalently immobilized onto controlled-pore glass beads. The immobilized matrix contained 66 micrograms of gangliosides per gram beads.

The gangliosides affinity technique is a unique and novel method to purify BLF and BTF. In addition to BLF and BTF, immobilized ganglioside can be used to purify other proteins that interact with eukaryotic cell walls. This ganglioside affinity method presents several benefits in comparison with the methods used previously. This affinity chromatography procedure provides a rapid and efficient isolation of BLF and BTF without the need to pretreat whey. The inclusions of pH difference and sodium chloride in the chromatography buffers remove some of the nonspecifically absorbed whey proteins before elution of BLF or BTF. Gangliosides coupled to CPG beads have good mechanical properties and are stable to organic solvents, acidic solutions, and drying. The immobilized ganglioside column has been used repeatedly without apparent decrease in BLF binding capacity for 6 to 12 mo. The support can be cleaned in ethanol, urea, or high salt buffers.

Bovine lactoferrin (BLF) was purified by affinity chromatography using immobilized gangliosides. After the matrix was loaded with whey protein (WPI or WPC), the matrix was washed with sodium phosphate buffer (pH 7) followed by sodium acetate (pH 4) before elution of lactoferrin with 1 M NaCl in sodium acetate buffer. Lactoferrin was identified by N-terminal protein sequencing. From the intensities of the protein bands in SDS-PAGE, lactoferrin constitutes a minimum of 40% of the total protein in the salt eluted sample. Pretreated whey isolate (1% wt/vol) showed the highest BLF purity among protein sources, and whey protein isolate (10% wt/vol) showed the highest recovery of BLF.

Bovine transferrin (BTF) was fractionated from bovine whey using ganglioside affinity chromatography. After the immobilized matrix was loaded with a 2% whey solution, the matrix was washed with sodium acetate buffer at pH 4 containing 1 M NaCl before elution of BTF with sodium phosphate buffers at pH 7. Con-A affinity and Mono-Q anion exchange chromatography were used for further purification. The ganglioside column showed a 74.2% BTF recovery from whey and BTF was enriched to 61% purity with Mono-Q chromatography. Bovine transferrin was identified by SDS-PAGE analysis, Western analysis, and isoelectrofocusing.

Bovine lactoferrin and bovine transferrin are of commercial interest because of their medical and nutritional benefits. Therefore, BLF and BTF purified using our affinity column could be applied to infant formulas to strengthen the immune system, to specialty dietary formulations to assist iron absorption, to pharmaceuticals to treat harmful bacteria, and to personal health items such as antibacterial toothpaste or anti-microbial cosmetics.

In conclusion, immobilized gangliosides can be used to purify BLF and BTF from bovine whey. These affinity purification procedures are suitable to obtain BLF and BTF from whey.

APPENDIX

AN ATTEMPT AT THE PURIFICATION OF BOVINE PROSAPOSIN

FROM WHEY

INTRODUCTION

Prosaposin is a 66 kDa glycoprotein found in milk at a concentration of 6 $\mu\text{g/ml}$ (9), contains 20% carbohydrate, and has an isoelectric point of 5.4. (1, 3). Prosaposin is the precursor of the lysosomal saposin proteins, which are required for hydrolysis of glycosphingolipids, but is also found in its unprocessed form in milks. This protein plays a broad role in the development, maintenance, and repair of the nervous system (4, 8). Hiraiwa et al., (6) have demonstrated that prosaposin displayed a strong affinity to gangliosides at pH 4.0 a greatly reduced affinity at neutral pH value. Prosaposin in human milk was isolated using several steps such as ion exchange, size exclusion, Con-A sepharose, and immunoaffinity chromatography (5, 6). Recently, bovine prosaposin was isolated from bovine milk and whey protein concentrate (WPC) with multiple steps such as Con-A sepharose, cellulose anion exchange, and immunoaffinity chromatography (9).

In this study, I tried to purify bovine prosaposin by biospecific binding using gangliosides affinity chromatography as well as by traditional methods including Con-A sepharose, Mono Q anion exchange, and isoelectricfocusing.

Approach 1

The interactions between prosaposin and gangliosides were found to be maximal at acidic pH values and greatly reduced at neutral pH values (6). A 1% solution of WPI in 50 mM sodium acetate buffer at pH 4 was applied to the immobilized gangliosides matrix. Prosaposin was purified by batch mode using four different buffers; A=sodium acetate, pH 4 containing 1 M NaCl; B= sodium acetate, pH 4; C= sodium phosphate, pH 7; D= sodium phosphate, pH 7 containing 1 M NaCl. Whey proteins not bound to the

column at pH 4 were reapplied to the column after washing the matrix with buffer B. Lactoferrin (3% of whey) and BSA (10% of whey) are more abundant than prosaposin (0.1% of whey) (2, 9, 10). Since BSA and lactoferrin were bound to the matrix, BSA and lactoferrin were removed at pH 4 with 1 M NaCl before eluting proteins at pH 7 (Figure 1). The eluting proteins were collected and each fraction was analyzed by nonreducing SDS-PAGE to distinguish prosaposin from BSA, which has a similar molecular weight of 66 kDa (9). Successive elution of proteins at pH 4 containing salt removed a significant amount of BSA, lactoferrin, β -lactoglobulin, and α -lactalbumin from the whey sample (Figure 1, lanes B). Specific elution of proteins at pH 7 with and without salt is shown in lanes C and D. There is an enrichment in proteins between 80 kDa and 60 kDa which are not visible in the starting sample (Figure 1, lane W).

