ISOLATION OF IMMUNOGLOBULINS FROM CHEESE WHEY AND MILK USING ULTRAFILTRATION AND IMMOBILIZED METAL AFFINITY CHROMATOGRAPHY

by

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B.Sc. (Agr.), The University of British Columbia, 1989

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF

THE REQUIREMENTS FOR THE DEGREE OF

MASTER OF SCIENCE

in

THE FACULTY OF GRADUATE STUDIES

(Department of Food Science)

We accept this thesis as conforming

to the required standard

THE UNIVERSITY OF BRITISH COLUMBIA

September 1992

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Date October 6, 1992

DE-6 (2/88)

ABSTRACT

Cheddar cheese whey was concentrated using an ultrafiltration (UF) membrane with a 50 kDa molecular weight cut-off (MWCO). The concentrated whey was applied to an immobilized metal affinity chromatography (IMAC) column to isolate IgG. With a 50 ml IMAC column loaded with 17.3 ml of 50 mM $CuCl_2$ to get 2/3 Cu saturation, 1240 mg of IgG could be isolated with 56% purity. About 152 mg of lactoferrin and 90 mg of lactoperoxidase could be isolated along with the IgG. By using concentrated whey instead of unconcentrated whey, the time required to apply the whey was reduced proportionally to the concentration factor.

Whey could be concentrated up to 30 times without any decrease in recovery or purity of IgG obtained by IMAC. UF of whey with a 50 kDa or 100 kDa MWCO membrane at 4°C or 45°C did not affect IgG recovery or purity obtained by IMAC. Diafiltration also had no effect. By using 50 mM glycine instead of 1.0 M NH_4Cl in the eluting buffer, elution was more efficient. The type of whey used could affect the recovery and purity of IgG obtained by IMAC. IMAC was run at room temperature or 7°C without any loss in recovery or purity of IgG, but the elution profile was altered. IgG could be recovered from a whey concentrated commercially by UF as well. A method was also developed to isolate IgG from raw skim milk using UF and IMAC.

Ion exchange chromatography was compared to IMAC as an alternative method to isolate IgG from concentrated whey. Ion exchange chromatography gel was about 31 times less expensive than IMAC gel. The capacity of an ion exchange chromatography column for IgG was 16 times lower than for an IMAC column, and the purity of IgG recovered was

lower. Taking the capacity into account, the overall cost of IMAC was about 2 times more than ion exchange chromatography. Considering the high purity of IgG obtainable and the advantages in working with smaller columns, IMAC appeared to be a better method of isolating IgG.

A small-scale shelf life study of IgG in UHT milk was also done. A thermal resistance curve for IgG in UHT milk was constructed assuming first-order destruction of IgG at 62°, 66°, 70°, 74°, 78° and 80°C. D values were obtained from the first-order destruction curves. The D values were used to plot a thermal resistance curve, and D values were extrapolated for temperatures used for the shelf life study. D values extrapolated were 1.77×10^{14} yr at 4°C, 3.85×10^{7} yr at 25°C, and 1.94×10^{5} yr at 35°C. IgG isolated by IMAC was membrane sterilized using prefiltration and final filtration with a 0.2 μm membrane. Membrane sterilization did not affect IgG activity. Membrane sterilized IgG was aseptically injected into 250 ml Tetra Brik cartons containing 2% white UHT milk. The cartons were stored at 4°, 25° and 35°C. Over 5 months of the shelf life study, no change was observed in IgG concentration as predicted.

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ACKNOWLEDGEMENTS

I would like to thank Dr. S. Nakai and Dr. E. Li-Chan for their advice and support for this study.

Thanks to my committee members, Dr. D.D. Kitts, Dr. B.J. Skura and Dr. J. Vanderstoep of the Food Science Department, and Dr. B.D. Owens of the Animal Science Department, for their help. Special thanks to Dr. Skura for all his suggestions regarding the microbial aspects of this study.

I would also like to thank my family and friends for all their support and encouragement. Thank you, Doug, for your support and help.

I would like to express my appreciation to many of the people in the Food Science Department who have made my studies a lot easier and enjoyable. Special thanks to Sherman Yee and Val Skura for their assistance. To the many people who have given me helpful advice, I thank all of you.

Finally, I would like to thank Dairyland Foods for their participation in this project. I would also like to acknowledge the financial support of the Science Council of B.C., the Natural Sciences and Engineering Research Council of Canada, and the Dairy Bureau of Canada.

I. INTRODUCTION

Milk is the main source of nutrition for newborn mammals. Besides providing nourishment, milk also provides passive immunity. Many immunologic agents have been found in the milk of various mammals including humans and cows. Mother's milk is, therefore, the best food for an infant. In cases where mother's milk is not available, infant formulas can meet the nutritional needs of an infant, but are deficient in immunologic activity. Researchers have proposed fortifying formulas with immunologic agents obtained from cow's milk (Goldman, 1989; Nakai, 1991). Immunologic agents in cow's milk include immunoglobulins (Ig), lactoferrin, lactoperoxidase, lysozyme, and xanthine oxidase. Cow's milk immunoglobulins have potential for immunological fortification purposes.

Studies have found ingestion of immunoglobulins, mainly IgG, from cow's colostrum can prevent enterotoxigenic Escherichia coli and rotavirus infection in infants (Hilpert et al., 1977; Ebina et al., 1985; Brüssow et al., 1987). Tacket et al. (1988) found Ig could prevent traveler's diarrhea in adults. Animal studies have found cow's milk immunoglobulins can prevent infections when fed to piglets, calves, lambs and foals (Saif et al., 1983; Bridger & Brown, 1981; Clarkson et al., 1985; Holmes & Lunn, 1991). In many of these studies, pregnant cows were immunized against the specific pathogen studied, and colostrum was used. Yolken et al. (1985) showed that milk from nonimmunized cows has activity against rotavirus. It is likely that milk from nonimmunized cows also has activity against other pathogens. Colostrum has a high concentration of Ig, but is not available in large amounts. Milk or whey would be better sources of Ig since they are available in large volumes and are acceptable for food use. Whey would especially be a good source of Ig since it is a waste

product of casein or cheese manufacture. The concentration of Ig in these sources is low, though, so a method is needed to isolate the Ig. The isolated Ig could be used in infant formulas, animal feeds, or prophylactic treatments for older children or adults against infections.

Many methods exist to isolate immunoglobulins from milk or whey. Precipitation with ammonium sulfate or solvents, affinity chromatography and ion exchange chromatography can all be used. For large-scale extraction of Ig for food use, a method that is fast, simple, relatively cheap, and nontoxic; and gives high purity and yield is needed. Ultrafiltration (UF) and diafiltration have been used to concentrate Ig and remove small proteins in whey to obtain an Ig concentrate. UF is a gentle process which is suitable for large-scale use. A 12% Ig solution can be obtained from whey using UF and diafiltration (Stott and Lucas, 1989). A 12% Ig solution may be adequate for animal use. A higher purity of Ig would be desirable for other applications, since more Ig would be delivered in a smaller dose. In addition, other more abundant whey proteins like β-lactoglobulin can be allergens for some individuals (Taylor, 1986). The functional properties of other whey proteins may also affect the properties of the products the Ig solution is added to. Li-Chan et al. (1990) used immobilized metal affinity chromatography (IMAC), which is also known as metal chelate interaction chromatography (MCIC), to isolate IgG from whey with 75-95% purity. Large volumes of whey had to be used, though, which made the process inefficient.

One objective of this study was to find a method to isolate Ig from whey and milk that was simple and efficient, and could be done on a large-scale. UF and IMAC were selected as methods to use. Another objective was to membrane sterilize the IgG obtained

and aseptically add it to UHT milk. Since membrane sterilization should not affect IgG activity, unlike heat sterilization, this method of sterilization was chosen. Immunoglobulins in milk are heat labile and are denatured by heat at 70°C (Glover, 1985). Commercial systems are also available for aseptically injecting solutions into UHT products prior to packaging. Such a system could be used to aseptically add IgG to UHT sterilized infant formulas or other UHT products. The final objective of this study was to conduct a shelf life study of membrane sterilized IgG in UHT milk to examine IgG stability.

II. LITERATURE REVIEW

A. THE IMPORTANCE OF MILK IN PREVENTING INFECTION

Besides providing nourishment to infants, mother's milk provides protection against infection. Mata and Wyatt (1971) mentioned several studies showing the benefits of human milk for infants. One study mentioned was a British study which found that bottle-fed infants had greater morbidity rates for gastrointestinal disorders, respiratory infections and otitis media than breast-fed infants. The infections also tended to be of longer duration, and mortality was greater for bottle-fed infants. Another study discussed found that the incidence of acute infections, otitis media, febrile upper respiratory infections and acute diarrhea was lower in breast-fed than bottle-fed infants ranging in age from 3 months to one year. Studies have also found that there is a lower incidence of diarrheal diseases during the early months of exclusive breast feeding, but the rate increases as weaning progresses (Mata & Wyatt, 1971). Hanson and Winberg (1972) discussed infections in newborns in relation to feeding. They mentioned a study that found that the administration of unprocessed breast milk to newborns resulted in a rapid cessation of an enterocolitis epidemic caused by Escherichia coli O111-B4. They also mentioned that there is some indirect evidence that breast milk may protect against septicemia and meningitis. Arnon et al. (1982) found infants who died suddenly and unexpectedly from infant botulism were formula-fed at death. In contrast, breast-fed infants were disproportionately represented among cases hospitalized with infant botulism. This suggested human milk may have moderated the severity at onset of infant botulism allowing time for hospitalization, whereas infant formula may have permitted sudden onset and unexpected death (Arnon et al., 1982).

All the above studies were done in populations with adequate sanitation. In

populations where malnutrition and sanitation are problems, breast milk may not have as great of an effect. Studies have shown that if mothers are malnourished, milk volumes tend to decrease and so the protective nature of milk decreases (Hanson et al., 1977). The mechanism by which human milk can offer protection has not been fully established, but many immunologic agents are involved which may promote normal bacterial colonization of the gastrointestinal (GI) tract and suppress the invasiveness of certain pathogenic microorganisms (Hanson & Winberg, 1972).

B. IMMUNITY

In animals, immunity against foreign organisms or toxins is provided mostly by the immune system, but some protection is provided by general processes known as innate immunity. Innate immunity includes phagocytosis by white blood cells and tissue macrophage system cells, destruction by acid secretions and digestive enzymes in the stomach, resistance by the skin, and inactivation of bacteria by chemical compounds like lysozyme and basic polypeptides (Guyton, 1986). Immunity provided by the immune system is known as acquired immunity.

Lymphoid tissue is responsible for acquired immunity. Lymphoid tissue is usually found in lymph nodes located in the spleen, tonsils, adenoids, submucosal areas of the GI tract and bone marrow. There are two basic types of acquired immunity: humoral immunity and cell-mediated immunity (Guyton, 1986). Humoral immunity involves antibodies or immunoglobulins whereas cell-mediated immunity involves activated lymphocytes. The immune system is activated by the invasion of a foreign organism or toxin. Usually there

are one or more specific chemical groups on the foreign object that are different from all other compounds in the host and can elicit a response. These groups that can elicit a response are known as antigens. Most antigens have molecular weights greater than 8000 Da and are often proteins or large polysaccharides.

When an antigen enters the body, lymphocytes in the lymphoid tissue are activated. There are two major types of lymphocytes: T and B. T lymphocytes are preprocessed by the thymus gland. B lymphocytes are preprocessed in some unknown area of the body probably the liver during midfetal life, and in the bone marrow in late fetal life and after birth (Guyton, 1986). Only T and B lymphocytes specific to the antigen are activated. Considering that there are one million or more different types of preformed B and T lymphocytes in the body, there will be a specific lymphocyte to the antigen or a similar antigen. During the preprocessing of lymphocytes, a large RNA molecule, which codes for the portion of the antibody or T cell marker that gives specificity, is cut and spliced resulting in the large number of lymphocytes types. T lymphocytes that are activated by the antigen provide cell-mediated immunity. B lymphocytes specific for the antigen are activated and enlarge to form lymphoblasts. The lymphoblasts differentiate to form plasmablasts which are precursors to plasma cells. Plasma cells produce immunoglobulins which provide humoral immunity. In addition, some new B lymphocytes are formed which are specific for the antigen and are called memory cells. Upon subsequent exposure to the antigen, the memory cells are activated causing a rapid antibody response.

The milk of humans and other mammals contains many immunologic agents which provide passive immunity to the young. Passive immunity can be defined as temporary immunity conferred to a person without exposure to an antigen by transferring antibodies or

lymphocytes from someone or an animal that has been actively immunized against the antigen (Guyton, 1986). In the case of milk, the antibodies or lymphocytes are transferred from the mother to infant via the milk. The mother's immune system produces antibodies and lymphocytes which enter the milk. Arnon et al. (1982) mentioned that the enteromammary immune system is one mechanism by which antibodies can enter milk. In the enteromammary immune system, lymphocytes in the maternal gut-assosicated lymphoid tissue will recognize antigens present in the intestine, migrate to the breast, and there will produce antigen-specific immunoglobulins (sIgA).

C. STRUCTURE AND CHARACTERISTICS OF IMMUNOGLOBULINS (Ig)

Immunoglobulins (Ig) are globulins and glycoproteins which are associated with the γ-globulin fraction of serum (Stewart, 1984). They vary in molecular weights from 150-900 kDa (Guyton, 1986). Immunoglobulins are heterogeneous and can be divided into various classes based on their structural differences. In humans, five classes of immunoglobulins have been characterized: IgG, IgM, IgA, IgD and IgE (Stewart, 1984). Other species have similar classes of immunoglobulins. All immunoglobulins basically consist of 2 heavy and 2 light chains linked by covalent disulfide bonds and other non-covalent forces. A simple diagram of the basic Ig unit is shown in Figure 1. The heavy chains have about 450 amino acid residues with a molecular weight of 50 kDa, and light chains have 220 amino acid residues with a molecular weight of 23 kDa. The N-terminal end of a light or heavy chain is known as the variable region, and the rest of the chain is called the constant region. The variable region's name results from the variability of the

amino acid sequence in the region. The variable region of light and heavy chains is specific for the antigen. The constant region determines much of the physical and chemical properties of the Ig. The arms of the Y-shaped Ig can swing to greater than 100° giving flexibility to the protein. Since Ig has two arms it can bind at two sites. Using papain digestion, Ig can be broken down into 3 fragments. One fraction is called the F_c fragment and there are 2 identical F_{ab} fragments. F_c stands for fragment crystallizable and F_{ab} stands for fragment antigen binding. The F_{ab} fragment binds to the antigen it is specific for. With pepsin digestion, a bivalent antibody fragment is obtained which is called $(F_{ab}')_2$.

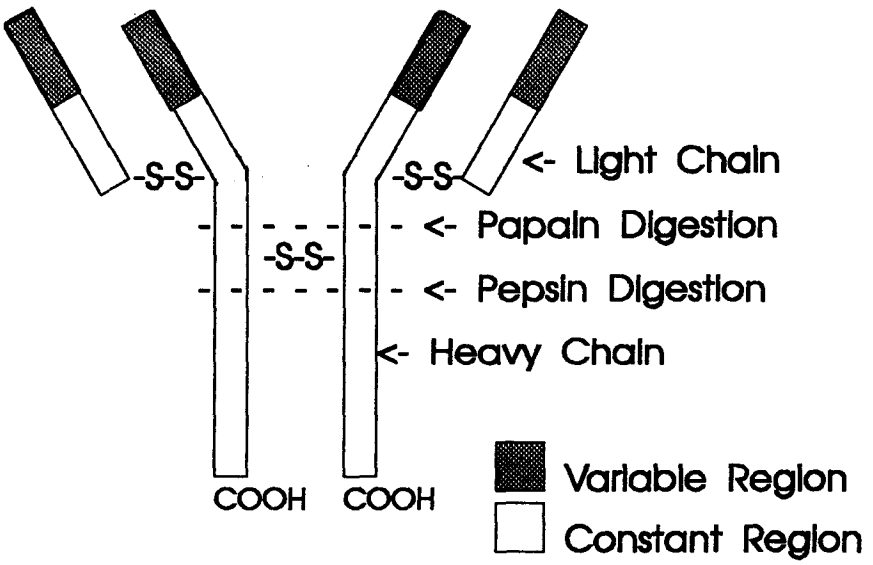

Figure 1. The basic four chain structure of immunoglobulins.

All classes of Ig have the four chain structure with similar light chains, but the heavy chains are specific for each class (Stewart, 1984). γ heavy chain is specific for IgG, α heavy

chain for IgA, μ heavy chain for IgM, δ heavy chain for IgD and ε heavy chain for IgE. IgG consists of the basic monomeric form; and functions to neutralize toxin and viruses, and to bind to and opsonize bacteria. IgG is the dominant class in serum. IgA occurs as a monomer and also as polymers, which are stabilized by inter-monomer disulfide bonds and a J (joining)-chain. In colostrum and milk, about 90% of IgA exists as secretory IgA or sIgA. sIgA is a dimer with one J-chain and an additional antigenically-distinct glucopeptide called secretory component. Secretory component (SC) has a molecular weight of 70 kDa and is synthesized by epithelial cells. SC covers the degradation-sensitive F_c portion of the IgA monomers which makes sIgA resistant to enzymatic degradation and pH changes (Hanson et al., 1977). The structure of sIgA allows it to be a specialized antibody for mucosal defense. It can block the adherence of pathogens to the intestinal mucosa. Studies have found that sIgA has activity against enterobacteria such as E. coli, Vibrio cholerae, Salmonella and Shigella (Hanson et al., 1982). The specificity will be determined by the presence of the bacteria in the maternal environment. IgM consists of 5 monomeric units and one J-chain linked by disulfide bonds. IgM in milk shows activity against gram-negative pathogens such as E. coli and Salmonella (Nakai, 1991). IgD is similar to IgG, but is slightly larger. IgE has a molecular weight of 188 kDa and is heat labile unlike IgM and IgG.

sIgA, IgG, IgM and IgD are all found in human milk. sIgA is found in the greatest concentration at 17 mg/ml in initial colostrum and 1 mg/ml in 4-day colostrum (Mata & Wyatt, 1971). IgG is found at 0.4 mg/ml in initial colostrum and 0.04 mg/ml in 4-day colostrum. IgM is found at 1.6 mg/ml in initial colostrum and 0.1 mg/ml in 4-day colostrum. The decrease in immunoglobulin concentration is due in part to an increase in milk production. However, the higher initial levels probably provide immunity to the infant until

the infant's own immune system becomes established. Jatsyk et al. (1985) found that IgA appeared in the feces of breast-fed infants soon after breast-feeding began. In bottle-fed infants, only 30% had IgA in the feces at 3-4 weeks and only 70% at 1.5 months. This study concluded that an infant's immune system does not start functioning effectively until after the first month of life, so high levels of Ig fed to the infant prior to this time provide protection. Although, studies have shown that Ig concentrations are high in colostrum and then decrease for the first several weeks after the start of lactation; afterwards, the concentrations tend to level off and will remain constant even after a year or more from the start of lactation. Goldman et al. (1982) suggested that the changes in immunologic composition in milk were unique for the early, mid and late phases of lactation, and were being regulated by some as yet unknown controls.

The structure of immunoglobulins provides some clues on how Ig can provide protection against foreign substances, but the actual mechanism is probably more complicated. Packard (1982) noted that immunoglobulins in milk are believed to provide protection by:

1) causing bacteria to agglutinate, so they can be cleansed from the intestinal cavity,
2) interfering with the process by which bacteria adhere and colonize the intestinal lining,
3) aiding other immune agents (opsonization),
4) being involved with further specialized antibody action, namely, complement fixation. Complement is a term to describe a system of about 20 proteins, many of which are enzyme precursors (Guyton, 1986). These proteins can be activated sequentially by antibody-antigen reactions leading to the prevention of damage by the foreign object.
5) neutralizing toxin,
6) killing viruses.

D. OTHER IMMUNOLOGIC AGENTS IN MILK

Immunoglobulins in milk provide specific protection, however, there are other agents

in milk which can provide non-specific protection. In human milk, immunologic agents besides Ig include bifidus growth factor, antiviral lipids, oligosaccharides-glycoconjugates, lysozyme, lactoferrin, neutrophils and macrophages (Goldman, 1989). The bifidus growth factor is a N-containing carbohydrate (methyl-N-acetyl-D-glucosaminide) that promotes the growth of Lactobacillus bifidus (Hanson & Winberg, 1972). The bifidobacteria flora in the GI tract is antagonistic to certain pathogens such as Shigella (Mata & Wyatt, 1971). Antiviral lipids disrupt enveloped viruses (Goldman, 1989). Oligosaccharides-glycoconjugates compete with mucosal receptors for the binding of enteric pathogens and toxins (Goldman, 1989). Lysozyme (muramidase) disrupts the cell walls of susceptible bacteria by hydrolysing peptidoglycans (Goldman, 1989). Lysozyme from human and bovine milk is active against gram-negative and gram-positive bacteria, but chicken egg lysozyme is only active against gram-positive bacteria (Nakai, 1991). Lactoferrin is an iron-binding protein which has a strong bacteriostatic effect on E. coli, Candida albicans and other organisms that require iron. Gram-negative bacteria tend to require more iron than lactobacilli which are beneficial to the human intestinal tract. In this manner, lactoferrin promotes the growth of beneficial microorganisms while destroying harmful ones. The effect of lactoferrin is absent when it is saturated with iron (Hanson & Winberg, 1972). Lactoferrin can resist trypsin, pepsin and chymotrypsin activity in the unsaturated form (Nakai, 1991). Neutrophils and macrophages have phagocytic activity and so can protect against invasion by foreign substances (Hanson & Winberg, 1972). Other immunologic agents in milk are leucocytes which prevent infection in the maternal gland and the infant's GI tract, and a fatty acid resistance factor which is active against staphylococci (Hanson & Winberg, 1972).

E. FORTIFYING INFANT FORMULAS WITH IMMUNOLOGIC AGENTS

The nutritional, physiological, psychological and immunological benefits of breast-feeding indicate mother's milk is the best food for infants. Packard (1982) stated that 90% or more of women should be able to breast feed successfully. However, for the 10% who cannot, and also due to the risk of environmental pollutants, medicines and drugs entering milk, there is a need for infant formulas. Infant formulas are formulated to meet the nutritional requirements of infants, but do not provide any immunologic protection. Researchers have suggested fortifying infant formulas to provide protection. It may also be useful to fortify products for older children or adults, and young animals. Fortifying formulas with immunologic agents may make formulas more closely simulate human milk, but due to the complexity of human milk the formulas still may not meet all needs.

Goldman (1989) gave several principles to use in selecting an immunologic agent. First, the physico-chemical structure should be known, it should be free of contaminants, its immunologic activity should be well characterized, appropriate quantities should be available, its fate should be determined in experimental animal models (dosages should be equivalent to those given to humans, young and older animals should be used, and the route should be the same), and the effect on experimental animals should be determined (the growth, development, general health, GI functions and innate immunologic activities of the experimental animals should not be impaired). There are also potential risks of using immunologic agents which should be examined as well. The risks include acute allergic disorders, other inflammatory reactions, competition for binding sites with endogenously produced factors, and disruption of normal development of the analogous agent or other host resistance factors (Goldman, 1989). It should be emphasized that in infants that are

nutritionally compromised, immunologic agents may not have much effect in preventing infection. If fortifying products with immunologic agents is desirable, sources for these agents would have to be found.

Goldman (1989) suggested using human milk, human serum and bovine milk as possible sources of immunologic agents. Human milk would be an ideal source due to functional compatibility and a lack of allergenicity. However, large supplies of human milk are not available, and human milk can be contaminated with microorganisms or environmental toxins (Packard, 1982). It has been suggested that some low molecular weight agents in human milk could be synthesized in the laboratory or by using recombinant DNA technology. This may not be feasible for complex agents like sIgA, though. Goldman (1989) cited studies that have shown human serum IgG could protect low birth weight infants from rotavirus infection and decrease cases of necrotizing enterocolitis. Another study found feeding human serum IgA-IgG could decrease necrotizing enterocolitis in low-birth-weight infants (Eibl et al., 1988).

Bovine milk would be a more reliable source of immunologic agents since large volumes are available. In addition, cows along with goats, pigs and horses belong to a group of animals which get the most transfer of immunity from the mother to the young after birth (Nakai, 1991). In comparison, a group of animals including humans, monkeys, rabbits and guinea pigs get the most transfer prior to birth. Therefore, cow's milk should be more immunologically complete than human milk. The composition of cow's milk differs from human milk, though, which prevents the direct substitution of cow's milk for human milk. Human milk contains 12.4% total solids, 3.8% fat, 1.0% protein, 7.0% lactose and 0.2% ash. Cow's milk contains 12.7% total solids, 3.7% fat, 3.4% protein, 4.8% lactose and 0.7% ash.

The types of proteins also differ. In human milk, whey proteins make up 60% of total proteins, but whey proteins only make up 20% of total proteins in cow's milk. Human casein is predominantly β-casein with 15% κ-casein and 3% of highly glycosylated phosphoprotein. Bovine casein has 45-55% α_s-casein, 25-35% β-casein and 8-15% κ-casein (Li-Chan & Nakai, 1989). The composition of whey proteins also varies as seen in Table I. Some proteins like α_s-casein and β-lactoglobulin are found in cow's milk but not human milk. These proteins can be potential allergens for a small number of individuals (Taylor, 1986).

There are many potential immunologic agents in bovine milk such as IgG, lactoferrin, lysozyme, xanthine oxidase, lactoperoxidase, and contact blocking viral factor (CVI) (Goldman, 1989). The concentration of lactoferrin and lysozyme are much lower in cow's milk than in human milk as seen in Table I. Bovine apo-lactoferrin is also less resistant than human apo-lactoferrin to trypsin digestion. In addition, if the infant formula is fortified with iron, lactoferrin may have low activity. Xanthine oxidase catalyzes the oxidation of hypoxanthine and xanthine to uric acid. It can also generate atomic oxygen and hydrogen peroxide by the oxidation of biomolecules. Hydrogen peroxide can inhibit bacteria. Lactoperoxidase is found in cow's milk, but not in human milk so it may not be desirable to use. Lactoperoxidase is active against gram-negative and catalase-positive bacteria like E. coli, Pseudomonas fluorescens, P. aeruginosa, Salmonella typhimurium and Klebsiella aerogenes (Nakai, 1991). To be active, lactoperoxidase requires hydrogen peroxide and thiocyanate to form hypothiocynate ions. Thiocynate can come from the milk or stomach, and hydrogen peroxide can be generated by certain bacteria. Hypothiocynate ions have rapid chaotropic effect on the inner membranes of bacteria. CVI acts by reversibly inhibiting the attachment of viruses onto target cells (Goldman, 1989). It is not species-specific and so can

be used against many viruses. CVI is resistant to acid and proteolytic digestion. Of all the potential immunologic agents in cow's milk, the most promising is IgG.

Table I. Whey protein composition in human and cow's milk in mg/ml (Hambraeus et al., 1977; Glover, 1985).