Approach 2

Con-A sepharose column was used to purify the protein eluting with buffer C (which is from the gangliosides matrix) because prosaposin is glycoprotein. Lane Cc in Figure A-1 shows the glycoproteins present in this sample. The two glycoprotein bands between BSA and lactoferrin were separated by preparative electrophoresis and subjected to Western analysis using human anti-saposin C antibody (9) (Figure A-2). Under reducing conditions, two bands have approximately the same molecular weight, but under nonreducing condition, they show different mobility (Figure 4). Prosaposin is immunologically undistinguishable because the saposin C antibody showed crossreactivity to BSA and lactoferrin as well as prosaposin (Figure A-2). The two bands were purified by preparative electrophoresis (Figure 4, lanes 1 and 2) and N-terminal protein was sequenced for identification. The two bands contained several proteins which showed 80% and 75% homologies with transferrin and β -lactoglobulin, respectively. The upper protein band of two bands showed about 75% homology with

the amino acids of prosaposin. This result suggests that a single band of prosaposin could not be separated by methods using size difference of protein such as preparative electrophoresis, size exclusion.

Approach 3

Con-A fraction of elutant at pH 7 (Figure A-1, lane Cc) was applied to Mono Q anion exchange column for charge separation and collected to analyze by SDS-PAGE (Figure 5). Anion exchange chromatography resulted in a significant separation of whey proteins and the enrichment of two bands, which have the expected location of prosaposin (Figure 5, lanes 5, 6, and 7). Prosaposin is known to convert from 66 kDa to 50 kDa after deglycosylation with N-deglycosidase F (1). Fractions 5, 6, and 7 which contained mainly two protein bands were deglycosylated with N-deglycosidase F to distinguish prosaposin from protein mixture with the same molecular weights by reduction of protein size. Unfortunately, after deglycosylation, the decreased size of commercial transferrin was similar to those of the two bands. Native glycosylated and deglycosylated fractions 5, 6, and 7 were analyzed by SDS-PAGE for immunodetection using human anti-saposin C antibody. On the Western blot, all samples without and with treatment by N-deglycosidase F showed weak corresponding to saposin C antibody. However, this result could not be considered as a meaningful one in that saposin C antibody crossreacts with transferrin as well as with BSA and lactoferrin. Fractions 5, 6, and 7 were focused on isoelectrofocusing gel with PI 5/8 for further separation of the two bands (Figure 6). Fraction 5 contained only transferrin with PIs of 5.8 and 6.0, but fraction 6 and 7 contained other protein bands including transferrin within PI 5.5 to 6.0 (Figure 6, lanes 5, 6, and 7). The Western analysis of isoelectrofocused was not tried since unknown proteins in fractions showed PI values similar to each other, and saposin C antibody did not have special crossreactivity to detect prosaposin.