Protein	Human milk	Cow's milk
α-lactalbumin	2.6	0.9
β-lactoglobulin	---	3.0
Lactoferrin	1.5	traces
Lactoperoxidase	---	0.03
Lysozyme	0.5	0.0001
Serum albumin	0.5	0.3
IgA	1.0	0.03
IgG	0.01	0.6
IgM	0.01	0.03

F. BENEFITS OF COW'S MILK IgG

1. Benefits for humans

In contrast to human milk where sIgA is the dominant Ig, IgG is the dominant Ig in cow's milk. IgM and IgA are also present in cow's milk. IgG makes up 85-90% of Ig in milk, IgM makes up less than 10% of Ig in milk, and IgA makes up the remaining portion

(Butler, 1969). In cow's colostrum, the IgG concentration is 50 mg/ml and in milk, it is 0.6 mg/ml (Jenness, 1988). Nakai (1991) stated evidence in the literature indicates IgG can replace IgA in most antibody functions as long as it remains intact.

Gonzaga et al. (1963) did one of the earliest studies to show the ability of cow's milk Ig to prevent infection in infants. Four cows were vaccinated against Type 2 poliovirus. Colostrum was collected from these cows and a group of nonimmunized cows. Forty-three infants who were not being breast-fed were divided into 4 groups and on the 3rd day of life all were fed Type 2 Sabin vaccine. One group was fed colostrum from immunized cows for 24 hrs before and 24 hrs after the vaccine. The second group which served as a control was fed colostrum from nonimmunized cows at the same time intervals. A third group received colostrum from immunized cows 2 hrs after the ingestion of the vaccine while a fourth group received the same colostrum 48 hours after the vaccine. In the first group, 8 of 11 infants showed no sign of virus in the feces. Two of the 11 showed signs of virus on the 13th day after the vaccine while 1 of the 11 showed signs of virus from the 7th to 17th days after the vaccine. In all other groups, virus was detected in the feces. In babies fed colostrum from nonimmunized cows, virus was found in the feces after 3 days in all cases. Giving colostrum from immunized cows after the vaccine did not affect the incidence of infection, but delayed the establishment of the virus or temporarily interfered with its isolation from the feces. It was concluded that the ingestion of bovine colostrum from immunized cows could prevent viral infection. No deleterious effects were seen due to the vaccine or colostrum, except for a milk induced transient diarrhea.

Several studies have shown that the oral intake of bovine immunoglobulins can prevent enterotoxigenic E. coli and rotavirus infection. Enterotoxigenic E. coli frequently

causes infantile gastrointestinal infection. Hilpert et al. (1977) stimulated antibody activity in pregnant cows by actively immunizing the cows with a polyvalent E. coli vaccine. The milk during the first 5-6 days after calving was collected and a colostral whey protein (CWP) mixture was made. The CWP was produced by precipitating casein using rennin, then taking the lactoserum and clarifying it by centrifugation and filtration. The whey was then concentrated using ultrafiltration, diafiltered, sterilized by filtration and lyophilized. The CWP was composed of 75±5% protein of which 40±5% was Ig, 15±5% was α-lactalbumin, 35±5% was β-lactoglobulin, 3±2% was serum albumin and 5±2% were other minor proteins.

Antibody activity was measured in vitro by passive hemagglutination and in vivo by a passive mouse protection test. The mouse test revealed that 100 μg of anti-E. coli CWP protected mice challenged with up to 50 000 colony forming units of E. coli O111:B4 bacteria. Normal CWP from nonimmunized cows gave no protection. In vitro tests revealed that anti-E. coli antibodies inhibited the growth of E. coli. Studies also revealed that Ig with specific anti-bacterial activity can pass through the GI tract of infants without being completely destroyed. Studies revealed the presence of intact Ig as well as fragmented Ig in the feces. $F(ab')_2$ is likely to result from pepsin digestion. Studies showed $F(ab')_2$ exhibited activity, but Fab had a considerable loss in activity. Therapeutic trials of CWP were conducted in Barcelona, Spain and Lille, France. One gram of anti-E. coli CWP/kg body weight/day was fed to infants who were less than 7 months old and suffering from gastroenteritis. Compared to controls fed nonimmunized CWP, the test group showed 48.7% bacteriological and clinical success compared to 27.7% success in controls. The controls showed 67.4% bacteriological and clinical failure compared to 23.1% failure in the test group.

Tacket et al. (1988) assessed the protection of milk immunoglobulin concentrate

against oral challenge with enterotoxigenic E. coli which is a common cause of traveler's diarrhea. A double-blind controlled trial in which a bovine milk immunoglobulin concentrate with high titer of antibodies against enterotoxigenic E. coli serotypes and fimbria types, E. coli heat-labile enterotoxin, and cholera toxin was used as a prophylaxis against E. coli challenge in 20 healthy adult volunteers. Controls were given an Ig concentrate with no anti-E. coli activity. Volunteers were given 3.55 g of Ig concentrate and 550 mg of antacid 3 times a day for 7 days. The antacid was added to prevent breakdown of the Ig in the stomach. No side effects were noted after ingesting the mixture. The concentrate contained 85% protein of which 45% was Ig. On the third day of Ig prophylaxis, the volunteers were given 10^9 colony forming units of enterotoxigenic E. coli (O78:H11). None of the 10 volunteers receiving Ig concentrate against E. coli had diarrhea, but 9 of the 10 controls did. Ig concentrate may be a better alternative to current prophylaxis for traveller's diarrhea such as antimicrobial agents and bismuth subsalicylate. Antimicrobial agents are not ideal due to the possibility of bacteria developing resistance, and bismuth subsalicylate has side effects.

Ebina et al. (1985) immunized 8 pregnant cows with human rotavirus, Wa strain, and obtained colostrum containing neutralizing antibody to human rotavirus (Rota colostrum). Rotavirus accounts for 1/2 of pediatric hospital admissions with acute diarrhea in Western Europe and the U.S., and is a major cause of infectious gastroenteritis throughout the world. Although, the use of a rotavirus vaccine is possible, problems arising with its safety for use in small children makes it questionable. Passive immunity against rotavirus seems to be a better alternative. To test the therapeutic effect of Rota colostrum, 18 patients with acute gastroenteritis were given 20-50 ml of Rota colostrum and 26 were routinely treated. There was no significant difference between controls and treated patients. To test the prophylactic

effect of Rota colostrum, 6 infants were given 20 ml of Rota colostrum each morning and 7 infants were given 20 ml of market milk as a control. After one month, 6 of the 7 infants given milk contracted rotavirus associated diarrhea and 1 of the 6 infants given Rota colostrum developed diarrhea. Rota colostrum significantly protected infants from diarrhea. The Rota colostrum prevented the outbreak of diarrhea, but did not prevent natural immunological responses to the rotavirus infection as noted by rises in complement fixation titers in 2 out of 5 Rota colostrum recipients.

Brüssow et al. (1987) found that pregnant cows could be hyperimmunized against all four human rotavirus serotypes resulting in a 100 times increase in neutralizing milk antibody titers over control milk from nonimmunized cows. Even immunizing against a single human rotavirus strain or with simian rotavirus SA11 could give good neutralizing activity against all serotypes. An immunoglobulin concentrate was made using the method of Hilpert et al. (1977). Hilpert et al. (1987) used the concentrate obtained to therapeutically treat infants hospitalized for acute rotavirus gastroenteritis. The daily dose given was 2 g of concentrate/kg body weight/d for 5 days. There was a significant (P=0.008) reduction in the duration of excretion of virus in infants given a 10% solution with a 1:6000 mean neutralizing titer against 4 human rotavirus serotypes, but not in infants given lower neutralizing titers (1:330 and 1:1100). Approximately 10% of orally administered antiviral activity was recovered from the stools of milk Ig concentrate-treated infants. This suggested that Ig was not totally destroyed in the GI tract of infants.

2. Benefits for animals

Many animal studies using animals such as calves, rats, rabbits, piglets, lambs, and

foals have shown that feeding bovine Ig from cow's milk can provide passive immunity against infection. Saif et al. (1983) collected colostrum from cows immunized with adjuvanted modified-live rotavirus vaccine, or modified-live rota-coronavirus vaccine, and from nonimmunized cows. Samples of these colostrums were supplemented in cow's milk infant formula. The formula was given to 28 newborn, unsuckled, antibody-seronegative, male Holstein calves. The calves were then orally challenged with virulent bovine rotavirus. Eight out of 8 calves given colostrum from the rotavirus vaccine-treated cows were protected from rotavirus diarrhea and shedding at 1% supplementation. None of the 8 controls fed colostrum from nonimmunized cows or 12 calves fed other colostrum showed protection at 1% supplementation. Six calves fed 0.1% supplemental colostrum from rotavirus vaccine-treated cows and 2 calves fed 10% and 50% supplemental colostrum from nonimmunized cows showed partial immunity. Partial immunity was identified as delayed onset and shortened duration of rotavirus-associated diarrhea and virus shedding. Saif et al. (1983) also mentioned that several studies showed that immunization of pregnant cows with E. coli K99 pili antigens can boost colostrum and milk antibody levels, and protect calves against E. coli diarrhea.

In another study, Michalek et al. (1987) hyperimmunized a group of cows with a multivalent vaccine of whole cell antigens of seven strains of Streptococcus mutans. Whey was made from the milk of cows showing the highest serum antibody activity. Gnotobiotic rats were monoinfected with different serotypes of S. mutans and given a caries-promoting diet containing whey (immune and control). Rats given immune whey had lower plaque scores, numbers of streptococci in plaque, and degree of caries activity compared to those given control whey from the milk of nonimmunized cows. Using ion exchange

chromatography, a fraction was prepared from the immune whey which contained high levels of IgG_1 anti-S. mutans antibody activity. 0.1% of the fraction offered similar protection to 100% immune whey. 0.01% of the fraction offered some protection. IgG_1 was therefore established as the active component in the whey. IgG_1 is the major subclass of IgG in cow's milk (Butler, 1969). Michalek et al. (1987) also mentioned that volunteers using immune whey as a daily mouth rinse showed a decrease in the number of S. mutans cells in plaque compared to volunteers using a control whey mouth rinse.

McClead and Gregory (1984) immunized pregnant cows with cholera enterotoxin and collected the colostrum. They found that 3% of total colostral IgG_1 had activity against cholera enterotoxin. Even after exposure to intestinal enzymes, the antibody remained active. The antibody from colostrum inhibited intestinal fluid secretion in infant and adult rabbits exposed to cholera enterotoxin.

Bridger and Brown (1981) fed bovine colostrum from nonimmunized cows to piglets. The colostrum showed rotavirus-neutralizing activity (1:640) against both porcine and bovine rotavirus. The piglets were divided into 3 groups. The first group was fed bovine colostrum for 10 days. The second group was fed colostrum for 10 days and then inoculated intranasally with a porcine rotavirus. The last group did not receive colostrum and was inoculated with rotavirus. The last group developed diarrhea, excreted rotavirus in the feces and died 6 days after infection. The second group remained healthy and developed antibody to rotavirus. Twenty-seven days after the initial inoculation, the first and second groups were inoculated with rotavirus. The first group became clinically ill and excreted rotavirus. The second group remained healthy. It was concluded that the bovine colostrum protected piglets from the clinical effects of porcine rotavirus and allowed the animals to develop immunity

which prevented subsequent infection. This study supports the idea that immunization of cows may not be necessary to get pathogen specific antibodies. In addition, strong cross reactivity of antibodies of one species with pathogens specific to another species was evident.

In other studies, Menšík et al. (1978) found that colostrum from cows immunized with a polyvalent vaccine against enteropathogenic strains of E. coli could give protection to piglets and calves from enteric E. coli infection. Clarkson et al. (1985) immunized pregnant cows with commercial multicomponent clostridial sheep vaccine and collected the colostrum. The colostrum was fed to 12 lambs. The colostrum offered some passive protection to the lambs as indicated by little or no production of antibody when they were vaccinated with clostridial vaccine. Holmes and Lunn (1991) fed bovine colostrum to foals to determine if bovine Ig could be used as an alternative source of Ig for newborn foals. Their study showed bovine Ig was taken up by foals and the mean half-life in the body was 7.4 days. Endogenous Ig production was earlier and more rapid in foals given bovine colostrum. Although, the study showed that bovine Ig was taken up by foals; the protective nature of the bovine Ig was not examined. These human and animal studies along with many others in the literature suggest bovine immunoglobulins can be used to prevent infection without causing any detrimental side effects.

3. Using cow's milk immunoglobulins from nonimmunized cows

In many of the studies mentioned on the benefits of ingesting cow's milk immunoglobulins, colostrum from cows immunized against a particular pathogen was used. Immunization of the cows caused the formation of antibodies specific to an antigen in high titers in the colostrum or milk. In the studies, subjects fed colostrum or Ig concentrate from

immunized cows were protected from infection, but subjects fed controls had no protection. These studies do not prove that immunoglobulins from nonimmunized cows can offer no protection against specific pathogens, since little information was given about the controls used. For example, in the study by Hilpert et al. (1977), there was some indication that the control Ig concentrate may have offered some protection. The study suggested that some infants fed Ig from nonimmunized cows had some protection against enterotoxigenic E. coli infection. This might indicate that there was some activity against E. coli in the colostrum of nonimmunized cows, although the titer may not have been high. No information on the titer against E. coli in the control Ig concentrate was given to substantiate this conclusion, though.

In other studies, controls were chosen that were not representative of immunoglobulins that might be obtained from nonimmunized cows. As a control, Tacket et al. (1988) used an immunoglobulin concentrate made from the milk of cows immunized against human rotavirus and having negligible anti-E. coli activity. By immunizing against human rotavirus, any immunoglobulin activity against E. coli naturally present might be diluted out giving negligible anti-E. coli activity. Ebina et al. (1985) used milk as a control whereas colostrum from immunized cows was used on test subjects. Colostrum has much higher concentrations of Ig than milk. If colostrum from nonimmunized cows was used, low levels of anti-rotavirus activity may have been detected which might have offered some protection. This was not studied, though.

Considering that a cow in any environment would be exposed to a wide variety of antigens, the cow should produce antibodies against a wide range of antigens, so it may not be necessary to immunize cows against specific antigens. In addition, bovine Ig against a

particular bovine pathogen can cross react with similar pathogens of other species like humans. The titers against specific pathogens may be low, though, but the doses needed to provide protection could be determined. It would also be better not to immunize because it is expensive, time critical and may not be ethically acceptable. Several studies have indicated that Ig from nonimmunized cows can offer protection as well.