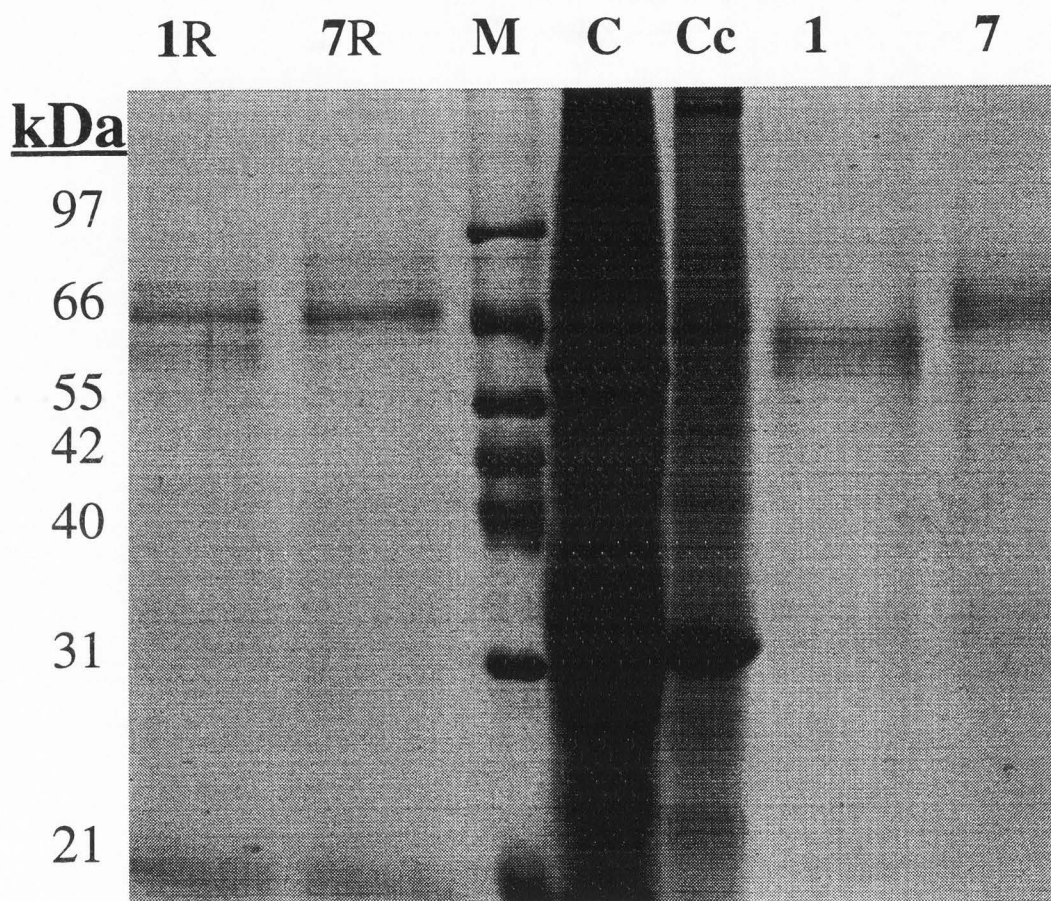


Figure A-1. SDS-PAGE of purified whey samples. All samples are in nonreducing condition but samples in lanes 1R and 7R. Lanes 1R and 7R, preparative electrophoresis purified fractions under reducing condition; lanes 1 and 7, preparative electrophoresis purified fractions; lane C, elution of proteins at pH 7 from ganglioside column; lane Cc, Con-A fraction of lane C sample; lane M, protein standard marker.

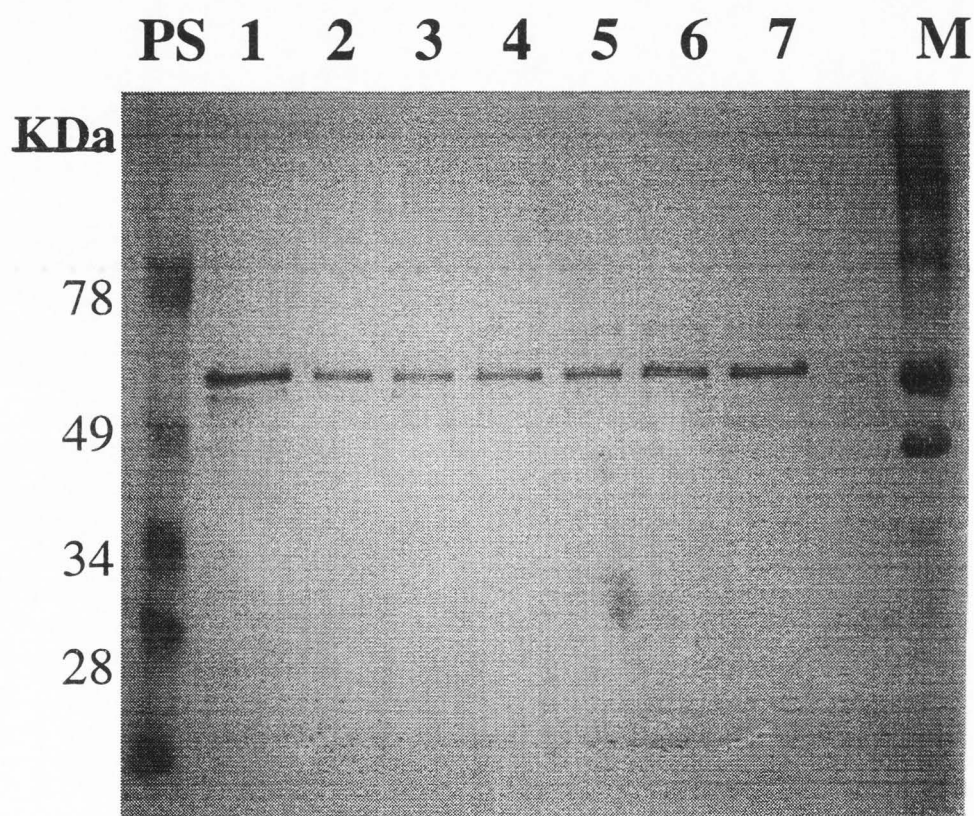


Figure A-2. Western analysis of preparative electrophoresis purified whey sample. Lanes 1-7, preparative electrophoresis purified whey samples were western analyzed using human anti-saposin C antibody; lane PS, prestained protein standard marker; lane M, protein standard marker.

Due to time limitations and the lack of detection equipment for prosaposin, further investigation was terminated. Immobilized gangliosides developed from approach 1 used to purify lactoferrin and transferrin as bioactive proteins, present in milk. Traditional protein separation methods from approaches 2 and 3 contributed mainly to isolate transferrin from bovine whey.

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