The study by Bridger and Brown (1981) showed colostrum from nonimmunized cows had rotavirus-neutralizing activity, and that the colostrum could protect piglets against a porcine rotavirus. Saif et al. (1983) noted that 10% and 50% supplementation with colostrum from nonimmunized cows provided partial immunity to calves. Yolken et al. (1985) examined the presence and effectiveness of rotavirus antibody in raw milk, pasteurized milk and infant formulas. Raw and pasteurized milk had detectable levels of IgG_1 antibody directed against rotavirus, but little or no anti-rotavirus antibody was detected in commercially available infant formulas or sterile milk preparations due to the sterilization process. Raw and pasteurized milk was capable of inhibiting replication of simian, bovine and human rotaviruses in tissue culture, and protecting mice from infection and disease in a murine model of rotavirus infection. Antibody titer in raw milk averaged 539 ng/ml and in pasteurized milk was 230 ng/ml. The level of IgG_1 was stable over 14 d at 4°C. It was established that neutralizing activity was exclusively found in fractions containing IgG_1. In mice tests, all 16 untreated animals developed detectable gastrointestinal rotavirus antigen, but none of the 8 fed raw milk did and 1 of the 8 fed pasteurized milk did. Treatment of the mice with IgG_1 fractions purified from raw and pasteurized milk also resulted in a significant decrease in the rate and extent of rotavirus infection. These results also reinforce the idea that immunoglobulins are responsible for viral neutralizing activity.

Stott and Lucas (1989) concentrated immunoglobulins from the whey of nonimmunized cows. They found that immunoglobulins had activity against infectious Bovine rhinotracheitis, Clostridium perfringens, Escherichia coli, Haemophilus somnus, and Salmonella dublin among other organisms. To test the effectiveness of their product in protecting newborn calves, calves were divided into five groups and fed 1 of 5 diets. The diets consisted of milk with near 0 g total Ig ingested, colostrum with 200-720 g of total Ig ingested, 600 g of product with 42 g of total Ig ingested, 200 g of product with 14 g of total Ig ingested, and 100 g of product with 7 g of total Ig ingested. Calves fed milk had a long term health/growth rate much worse than the control group fed colostrum. The group fed 100 g of product was much worse than the control group fed colostrum, but better than the group fed milk. The group fed 600 g of product was better than the control group, and the group fed 200 g of product was as good as the control group. It, therefore, appeared the dose of Ig was important in determining the level of protection. Since calves fed high doses of product with less Ig than colostrum did better than the controls, Stott and Lucas (1989) suggested that the product had antibodies against a greater number of pathogens than the colostrum from any one cow. This seems reasonable since pooled milk was used to make the whey for the product. A similar experiment was repeated under severe weather conditions. In this case, it was shown that higher levels of total Ig ingested from the product (54 g) seemed to give better protection. The product was also fed on a continuous basis as a food supplement to calves. In general, calves fed supplement were healthier and had higher growth rates and lower morbidity than controls not given supplement.

These four studies indicate Ig from the colostrum and milk of nonimmunized cows can provide protection. Titers against particular pathogens are not as high as when

immunization is used, though. By determining dose responses, suitable quantities of Ig could be given to provide protection. Since colostrum is difficult to obtain on a large-scale, milk or whey would be better sources of Ig. Whey especially would be a good source since it is a waste product of cheese or casein manufacture. The concentration of Ig in milk or whey is low compared to that in colostrum. Colostrum has about 50 mg of IgG/ml compared to 0.6 mg of IgG/ml in milk or whey (Jenness, 1988). A method is therefore needed to isolate Ig from milk or whey to get sufficient quantities for fortification purposes. The method should be simple, efficient, relatively cheap and nontoxic, and give high purity and yield. By finding such a method, a solution of immunoglobulins with specific activities against a wide range of pathogens could be blended and added to various products. The Ig could be used in infant formulas, in products for older children or adults as a prophylaxis against infection, or in animal feeds. It might also be possible to use the product for therapeutic purposes.

G. METHODS TO ISOLATE IMMUNOGLOBULINS

Conventional methods to isolate Ig from milk consist of producing whey by acid precipitation of casein. Ammonium sulfate can be used to precipitate Ig from the whey. Ion exchange chromatography or gel filtration can also be used (Butler & Maxwell, 1972). Other methods that can be used are solvent precipitation and affinity chromatography. Precipitation methods are batch processes which are difficult to implement on a large-scale. Affinity chromatography at the present time is expensive due to the high cost of the matrix support and antibody. Other chromatographic methods tend to have low capacity or limited

selectivity. For this study, ultrafiltration (UF) and immobilized metal affinity chromatography (IMAC) were chosen to isolate Ig. Ultrafiltration was chosen since it is suitable for large-scale operations and is a gentle process. IMAC was used since previous studies have found it has high capacity and high selectivity for IgG.

H. ULTRAFILTRATION (UF)

1. UF Theory

Ultrafiltration is defined by Renner and Abd El-Salam (1991) as a pressure-driven membrane process that can be used in the separation and concentration of compounds with molecular weights between 10^3-10^6 Da which corresponds to 0.01-0.02 μm. Pressures between 1-15 bars are used for UF. Most proteins fall within the range for UF. To put UF into perspective compared to other membrane processes, it falls between microfiltration and reverse osmosis. Microfiltration separates particles between 0.1-10 μm and so can separate bacteria and milk fat globules. Pressures less than 2 bars are used for microfiltration. Reverse osmosis separates small molecules and ions less than 1000 Da, or less than 0.001 μm from solvent. The operating pressures for reverse osmosis are greater than 20 bars.

The setup for UF is quite simple. It consists of passing a solution under pressure over a supported membrane usually using a pump. The applied pressure gradient across the membrane causes solvents and components smaller than the molecular weight cut-off (MWCO) of the membrane to pass through. Compounds and solvent that pass through the membrane are termed the permeate or ultrafiltrate. The solution retained by the membrane is termed the concentrate or retentate. The membrane is the most important aspect of an UF

system.

Several types of UF membranes are available. The first UF membranes were made of cellulose acetate and had several limitations. Cellulose acetate could only be used between pH 3-7, it was subject to microbial degradation, it could only be used at temperatures less than 35°C, and certain cleaning agents like chlorine could not be used with it (Glover, 1985). The development of other polymeric membrane materials such as polysulfone, polyacrilonitrile, polyvinylidenefluoride, and polyamide solved many of the limitations of cellulose acetate. Presently, many new types of membranes are appearing on the market. Most UF membranes are made asymmetrical or anisotropic which means the membrane forms a skin on top of a spongy support (Dziezak, 1990). Membranes can be arranged in a number of configurations such as spiral wound, hollow fiber, tubular, plate-and-frame, and pleated cartridge (Dziezak, 1990). The particular choice of membrane material and configuration will depend on the type of solution to be processed. The configurations of UF membranes are designed to give cross-flow filtration, where the feed passes parallel or tangentially to the membrane surface. This prevents severe clogging that occurs in conventional filtration, where the feed passes perpendicular to the membrane surface.

The molecular weight cut-off (MWCO) of an UF membrane will determine what compounds will pass through the membrane. Different manufacturers produce membranes with different MWCOs. Some typical MWCOs are 5, 10, 30, 50, 100 and 500 kDa. The molecular weights of some milk proteins are: 18 277 Da for β-lactoglobulin; 14 174 Da for α-lactalbumin; 66 267 Da for bovine serum albumin; 10^7-10^9 Da for casein micelles; 150 000 Da for IgG; 77 000 Da for lactoferrin; 89 000 Da for lactoperoxidase; and 80 000-900 000 Da for immunoglobulins (Glover, 1985; McL. Whitney, 1988; Yoshida, 1988). Based on

these molecular weights, more abundant whey proteins like β-lactoglobulin and α-lactalbumin should be separable from IgG using an UF membrane with a suitable MWCO. The MWCO of a membrane is somewhat arbitrary and is usually determined using linear dextran molecules. The MWCO therefore tends to be diffuse rather than sharp for proteins which can come in a variety of shapes. Since the MWCO is diffuse some compounds smaller than the MWCO will be retained and some compounds larger than the MWCO will pass through. The ability of a membrane to retain a compound is termed the retention and can be measured from 0-100%. Many membranes have a high retention for proteins which makes separation of proteins using UF difficult.

Renner and Abd El-Salam (1991) and Glover (1985) give detailed explanations on the flow characteristics of a solution through an UF membrane. A simplified explanation is given here. To get efficient UF, the flux or permeation rate should remain high. Flux is measured in kg or liter per membrane area unit (m^2) per time unit (h). The flux for an UF system can range from 30-300 liters/m^2h. The flux can be controlled by changing the inlet pressure or outlet pressure. The transmembrane pressure, which is the pressure gradient that exists through the membrane along the surface of the membrane, should remain below the maximum allowable for the membrane. The transmembrane pressure is approximately equal to the average of the inlet and outlet pressures. Above a certain transmembrane pressure, the flux will not increase further. The temperature of the feed also affects flux. At higher temperatures, flux is greater due to the lower viscosity of the solution. Other factors which affect flux were described by Renner and Abd El-Salam (1991) and Glover (1985). Initially, the flux is high, but decreases during the course of UF due to concentration polarization and fouling. Concentration polarization refers to the formation of a polarized layer or gel layer

at the surface of a membrane which disperses once the system is shut down. Fouling refers to the buildup of a polarized layer which offers resistance and interferes with flux, and does not disperse after shutdown, so the membrane must be cleaned.

UF can be done efficiently by keeping flux high. Permeate can be collected and measured to determine the degree to which the solution is concentrated. Most large-scale UF systems are able to concentrate continuously which increases efficiency. Rather than just concentrating the feed, diafiltration can also be done. During diafiltration, solvent is added to the feed to help remove low molecular weight compounds from the feed. Diafiltration can be done batchwise or continuously.

Since the advent of polymeric membranes, UF has been applied to many industrial processes. In the food industry, UF has been used extensively in the dairy industry. France, Denmark, New Zealand, Australia, Ireland and the U.S.A. are countries using UF commercially for dairy products (Glover, 1985). The main uses of UF in the dairy industry are for preconcentrating milk for cheesemaking and for the manufacture of whey protein concentrates (WPC). Using UF, whey can be concentrated 20-30 times to produce a WPC with a protein concentration of 35-80% dry matter.

2. Using UF to isolate Ig from milk

In many studies using bovine Ig to prevent infection, Ig concentrates were produced using UF. Several patents also describe the use of UF to produce Ig concentrates. Abraham (1988) described a process to prepare Ig against E. coli K-99 antigen from bovine milk. The Ig product could prevent enterotoxigenic E. coli infection in calves. To make the Ig product, pregnant cows were vaccinated with inactivated E. coli strain 168. Colostrum was collected

from these cows. The fat in the colostrum was separated out and the casein was clotted using enzyme. The resulting whey was ultrafiltered with a 22-30 kDa MWCO membrane until 50-70% of the water was removed. The concentrate was then sterilized and lyophilized.

Kothe et al. (1987) described a method to obtain a lactic or colostric Ig solution using UF. Colostrum or milk was acidified to pH 4.0-5.5 and then diluted 2 times with 0.9% NaCl. The mixture was subject to cross-flow filtration through a 0.1-1.2 μm membrane. The permeate was ultrafiltered through a 5-80 kDa MWCO membrane, preferably a 10 kDa MWCO membrane. The solution was concentrated, diafiltered with 0.9% NaCl and concentrated again before being filter sterilized. The resulting solution contained 77.4% Ig of which 85% was IgG when colostrum was used. A 0.5 ml dose of a 5% solution could protect mice from infections after they had been infected with 1×10^7 colony forming units of P. aeruginosa.

The patent by Stott and Lucas (1989) outlined a method to produce an immunoglobulin solution from milk. Raw milk was flash pasteurized. The milk was then prepared for cheesemaking and the whey byproduct was separated out. The whey was filtered at 49-54°C with a 10-100 kDa MWCO membrane. The concentrate was diafiltered and concentrated again. Reverse osmosis could be used to remove some water before the concentrate was lyophilized or spray dried. Spray drying conditions were chosen that would preserve Ig activity. To obtain a higher immunoglobulin concentration, a 50% Ig solution obtained by ion exchange chromatography could be blended in.

Hilpert (1984) also used UF to produce a milk immunoglobulin concentrate (MIC). To make MIC, fat was removed from milk and then casein was removed by acid or rennet treatment. The whey was preconcentrated 3 times, diafiltered with a volume of water equal

to the concentrate and concentrated again using a 6000 Da MWCO membrane. The concentrate was filter sterilized, evaporated and lyophilized. Using milk from the first 30 days of lactation, the MIC had a 75% protein content with 40% being Ig. Of the Ig, 75% was IgG. Hilpert mentions that the high Ig content was in part due to the high Ig content in the milk. The milk contained 0.42% total Ig. Removal of lactose, fat and minerals alone would give a protein fraction containing 10.5% Ig. The removal of casein would then give a protein fraction containing 35% Ig.

These studies show that UF can be used to concentrate Ig present in whey. The studies indicate that even though diafiltration was used, most of the more abundant whey proteins remained, but most lactose and minerals were eliminated. The high retention of proteins may have been due in part to the low MWCO of the membranes used. However, since most UF membranes have high retention of proteins, purifying Ig extensively from milk or whey using UF alone is not feasible at this time. Using diafiltration, large volumes of water would also have to be used which can create problems in disposing the permeate. Depending on the use of the Ig, further purification may not be necessary. However, in some applications, the other proteins may cause problems. For example, if Ig is to be added to infant formula or other nutritional products, the extra protein may upset the balance of nutrients. The other proteins may also affect the functionality of the products they are added to. Some proteins like β-lactoglobulin are also potential allergens for some individuals (Taylor, 1986). By further purifying Ig, more immunologic activity can be obtained in a smaller dose. Li-Chan et al. (1990) used immobilized metal affinity chromatography to obtain a 75-95% pure Ig solution from whey and so, this method was chosen to further purify Ig from whey concentrated by UF.

I. IMMOBILIZED METAL AFFINITY CHROMATOGRAPHY (IMAC)

1. IMAC Theory

In 1961, Helfferich (1961) published a paper on ligand exchange as a separation technique. Helfferich described the method as an ion exchanger containing a complexing metal ion which is used as a solid sorbent. Potential ligands are sorbed from solutions or gases and form complexes with the metal in the resin or displace other ligands that have previously complexed. Ligands that were isolated or separated included ammonia, organic amines, polyhydric alcohols, olefins, acetylene derivatives, anions of organic acids, and amino acids. Porath et al. (1975) described a similar technique called metal chelate affinity chromatography as a method to fractionate proteins in serum. They noted histidine and cysteine can form stable complexes with zinc and copper ions in near neutral aqueous solutions. These amino acids should also form complexes with other ions of transition elements such as Cd, Hg, Co and Ni. Using agarose derivatives, Porath et al. (1975) were able to synthesize chelate forming adsorbents for proteins.

Since many names exist for the technique, Porath and Olin (1983) suggested using the term immobilized metal affinity adsorption (IMA adsorption) to cover all types of interactions between solutes and immobilized metals of whatever form. Ligand exchange chromatography is not a good name since ligand has a different meaning in immunoaffinity chromatography and immunoassays. There is also another mechanism besides ligand exchange that contributes to binding. The term metal chelate affinity chromatography is also restrictive since it only accounts for adsorbents where metal ions have been immobilized by chelate formation. IMA chromatography (IMAC) is a broader term which allows it to include interactions between solutes and immobilized heavy metals regardless whether the metals are

in the form of ions or not. It can include metal attached to supports by ionic or covalent bonds or even metal dispersed as a colloid in an insoluble matrix. The broader definition can also account for the observation that metals frequently exhibit characteristic affinity behaviour independent of their oxidation state. The terms ligand exchange, metal chelate, and immobilized metal ion affinity (IMIA) chromatography could be used if necessary or desirable as subdivisions of IMAC (Porath & Olin, 1983).

Sulkowski (1985) provided an explanation on the theory of IMAC. In aqueous solutions, metal ions are highly solvated due to the coordination of water molecules. The metal ion can be considered as a Lewis acid (electron-pair acceptor), and the water as a Lewis base (electron-pair donor). If the water is replaced by a stronger base, a metal complex results. Ligand (electron donor group/atom) is the term for the group combining with the metal ion. Ligand exchange refers to the replacement of one electron donor group by another. A donor atom (N, O, S) with a free pair of electrons is a simple or monodentate ligand. If two or more donor atoms are present on one ligand molecule, the ligand is polydentate and its coordination with the metal ion results in a metal chelate. The binding of a ligand to a metal ion is much stronger in a metal chelate than in a metal complex, and is referred to as the chelate effect.

IMAC exists as normal or reversed varieties (Sulkowski, 1985). Most IMAC is normal. In normal IMAC, the metal ion is immobilized, usually chelated, on a solid matrix support or liquid carrier. Incoming compound (protein) can bind to the metal ion(s) by electron donor grouping(s) resident on the surface of the compound (protein). In reversed IMAC, the metal ion is immobilized on the surface of the compound (protein). The metal ion can then establish a coordination bond to an electron donor grouping attached to a

support. For normal IMAC, the commonly used chelating ligand for immobilizing metal ions is iminodiacetic acid (IDA). To form a chelating gel, IDA is coupled to a support by a molecular arm. Tris (carboxymethyl) ethylene diamine (TED) can also be used.

IMAC can be used to purify proteins since amino acids form stable chelates with metal ions (Sulkowski, 1985). Both amino and carboxyl groups can participate in forming a chelate. In small peptides, the α-amino group and up to 3 peptide (amide) nitrogens can be involved. The amino acids, cysteine, histidine and arginine, have electron donor atoms in their side chains. Proteins bind to metal ions mainly by amino acid residues with electron donating side chains. Sulkowski (1985) mentioned that Porath postulated that histidine, cysteine and tryptophan residues of a protein were most likely to form stable coordination bonds with chelated metal ions at near neutral pH. Studying protein models, Sulkowski (1985) summarized several points of protein interaction with IMAC. These are:

1) Proteins lacking histidine/tryptophan residues on their surface are not retained on IDA-Cu^{2+} gel.
2) A single histidine residue on a protein's surface is sufficient to retain the protein on IDA-Cu^{2+} at neutral pH.
3) The strength of protein binding to IDA-Cu^{2+} correlates with the available histidine residues.
4) IDA-Cu^{2+} will recognize a tryptophan residue on the surface of a protein, but several tryptophan residues may be needed to bind the protein.
5) Retention of a protein on IDA-Zn^{2+} or IDA-Co^{2+} requires two proximal histidine residues.

There is still little information about the binding of chelated metal ions to proteins even though there is much information about the binding of free metal ions to proteins.

When using IMAC for proteins, there are generally two phases to consider: adsorption and elution (Sulkowski, 1985). For adsorption of protein to the column, a pH where the electron donor grouping(s) on the protein surface is at least partially unprotonated should be used. To elute proteins, protonation, ligand exchange or chelate annihilation can be used.

In protonation, the electron donor grouping(s) on the protein surface are protonated reversing the coordination to the IMAC gel and resulting in protein displacement. In ligand exchange, proteins are eluted by using competing electron donor solutes such as imidazole. In chelate annihilation, a chelating agent is added to release bound proteins by destroying the binding of metal ion to IDA. This method is not recommended since protein will be recovered with poor purity, unless only the protein of interest is bound to the column.

A wide variety of studies on the use of IMAC with proteins have been reported in the literature. Some studies analyzed the interaction of proteins with IMAC columns (El Rassi & Horváth, 1986; Belew et al., 1987; Hutchens et al., 1988; Hutchens & Yip, 1990; Porath, 1990). Other studies have focused on purifying proteins using IMAC (Andersson & Porath, 1986; Andersson et al., 1987; Le Grice & Grüninger-Leitch, 1990).

2. Using IMAC to Isolate IgG from Whey

Al-Mashikhi et al. (1988) used IMAC to separate Ig and lactoferrin from cheese whey. They recovered about 200 mg of IgG/25 ml of Cu-loaded gel from 1 liter of whey. The purity of IgG was 77.2% and 53.0% for acid whey and Cheddar cheese whey, respectively. Using a crude Ig solution, they reported a capacity of 101 mg of IgG/ml of Cu-loaded gel. Li-Chan et al. (1990) modified the method of Al-Mashikhi et al. (1988) by using competitive displacement of cheese whey proteins. They found that 100 mg of IgG could be recovered per ml of Cu-charged bed volume with 75-95% purity. Li-Chan et al. (1990) noted that the order of displacement of proteins in cheese whey was β-lactoglobulin, α-lactalbumin, bovine serum albumin, lactoperoxidase-lactoferrin, and Ig. Therefore, by continuously applying cheese whey to an IMAC column, Ig would competitively displace less tightly bound

proteins. Porath and Olin (1983) noted a similar effect when increasing the amount of serum applied to an IMAC column. By increasing sample load, the total amount of proteins adsorbed to the column increased. However, the amount of material adsorbed relative to that applied (expressed as a percent) decreased as the sample load was increased. This indicated that the adsorbent had varying affinities for different compounds present in serum. With an increase in sample load, the components with higher affinity for the adsorbent displaced those with lower affinity.

Li-Chan et al. (1990) studied the effect of pH, volume of whey applied, Cu loading, equilibrating buffer and eluting buffer on the capacity of the IMAC column for IgG. They found applying whey at pH 6.0 improved the binding and capacity of the column for IgG. Whey was applied to the column in a sufficient volume to lie between the breakthrough and saturation points for IgG binding. The breakthrough point is the volume or IgG content when IgG is first detected in the unbound fraction. The saturation point is the volume or IgG content when the IgG concentration in the unbound fraction is equivalent to that in the applied whey. When volumes of whey less than the breakthrough point were applied, IgG purity and recovery were low. Between the breakthrough and saturation points, there was little change in IgG capacity or purity. By increasing Cu load, the capacity of the column for IgG increased. When large volumes of whey were applied, though, immobilized Cu ions moved downwards. To prevent Cu leakage, the Cu load had to be adjusted. Tris acetate or sodium phosphate buffers or distilled water could be used as equilibrating buffers. NaCl in the equilibrating buffer did not increase capacity for protein adsorption, possibly because the ionic strength of whey was high. Use of a pH gradient from 6.1 to 2.8 to elute bound proteins resulted in the loss of IgG activity. Ig is unstable below pH 3 to 4 (Li-Chan et al.,

1990). Competing ligands such as NH_4Cl, glycine and imidazole were found to be effective in the eluting buffer. Although the recovery and purity of IgG obtained from whey were high using IMAC, the method was time-consuming since large volumes of whey had to be applied.

III. MATERIALS AND METHODS

A. ULTRAFILTRATION (UF)

1. Preparation of Samples for UF

Cheddar cheese whey (CCW) and white CCW were obtained from Dairyland Foods (Burnaby, B.C.). The whey was frozen in 3 liter batches and stored at -25°C until needed. The pH of the whey was adjusted to 6.0 with 1.0 N HCl or 1.0 N NaOH. pH measurements were made using a Fisher Accumet pH meter Model 620 (Fisher Scientific Co., U.S.). The whey was centrifuged at 10 000 × g for 15 minutes at 5°C to remove insoluble matter. When whey was to be concentrated 10 times or more, 5 liters of whey was used so the final volume of concentrate was greater than the minimum recirculation volume of 100 ml for the UF system. Since foaming became a problem below 200 ml, the volume of concentrate was kept above 200 ml if possible. Foaming can cause denaturation of proteins, including immunoglobulins.

Raw milk obtained from the University of British Columbia Dairy Unit was chilled to 5°C and then centrifuged at 4000 × g for 30 minutes at 5°C to remove fat. The skim milk was used for UF or was separated into whey and casein by acid precipitation of the casein. Whey was made by adjusting the pH of the skim milk to 4.6 using 1.0 N HCl and heating to 30°C for 10 minutes. Casein was removed by filtering through cheesecloth. The resulting whey was adjusted to pH 7.0 with 1.0 N NaOH.

2. Concentration Mode

Ultrafiltration was carried out using the CPS-1 Cartridge Pumping System with 8 inch

Harp™ hollow fiber ultrafiltration (UF) membrane cartridges purchased from Supelco Canada Ltd. (Oakville, ON). The CPS-1 Cartridge Pumping System consisted of a Masterflex peristaltic pump drive (Model No. 7520-00), Masterflex pump head (Model No. 7018-20), and Norprene tubing (Model 6404-18). All were from Cole Parmer Instrument Co. (Chicago, IL). The pump was linked to a UF membrane cartridge using a cartridge adapter kit. The cartridge adapter kit consisted of Delrin end caps, tubing ports and backpressure regulating valve. The backpressure regulating valve was used to control recirculation rate and transmembrane pressure. Pressure gauge assemblies were attached to the inlet and outlet caps of the cartridge adapter kit to measure inlet and outlet pressures. The Harp™ hollow fiber UF membrane cartridges were 20.3 cm in length with fiber inner diameter of 1.1 mm, and had a total membrane surface area of 0.03 m^2. Three cartridges were available with molecular weight cut-offs (MWCO) of 50, 100 and 500 kDa (Models HF0.3-43-PM50, HF0.3-43-PM100 and HF0.3-43-PM500, respectively).

The UF processing solution was heated to 45-50°C in a waterbath, and processed at an average transmembrane pressure of 14 psi, recirculation rate of 1800 ml/min and permeate flow rate of approximately 15 ml/min, unless otherwise stated. The permeate (ultrafiltrate) was collected while the concentrate (retentate) was recirculated. UF was continued until the desired concentration factor was reached. The concentration factor (CF) was calculated as: CF = initial volume/(initial volume - volume of permeate).

The UF system was cleaned with 0.1 N HCl according to the manufacturer's recommendations (Anonymous, 1989). The membrane was determined to be adequately cleaned once water flux was restored. Membranes were stored in 0.1% (w/v) sodium

benzoate at a slightly acidic pH to prevent microbial growth.

3. Diafiltration Mode

To run the UF system in diafiltration mode, the setup suggested by the manufacturer was used (Anonymous, 1989). The figure below illustrates the setup.

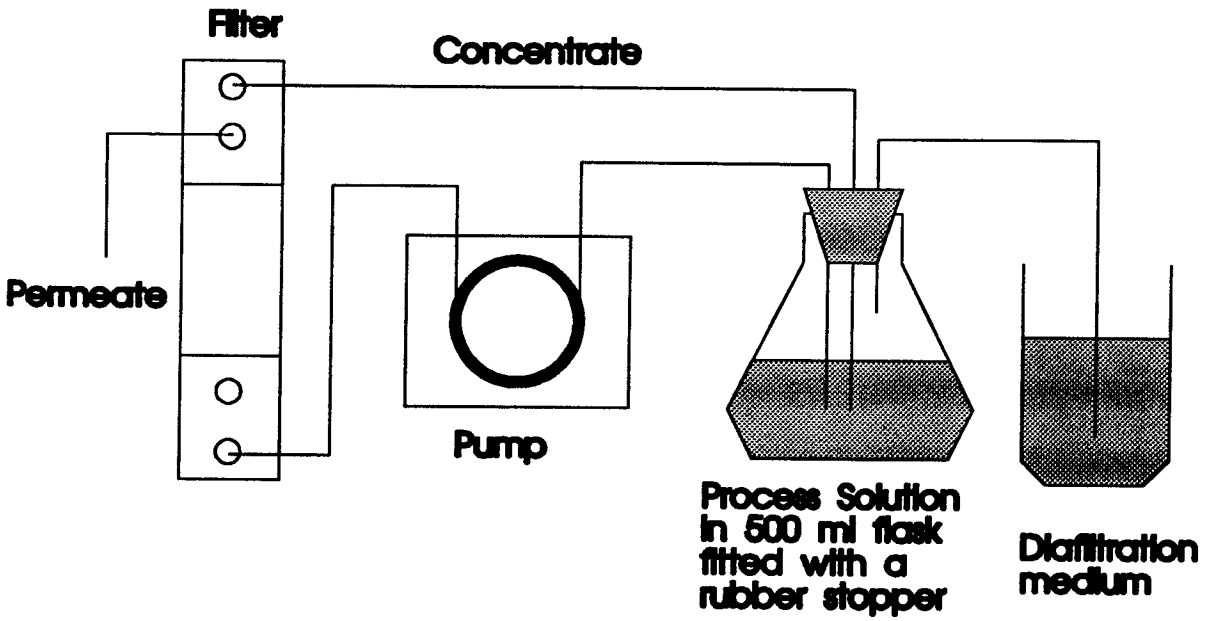

Figure 2. Setup for diafiltration.

Using this configuration, continuous diafiltration occurred. A vacuum, which formed in the flask containing the solution being processed, caused diafiltration medium to enter the system at the same rate that permeate was extracted. Glover (1985) stated that continuous diafiltration is more effective than batchwise diafiltration.

B. IMMOBILIZED METAL AFFINITY CHROMATOGRAPHY (IMAC)

The IMAC method of Li-Chan et al. (1990) was used. Chelating Sepharose Fast Flow (CSFF) and Chelating Sepharose 6B (CS6B) from Pharmacia Inc. (Uppsala, Sweden) were packed into columns with inner diameters of 1.4 cm and 2.5 cm for bed volumes of 10 ml and 50 ml, respectively. CSFF consists of iminodiacetic acid (IDA) groups on spacers coupled by stable ether linkages to highly cross-linked 6% agarose. CS6B consists of IDA coupled to Sepharose 6B after epoxy activation. The upper half to two-thirds of the columns were loaded with Cu using 50 mM $CuCl_2$. The bright blue colour of the column indicated Cu-loaded gel. Loading a 10 ml column with 3.0 ml of 50 mM $CuCl_2$ caused the column to be 1/2-3/5 Cu-loaded. Loading a 50 ml column with 17.3 ml of 50 mM $CuCl_2$ caused the column to be 2/3 Cu-loaded. The manufacturer specified that CSFF had a total capacity of 22-30 μmol Zn^{2+}/ml of gel. Hutchens et al. (1988) mentioned that the capacity for CSFF was 31-40 μmol Cu^{2+}/ml of gel. After the column was loaded with Cu, unbound Cu ions were removed by washing with distilled water. The column was then equilibrated with 5-6 column volumes of 50 mM sodium phosphate buffer at pH 6.0.

Sample was applied to the equilibrated column. A sufficient volume of sample was applied to the column to lie between the breakthrough point and saturation point for IgG binding. Li-Chan et al. (1990) defined the breakthrough point as the volume of applied sample (or corresponding IgG content) at which IgG was first detected in the unbound fraction. The saturation point was defined as the volume of applied sample (or corresponding IgG content) at which the IgG concentration in the unbound fraction was equivalent to that in the sample being applied. The saturation point was also referred to as the exhaustion

concentration when the column bed is judged ineffective. Ideally, the column should be loaded to the saturation point. However, there was no fast and simple method to determine when the saturation point was reached when a mixture of proteins was applied. Based on the estimated capacity of the IMAC column, sufficient volume was applied to at least pass the breakthrough point. Li-Chan et al. (1988, 1990) estimated the capacity for CSFF was 60 mg of IgG/ml of Cu-loaded gel, and for CS6B was 100 mg of IgG/ml of Cu-loaded gel. From preliminary trials in this study, the estimated capacity for CSFF was 38 mg of IgG/ml of Cu-loaded gel, and for CS6B was 42 mg of IgG/ml of Cu-loaded gel.

Before concentrated whey was applied to the column, it was centrifuged at 4000 × g for 30 minutes at 5°C, and then filtered through absorbent cotton to remove fat and insoluble matter. This reduced problems with fat binding to the column, but did not affect binding of IgG. The sample was applied at a flow rate of 0.4-0.5 ml/min for the 10 ml column, and 1.0-3.3 ml/min for the 50 ml column using a peristaltic pump Model SJ-1211 (Atto Chromatograph, Japan).

After the sample was applied, the column was washed with 50 mM sodium phosphate buffer at pH 6.0 to remove unbound or weakly bound proteins. The protein content was monitored manually by measuring the absorbance at 280 nm with a spectrophotometer Model UV-160 (Shimadzu Corp., Kyoto, Japan). Once the absorbance fell below 0.2, washing was stopped and elution was started. To elute bound proteins, either 1.0 M NH_4Cl or 50 mM glycine in 50 mM sodium phosphate buffer at pH 8.0 was used. Eluted fractions were collected using a fraction collector. The protein content of eluted fractions was monitored manually by measuring the absorbance at 280 nm. When absorbance fell below

0.6, elution was stopped. The column was then eluted with distilled water until the absorbance decreased below 0.6. To regenerate the column, Cu ions were stripped off with 0.033 M EDTA. 50 mM NaOH was used to remove any remaining protein or fat. The column was rinsed thoroughly with distilled water. If the column was not used for an extended period of time, the column was stored in 20% ethanol. The column was run at room temperature unless otherwise stated.

The various fractions collected from the column were dialyzed against distilled water using Spectra/Por Molecularporous dialysis tubing with a molecular weight cut-off of 6-8 kDa (Spectrum Medical Industries, Inc., Los Angeles, CA). The fractions were analyzed using SDS-PAGE, radial immunodiffusion (RID) assay for IgG activity and lactoferrin activity, biuret protein determination, Kjeldahl protein determination, and 2,2'-azino-di-(3-ethyl-benzthiazolinsulfonic acid) diammonium salt (ABTS) assay for lactoperoxidase activity.

C. CONDUCTIVITY

The conductivity of whey and milk was measured before and after ultrafiltration using a Model 31 conductivity bridge with a #3403 conductivity cell from Yellow Springs Instrument Co., Ltd. (Yellow Springs, OH). The temperature of the solution was measured, and the conductivity was corrected for a temperature of 18°C. A 2% difference in conductivity for every degree difference from 18°C was assumed (Davis, 1951). Conductivity was 2% higher for every degree difference above 18°C, and conductivity was 2% lower for every degree difference below 18°C.

D. LACTOSE DETERMINATION

The lactose content of whey and milk before and after UF was determined using the phenol-sulfuric acid method of Lawrence (1968). Briefly, the method consisted of diluting the sample by a factor of 1000 with water and preparing lactose standard solutions varying in concentration from 0-100 μg/ml. An aliquot of 1.0 ml of diluted sample or standard was mixed with 1.0 ml of 5% phenol solution and then 5.0 ml of concentrated sulfuric acid was added. The mixture was vortexed and allowed to cool at room temperature. The absorbance of the sample was measured in a 1 cm glass cell using a spectrophotometer at 490 nm. All standards were done in triplicate, and all samples were done in quadruplicate. A standard curve of absorbance versus lactose concentration was constructed using the standards. Using simple linear regression, the equation of the standard curve was determined, and the lactose concentration of samples was calculated.

E. SODIUM DODECYL SULFATE-POLYACRYLAMIDE GEL ELECTROPHORESIS (SDS-PAGE)

1. Preparing Samples

To prepare samples for SDS-PAGE, 0.1 ml or 1 mg of sample was combined with 0.7 ml of 10 mM sodium phosphate buffer at pH 7.0, 0.2 ml of 10% SDS in 10 mM sodium phosphate buffer at pH 7.0, 50 μl of 0.05% bromophenol blue tracking dye, and 20 μl of β-mercaptoethanol. If 0.1 ml of sample contained less than 1 mg of protein, the volumes of sample and buffer were adjusted to get 1 mg of protein in the final volume. The prepared samples were heated 5-10 minutes in a boiling waterbath and allowed to cool to room

temperature.

2. Running and Staining Gels

SDS-PAGE was done using the PhastSystem electrophoresis unit. PhastGel gradient 10-15% acrylamide gels with 2% cross-linking and PhastGel SDS buffer strips (0.55% SDS) were obtained from Pharmacia Inc. (Uppsala, Sweden). A sample volume of 1.0 µl was applied to the gel using a PhastGel 8/1 sample comb applicator from Pharmacia Inc. (Uppsala, Sweden). The gels were run at 250 V, 10 mA, 15°C for 63 volt hours.

The gels were Coomassie or silver stained using the PhastSystem protocol. Coomassie staining consisted of staining with 0.1% PhastGel Blue R solution in 30% methanol and 10% acetic acid, destaining in 30% methanol and 10% acetic acid, and preserving in 10% acetic acid and 5% glycerol. Silver staining consisted of washing in 10% ethanol and 5% acetic acid, sensitizing in 8.3% glutaraldehyde, washing with distilled water, staining with 0.25% $AgNO_3$, developing with 0.04% formaldehyde in 2.5% Na_2CO_3, stop bathing in 5% acetic acid, and preserving in 10% acetic acid and 5% glycerol.

2. PhastImage Gel Analyser

The PhastImage Gel Analyser from Pharmacia Inc. (Uppsala, Sweden) was used to analyze bands on SDS-PAGE gels to determine IgG concentration. Different concentrations of IgG standard were run along with samples for SDS-PAGE. Bovine IgG from serum (Sigma Chemical Co., St. Louis, MO) was used as a standard. The gels were scanned by the PhastImage Gel Analyser. The PhastImage Gel Analyser program was used to calculate the

slope volume of the IgG heavy chain band. The slope volume is defined as the volume of the band down to the baseplane indicated by the contours around the band. A standard curve of slope volume of IgG heavy chain band versus IgG concentration was calculated using simple linear regression. The IgG concentration of the samples was determined using the standard curve.

F. RADIAL IMMUNODIFFUSION (RID) TECHNIQUES

1. RID for IgG Activity

RID plates were prepared according to Ingild (1983) with modifications. Briefly, RID plates were prepared by combining 0.06-0.18 ml of anti-bovine IgG (whole molecule) developed in rabbit (Sigma Chemical Co., St. Louis, MO or ICN ImmunoBiologicals, Lisle, IL) with phosphate buffered saline (PBS) (0.15 M NaCl in 10 mM sodium phosphate at pH 7.0) to make a total volume of 2.0 ml. This solution was equilibrated in a waterbath at 55°C. Fifty mg of agarose Type IV (Sigma Chemical Co., St. Louis, MO) was mixed with 4.6 ml of PBS and 0.4 ml of 0.35% NaN_3, and boiled to dissolve the agarose. This mixture was also equilibrated in a 55°C waterbath. The agarose and antiserum solutions were combined and poured into a leveled radial immunodiffusion plate (ICN, Cleveland, OH) after removing air bubbles. The plate was allowed to solidify at room temperature for 15 minutes and was stored in a moist chamber at 4°C.

The plates were prepared for sample application by cutting 3.0 mm diameter holes into the agarose using a RID template (ICN, Cleveland, OH). A sample volume of 3.0 µl was

applied to each well. Duplicates were made for each sample. Bovine IgG from serum (Sigma Chemical Co., St. Louis, MO) was used as a standard. The bovine IgG was solubilized in 10 mM sodium phosphate buffer at pH 7.0 and the absorbance of the solution was measured at 280 nm. The concentration of IgG was determined using an extinction coefficient of 13.7 for a 1% solution in a 1 cm cell at 280 nm (Fasman, 1976). IgG solutions of varying concentrations were prepared from an IgG solution of known concentration. Five different concentrations of IgG standard were used in each RID plate. For a plate with 0.18 ml of antiserum, the standards varied in concentration from 0.5-1.5 mg/ml. For a plate with 0.06 ml of antiserum, the standards varied in concentration from 0.1-0.5 mg/ml. The RID plates were incubated at room temperature in a moist chamber until there was no further increase in precipitin ring diameter. This usually took about 48 hrs. The ring diameters were measured using a micrometer. Four measurements at various orientations were taken for each ring, and the average was calculated. A standard curve was plotted with the square of the diameter of precipitin ring versus IgG concentration. Using simple linear regression, the equation of the standard curve was calculated, and the IgG content of samples was computed.

2. RID for Lactoferrin Activity

a. Immunization Procedure for Chickens

Antibodies against bovine lactoferrin were developed in chickens. Three laying white leghorn hens from the University of British Columbia Poultry Unit were obtained. The chickens were housed and fed under normal conditions. The chickens received an initial injection of bovine lactoferrin solution in Freund's complete adjuvant (FCA). To prepare the

initial injection solution, a 5.0 mg/ml solution of bovine lactoferrin from milk (Sigma Chemical Co., St. Louis, MO) in PBS (0.15 M NaCl in 10 mM sodium phosphate buffer at pH 7.0) was prepared. Using an equal volume of Freund's complete adjuvant, the lactoferrin solution was added dropwise to the adjuvant while vortexing the adjuvant to form a water-in-oil emulsion. Each chicken was injected intramuscularly with 0.25 ml of the emulsion at four sites on the breast muscle to give a total injection of 1.0 ml.

After two weeks, the chickens were given a second injection using lactoferrin solution in Freund's incomplete adjuvant (FIA) (Difco, Detroit, MI.) using the same protocol. The same procedure was used to prepare the emulsion with FIA as with FCA. A third booster of lactoferrin in Freund's incomplete adjuvant was given after three months to maintain anti-bovine lactoferrin IgY levels.

b. Isolation of Anti-bovine Lactoferrin IgY from Egg Yolk

IgY was isolated from egg yolk using the method of Akita and Nakai (1992). The method consisted of separating the yolk from the white and rolling the yolk on a paper towel to remove any remaining egg white. Using a needle, the egg yolk membrane was punctured and the contents of the yolk without the vitelline membrane were collected. The egg yolk contents were diluted with 6-10 volumes of water acidified to pH 2.7-2.8 with 0.1 N HCl. The pH of the mixture was then adjusted to pH 5.0-5.2 using 0.1 N HCl or 0.1 N NaOH. The mixture was held at 4°C for at least 2 hrs and then centrifuged at 10 000 x g for 45 min at 5°C. The precipitate containing lipid was discarded. To the slightly cloudy supernatant containing IgY, $(NH_4)_2SO_4$ was added to a concentration of 33% (w/v). The IgY solution was

allowed to stand a minimum of 1 hr, or overnight at 4°C before being centrifuged at 10 000 × g for 30 min at 5°C. The supernatant was discarded and the precipitate containing IgY was collected. Water was added to the precipitate, and then the solution was dialyzed against water using Spectra/Por Molecularporous dialysis tubing with a molecular weight cut-off of 6-8 kDa. Finally, the dialyzed IgY was lyophilized.

c. Preparing RID Plates for Lactoferrin Activity

To prepare the anti-bovine lactoferrin IgY for RID, the lyophilized IgY was solubilized with a volume of PBS (1.5 M NaCl in 10 mM sodium phosphate buffer at pH 8.0) equal to 1/10 the initial egg yolk volume. This volume was chosen based on the results of Meisel (1988). Meisel prepared anti-bovine lactoferrin IgY from eggs and diluted IgY with 1/10 the initial yolk volume for use with RID. Preliminary RID trials also indicated that the anti-lactoferrin IgY activity of the isolated IgY was not high, so diluting with 1/10 the initial egg yolk volume would be suitable.

RID plates for lactoferrin activity were prepared in a similar manner as RID plates for IgG activity, except the composition of the PBS was changed. Papers in the literature have cited that IgY requires high salt concentrations to produce a precipitin ring (Crowle, 1973; Meisel, 1988). RID plates were prepared by combining 0.20 ml of anti-bovine lactoferrin IgY solution with PBS (1.5 M NaCl in 10 mM sodium phosphate at pH 8.0) to make a total volume of 2.0 ml. This solution was equilibrated in a waterbath at 55°C. Fifty mg of agarose Type IV was mixed with 4.6 ml of PBS and 0.4 ml of 0.35% NaN_3. The mixture was boiled to dissolve the agarose and then equilibrated in a 55°C waterbath. The agarose

and antiserum solutions were combined and poured into a leveled radial immunodiffusion plate after removing air bubbles. The plate was allowed to solidify at room temperature for 15 minutes and was stored in a moist chamber at 4°C.

The plates were prepared for sample application by cutting 3.0 mm diameter holes into the agarose using a RID template. A sample volume of 3.0 µl was applied to each well. Duplicates were made for each sample. Bovine lactoferrin from milk (Sigma Chemical Co., St. Louis, MO) was used as a standard. The lactoferrin was solubilized in 10 mM sodium phosphate buffer at pH 7.0 and then the absorbance of the solution was measured at 280 nm. The concentration of lactoferrin was determined using an extinction coefficient at 1% and 1 cm of 15.1 (Fasman, 1976). At least 5 standards ranging in concentration from 0.2-2.4 mg/ml were used for each RID plate. The RID plates were incubated at room temperature in a moist chamber until there was no further increase in precipitin ring diameter. This usually took about 48 hrs. The ring diameters were measured using a micrometer. Four measurements at various orientations were taken for each ring, and an average was calculated. A standard curve of diameter of precipitin ring squared versus lactoferrin concentration was made. Using simple linear regression, the equation of the standard curve was calculated and the lactoferrin content of the samples was determined.

G. PROTEIN DETERMINATION

1. Biuret Method

Protein concentration was measured by the biuret method using Sigma Diagnostics

Total Protein Reagent (Sigma Chemical Co., St. Louis, MO). The recommended protocol was modified, so readings could be made with an ELISA reader. Bovine serum albumin (BSA) protein standard (Sigma Chemical Co., St. Louis, MO) was used for the standard curve. The BSA standard was diluted to produce standards containing 2.0, 4.0, 6.0, 8.0 and 10.0 mg of protein/ml with 10 mM sodium phosphate buffer at pH 7.0. If necessary, samples were diluted with 10 mM sodium phosphate buffer at pH 7.0, so the protein concentration would lie between 0.0-10.0 mg/ml. In an Immulon 2 flat bottomed, polystyrene microtitre plate (Dynatech Laboratories Inc., Chantilly, VA), 50 µl of sample or standard was added to a well in triplicate. The procedure was repeated in a second ELISA plate. Total protein reagent (200 µl) was then added to the samples in one plate, while 200 µl of 0.6 N NaOH was added to the samples in the other plate. 0.6 N NaOH was used since the reagent consisted of 0.6 M NaOH, 12 mM copper sulfate, 31.89 mM sodium potassium tartrate, and 30.1 mM potassium iodide. The plates were allowed to incubate at room temperature for 10 min. The plates were then read at 550 nm in an ELISA reader Model EAR 400 (SLT-Labinstruments, Austria) using distilled water as the blank. Error caused by the cloudiness of a sample, if present, was corrected for by subtracting the readings of the samples with 0.6 N NaOH from the readings of the samples with reagent. Simple linear regression was used to calculate the equation of the standard curve of absorbance versus concentration of BSA standards, and the protein concentration of the samples was determined.

2. Kjeldahl Method

A volume of sample containing 6-10 mg of protein was added to a micro-Kjeldahl

flask. The sample was dried in an oven at 60°C for 1 day or until dry. The dry sample containing 6-10 mg of protein was digested using the micro-Kjeldahl method II of Concon and Soltess (1973). The nitrogen content of the samples was determined using a Technicon AutoAnalyzer II system (Technicon, Elmsford, NY). Protein content in the sample was calculated using N x 6.38.

H. LACTOPEROXIDASE ASSAY

The lactoperoxidase content of fractions was determined using the method described in the Sigma Chemical Co. (St. Louis, MO) Bulletin No. 8-84. The method was based on measuring the change in absorbance per minute of the reaction:

$$H_2O_2 + DH_2 \xrightarrow{\text{lactoperoxidase}} 2H_2O + D$$

where DH_2 is a colourless dye and D is a dye. In this assay, 2,2'-azino-di-(3-ethyl-benzthiazolinsulfonic acid) diammonium salt (ABTS) served as the colourless dye.

To perform the assay, the test sample was diluted with cold 0.2 M K_2HPO_4 solution. For a 5.0 mg/ml enzyme solution, the dilution ratio is 1:20 000. A volume of 0.02 ml of diluted sample was added to a mixture of 2.20 ml of 0.1 M potassium phosphate buffer (pH 5.5), 0.70 ml of 100 mM ABTS solution, and 0.10 ml of 30% H_2O_2 solution. The absorbance at 436 nm of the mixture was measured 30, 60, 90, 120 and 150 s after the start of the reaction. The average change in absorbance (abs) per minute was calculated and the volume activity was calculated as:

volume activity = 3.02/(29.3 x 1 x 0.02) x Δabs/min (units/ml of sample).

The Δabs/min should be between 0.005-0.015. To convert volume activity of lactoperoxidase to mg of lactoperoxidase/ml, a conversion factor of 250 units/mg of lactoperoxidase was used as stated in the specifications sheet for lactoperoxidase (Sigma Chemical Co., St. Louis, MO).

I. ION EXCHANGE CHROMATOGRAPHY

1. Column Preparation

Sixty ml of carboxymethyl (CM) cellulose grade 2 (HC-2) from Phoenix Chemicals Ltd. (Christchurch, N.Z.) was packed into a column with an inner diameter of 2.6 cm. The column was equilibrated with 6 column volumes of 50 mM citric acid-sodium citrate buffer at pH 5.4 or 50 mM citric acid-sodium citrate buffer at pH 5.0. Electrodialyzed concentrated whey was applied to the column at a flow rate of 2.0 ml/min. After applying the sample, the column was washed with 10 mM buffer at pH 5.4 or pH 5.0. The protein content was monitored manually by measuring the absorbance at 280 nm using a spectrophotometer. Washing was stopped once the absorbance dropped below 0.2. The bound protein was eluted with 250 mM NaCl in 10 mM buffer at pH 5.4 or pH 5.0. The protein content of the eluted fractions was monitored by measuring the absorbance at 280 nm. Elution was stopped when the absorbance dropped below 0.6. The elution procedure was repeated with 1.0 M NaCl in 10 mM buffer at pH 5.4 or pH 5.0 to remove any remaining protein. The column was cleaned with 10 mM buffer at pH 5.4 or pH 5.0. If the column was not used for an extended period of time, it was flushed with 20% ethanol prior to storage.

Fractions collected from the column were dialyzed against water. Spectra/Por

Molecularporous dialysis tubing with MWCO of 6-8 kDa was used. The fractions were then analyzed using SDS-PAGE, RID and the biuret method.

2. Electrodialysis of Concentrated Whey for Ion Exchange Chromatography

Electrodialysis was performed using the Micro Acilyzer G1 Desktop Desalinator (Asahi Chemical Industry Co., Ltd., Kawasaki, Japan) with an AC-230-10 cartridge. The cartridge had a MWCO of 300 Da for cations and 1000 Da for anions. The concentrated whey was given a first-order desalination using 0.02 A as the end current. First-order desalination was stated as giving maximum desalination of 300 ppm NaCl or equivalent by the manufacturer. The conductivity of the electrodialyzed sample was measured to determine the effectiveness of electrodialysis. The pH of the electrodialyzed sample was then adjusted to 5.4 or 5.0 with 1.0 N HCl depending on the pH of the buffer used.

J. SHELF LIFE STUDY OF IgG

1.Dairyland Foods Aseptic Injection System

The Dairyland Foods aseptic injection system consisted of a 0.2 μm Nylon 66 microfiltration membrane unit which aseptically fed and mixed sterilized solution into the flow of UHT product. The rate of injection of the microfiltered solution was 5-30 liters/hr. The UHT product was processed at a rate of 8000 liters/hr. A minimum of 1600 liters of UHT product must be processed. To process the minimum 1600 liters, 1-6 liters of microfiltered solution was required (1600 l / 8000 l/hr = 0.2 hr; 5-30 l/hr x 0.2 hr = 1-6 l).

The microfiltered solution would be diluted 267-1600 times after it was fed into the processing stream (8000 l/hr / 5 l/hr = 1600; 8000 l/hr / 30 l/hr = 267). Therefore, to get an IgG concentration of 1.0 mg/ml in the finished UHT product, the microfiltered IgG solution would have to have a concentration of 267-1600 mg/ml. Sixteen hundred grams of IgG was required to obtain 1.0 mg of IgG/ml in the final product using the aseptic injection system, and processing the minimum 1600 liters (1600 mg/ml x 1000 ml = 1600 g; 267 mg/ml x 6000 ml = 1602 g). Using the small-scale UF and IMAC systems available, only small amounts of IgG could be isolated which made use of the aseptic injection system unfeasible. In addition, difficulties were anticipated in solubilizing IgG at concentrations between 267-1600 mg/ml. Based on these considerations, it was concluded that a small-scale aseptic injection process must be used instead.

2. Design of a Small-scale Aseptic Injection System

A small-scale aseptic injection system was devised using 250 ml Tetra Brik cartons of UHT milk obtained from Dairyland Foods (Burnaby, B.C.). In a horizontal laminar flow hood Model EV424 (Envirco, Albuquerque, NM), the tops of UHT cartons were swabbed with 70% ethanol using sterile cotton swabs. Over the straw opening on the top of the carton, a piece of 19 mm wide Scotch Brand Magic Tape (3M Canada Inc., London, ON) was applied. The tape was swabbed with 70% ethanol. A plug of General Electric window and door silicone caulking (General Electric, Mississauga, ON) was placed on top of the tape. The caulking was allowed to cure for at least 24 hr. Samples were injected or withdrawn from the cartons through the silicone plugs using sterile disposable syringes attached to 20G

1.5 inch sterile needles both from Becton Dickinson & Co. (Rutherford, NJ). The silicone plug was swabbed with 70% ethanol before injection or withdrawal of sample. After injection or withdrawal of sample, a dab of silicone caulking was applied on top of the plug to ensure contamination would not occur.

3. Preliminary Trials to Test the Sterility of the Small-scale Aseptic Injection System

To test the maintenance of sterile conditions during injection and withdrawal of sample using the small-scale aseptic injection system, trials were conducted using membrane sterilized water. Six Tetra Brik cartons containing 250 ml of UHT chocolate milk from Dairyland Foods (Burnaby, B.C.) were prepared for injection as stated above. Using a sterile syringe, distilled water was passed through a presterilized 25 mm syringe filter with a 0.2 μm nylon membrane (Nalgene, Rochester, NY) connected to a sterile 20G 1.5 inch needle and sterile syringe. The sterilized water was injected into an autoclaved 25 ml microflex flask with a silicone septum (Kontes Scientific Glassware/Instruments, Vineland, NJ). The septum was swabbed with 70% ethanol. Eleven ml of sterilized water was withdrawn from the flask using a sterile disposable syringe attached to a sterile needle, and then injected into an UHT carton. Septums and silicone plugs were swabbed with 70% ethanol before injection and after withdrawal of the needle. A thin film of silicone was applied to the silicone plug to prevent contamination. All the above operations were done in a laminar flow hood.

Six packages were injected with sterile water and shaken. Three cartons were incubated at 30°C and three cartons were incubated at 35°C. Three cartons without water were also incubated at 30°C and three cartons without water were incubated at 35°C as

controls. After 1 and 2 weeks of incubation, packages were tested for microbial growth using the pour plate method with plate count agar (Difco, Detroit, MI) and 1.0 ml of sample to ensure no growth had occurred. Duplicates were done for each sample. Plates were incubated at 25°C for 48 hrs.

4. Membrane Sterilization

A method for membrane sterilizing IgG solution on a small-scale was devised after several trials. To prepare the IgG solution, lyophilized fractions of IgG from IMAC were pooled and solubilized with water. The IgG solution was diluted to varying degrees and filtered through a presterilized 25 mm syringe filter with a 0.2 μm nylon membrane. The maximum operating pressure of the filter was 40 psig and the surface area was 3.8 cm^2. Syringe filters were ideal for the small volumes involved and for maintaining aseptic conditions. It was noted that IgG solution with a protein concentration greater than 550 mg/ml was not completely solubilized. The protein solution was solubilized at a concentration of 400 mg/ml. Filtering IgG solution with a protein concentration of 133 mg/ml caused the 0.2 μm nylon membrane syringe filter to plug up immediately. The IgG solution contained about 50% IgG. At a protein concentration of 66 mg/ml, some solution passed through the filter. At a protein concentration of 33 mg/ml, the solution passed through easily. Due to the difficulty in filtering IgG solution even at low protein concentrations, it was concluded that prefiltration or centrifugation was required before the final filtration. This conclusion was supported by the results of Hilpert (1984).

Hilpert (1984) prepared a milk immunoglobulin concentrate (MIC) from cow's milk.

MIC solution containing 10% dry matter, 7-8% total protein and 2-3% Ig was membrane sterilized by passing it through a Seitz or Filtrox depth filter plate (type Supra EK) for prefiltration and then through a Millipore 0.45 μm pore size membrane filter for sterilization. The sterilized solution was lyophilized under sterile conditions to increase the Ig concentration.

To improve final filtration, centrifugation and prefiltration methods were investigated. Centrifuging IgG solution (50 mg of protein/ml) at 10 000 × g for 25 minutes at 5°C and then passing the solution through a 0.2 μm nylon membrane syringe filter facilitated passage of the solution through the filter. There did not appear to be a noticeable difference in IgG content before and after centrifugation.

Prefiltration methods were also examined. GF/A glass microfiber paper with a 1.6 μm pore size (Whatman Ltd., England) was held in a 25 mm filter holder and attached to a disposable syringe for prefiltration. A presterilized 37 mm syringe filter, Acrodisc 37 GF, was purchased from Gelman Sciences (Ann Arbor, MI). The Acrodisc 37 GF had a built in borosilicate glass fiber prefilter and a 0.2 μm Supor membrane. The membrane area of the Acrodisc 37 GF was 7.5 cm^2 and the maximum operating pressure was 75 psi. When an IgG solution containing 240 mg of protein/ml was filtered through an Acrodisc 37 GF syringe filter, the filter clogged immediately. Passing IgG solution with 55 mg of protein/ml through a GF/A prefilter and then through a 0.2 μm nylon membrane syringe filter, or directly through an Acrodisc 37 GF syringe filter caused some clogging. An IgG solution with a protein content greater than 70 mg/ml caused the GF/A prefilter to clog. Passing IgG solution with 55 mg of protein/ml through 3 layers of GF/A and then through an Acrodisc

37 GF syringe filter resulted in little clogging.

Based on these preliminary trials, a membrane sterilization procedure was established for small volumes of IgG solution. The procedure consisted of reconstituting lyophilized IgG from IMAC with distilled deionized water to get a solution with 60-70 mg of protein/ml. The solution was prefiltered through 3 layers of GF/A, and filtered through 2 Acrodisc 37 GF syringe filters. Two filters were used in case the first filter was defective. The membrane sterilized IgG solution was injected into an autoclaved 25 ml microflex flask with a silicone septum. The septum was swabbed with 70% ethanol before and after injection. All procedures for membrane sterilization were carried out in a laminar flow hood.

The effectiveness of the membrane filtration method was tested using spread plates with plate count agar (Difco, Detroit, MI). For spread plates, 0.1 ml of sample was used. To enumerate microorganisms in samples with high microbial counts, 1.0 ml of sample was serially diluted in 9.0 ml of sterile 0.1% peptone water (Difco, Detroit, MI). Plates were done in duplicate for each sample, and all plates were incubated at 25°C for 48 hrs. The spread plate method was chosen because when the pour plate method was used, it was found that microorganisms grew mainly on the surface of the plates. This indicated that the bacteria contaminating the IgG solution were mainly aerobes and probably Pseudomonas based on the odour. The spread plate method was the better method of enumeration in these circumstances. Samples of IgG solution were taken out before and after filtration steps to determine if IgG was lost.

5. Thermal Resistance Curve for IgG

The decimal reduction value (D value) of the membrane sterilized IgG in UHT milk at 4°, 25° and 35°C were extrapolated from a thermal resistance curve. To plot a thermal resistance curve, D values for IgG were determined for temperatures of 62°, 66°, 70°, 74°, 78° and 80°C. The D value is the time required for a ten-fold decrease in concentration (Ramaswamy et al., 1989). D values for IgG were based on the assumption that IgG follows first-order thermal destruction. Micropipets were used to determine D values for IgG in UHT milk. One hundred ml of white 2% UHT milk was aseptically transferred to an autoclaved 250 ml Erlenmeyer flask in a laminar flow hood. Membrane sterilized IgG solution was added to the milk to increase the IgG concentration to 1.0 mg/ml. The milk was then mixed well. One hundred µl of IgG fortified UHT milk was inserted into 100 µl micropipets (VWR Scientific Inc., London, ON) using a 5 ml sterile disposable syringe with a sterile 18G 1.5 inch needle. The milk was centered in the micropipet leaving space at either end. This prevented the milk from being heated when each end of the micropipet was heated with a bunsen burner to seal the ends. Twelve micropipets were prepared for each temperature trial.

A constant temperature waterbath Model MB-1120A-1 (Blue M Electric Co., Blue Island, IL) was used to heat the micropipets containing IgG fortified milk. A waterbath was used rather than an oil bath since temperatures were kept below 80°C, and the waterbath was easier to work with. The waterbath was set to one of the 6 temperatures used: 62°, 66°, 70°, 74°, 78° or 80°C. The temperature was monitored using a mercury thermometer with a temperature range of -20°-110°C. The thermometer was calibrated in ice water and boiling water to ensure its accuracy. The temperature of the waterbath fluctuated at most ±0.5°C.

String was tied to one end of the sealed micropipets to facilitate insertion and withdrawal of the micropipet from the waterbath. The micropipets were inserted into the waterbath and were withdrawn after a desired length of time taking the come-up time of the micropipet into consideration. The withdrawn micropipet was immediately immersed in ice water to stop heating. The IgG fortified milk was extracted from the micropipet and the IgG activity was measured using RID. The ELISA method of Kummer et al. (1992) for IgG was also used to compare with the RID results. From RID results, plots of the logarithm of IgG concentration versus time on a linear scale at each temperature were made. Using simple linear regression, the equations of the lines were calculated and the D values were determined. A thermal resistance curve for IgG in UHT milk could be plotted using the D values found.

The come-up time for the micropipets was determined using a DataTaker Field Logger Model DT 100F (Data Electronics Pty. Ltd., Austl.) connected to a Compaq computer loaded with the Decipher program (Data Electronics Pty. Ltd., Austl.). To measure come-up time, the DataTaker was connected to a T-type thermocouple and programmed to take temperature readings every 1 s using the Decipher program. The thermocouple was inserted into 100 µl of milk in a half sealed micropipet. The micropipet and thermocouple were inserted into a waterbath set at 62°C or 78°C, and the time required for the contents of the micropipet to reach the waterbath temperature was determined. The micropipet and thermocouple were then inserted into ice water to determine the time required for the temperature to drop. The results indicated that the average come-up time was 3.75 s at 78°C and 3 s at 62°C. The time required for the temperature to drop was 3 s at both 62° and 78°C. A come-up time of 4 s

was therefore assumed.

6. Sandwich ELISA for the Determination of Total IgG Content

The sandwich ELISA method of Kummer et al. (1992) was used to determine IgG content in samples. Immulon 2, flat bottomed, polystyrene microtitre plates (Dynatech Laboratories Inc., Chantilly, VA) were used. Briefly, the method consisted of coating the plate wells with 100 µl of coating solution (1 µg/ml of anti-bovine IgG antiserum developed in rabbit (Sigma Chemical Co., St. Louis, MO) in 0.015 M sodium carbonate coating buffer at pH 9.6). The plate was incubated for 60 min at 37°C and then washed two times with PBS-Tween at pH 7.4 (8.0 g NaCl, 0.2 g KH_2PO_4, 1.15 g Na_2HPO_4, 0.2 g KCl in 1 l of distilled water with 0.05% Tween 20). A volume of 250 µl of blocking agent was added (0.25% chicken egg ovalbumin in PBS at pH 7.4). The plate was incubated for 30 minutes at 37°C and then emptied. One hundred µl of sample or standard was then added and the plate was incubated for 60 min at 37°C. Samples or standards were done in triplicate. Standards were diluted in PBS-Tween so the concentration of IgG varied from 5-30 ng/ml. Samples were diluted by an appropriate factor so the concentration would lie within the standard curve. Three dilutions were made for each sample. The plate was then washed 3 times with PBS-Tween. One hundred µl of conjugate (0.43 µg/ml anti-bovine IgG alkaline phosphatase conjugate (Sigma Chemical Co., St. Louis, MO) diluted in 10 ml of PBS-Tween) was then added to the plate. The plate was incubated at 37°C for 60 min and was washed 3 times with PBS-Tween and rinsed with distilled water. One hundred µl of substrate (5 mg p-NNP (p-nitrophenyl phosphate, di-sodium) tablet (Sigma Chemical Co., St. Louis, MO) in

10 ml diethanolamine substrate buffer at pH 9.8) was then added. The plate was then incubated at 37°C and read at 405 nm with an ELISA reader Model EAR 400 (SLT-Labinstruments, Austria) after 30, 45 and 60 min.

7. Shelf Life Study of IgG in UHT Milk

Tetra Brik cartons containing 250 ml of 2% white UHT milk were obtained from Dairyland Foods (Burnaby, B.C.). The cartons were prepared for injection using the method outlined in "2. Design of a Small-scale Aseptic Injection System". Lyophilized IgG from several IMAC runs was pooled together. Distilled deionized water was used to solubilize the IgG, so that the protein concentration of the resulting IgG solution was between 60-70 mg/ml. The pH of the solution was checked. The protein concentration was determined using the biuret method and the IgG concentration was measured using RID.

The IgG solution was then membrane sterilized using the method outlined in "4. Membrane Sterilization". Based on the IgG concentration of the IgG solution, sufficient volumes of the sterilized solution were aseptically injected into the UHT cartons to give the milk an IgG concentration of approximately 1.0 mg/ml. This volume worked out to 8.0-9.0 ml. One mg/ml was chosen since changes in this concentration could be measured easily by RID. After IgG solution was added, the cartons were shaken and samples were withdrawn from the cartons. The samples were analyzed for IgG content using RID. Twelve cartons were injected with IgG solution. Cartons were then incubated at 4°C, 25°C and 35°C. Four cartons were incubated at each temperature. Four cartons not fortified with IgG were prepared for aseptic injection and incubated at each temperature as controls. At one month

intervals, samples were withdrawn from 2 injected cartons and 2 control cartons at each temperature. Samples were analyzed for IgG content using RID, and for bacterial growth using spread plates and pour plates with standard plate count agar incubated at 25° or 35°C for 48 hrs. For pour plates, 1.0 ml of sample was used, and for spread plates, 0.1 ml of sample was used. Samples were analyzed for yeasts and molds using pour plates with potato dextrose agar (Difco, Detroit, MI) acidified to pH 3.5 with 10% tartaric acid. These plates were incubated at 25°C for 48 hrs.

IV. RESULTS AND DISCUSSION

A. PRELIMINARY TRIALS

Preliminary trials were conducted to determine whether UF alone could be used to isolate IgG from whey. Whey was used for preliminary trials since problems with casein were eliminated. In one trial, 1000 ml of CCW was concentrated 2 times and then diafiltered with 2100 ml of distilled deionized water using a 100 kDa MWCO membrane. Some loss of α-lactalbumin and β-lactoglobulin were noted by SDS-PAGE of samples. In another trial, 3500 ml of acid whey was concentrated with a 100 kDa MWCO membrane. The whey was concentrated 10 times, and then diafiltered with 1900 ml of distilled deionized water. SDS-PAGE of the concentrate before and after diafiltration showed some loss of α-lactalbumin and β-lactoglobulin, but no loss of bovine serum albumin (BSA). In another trial, 2345 ml of acid whey was concentrated 10 times and diafiltered with 3000 ml of distilled deionized water using a 100 kDa MWCO membrane. SDS-PAGE of samples before and after diafiltration showed almost complete loss of α-lactalbumin and some loss of β-lactoglobulin. The volume of diafiltration solution to the volume of concentrate was 4 to 1, 5.4 to 1 and 12.8 to 1, respectively for the 3 trials. Extensive diafiltration did not seem to increase the purity of IgG to a great extent.

Kothe et al. (1987) developed a method of producing a lactic or colostric Ig solution using membrane filtration. In one of their experiments, 15 liters of colostrum was acidified to pH 4.82 and then diluted to 30 liters with 0.9% NaCl. Precipitated casein was removed by passing the solution through a 0.4 μm microfiltration membrane and diafiltering with 120 liters of 0.9% NaCl. The permeate containing IgG was filtered through a 10 kDa

MWCO membrane and concentrated to 25 liters. The solution was then diafiltered with 120 liters of 0.9% NaCl (4.8 times the concentrate volume), and then further concentrated before filtration sterilization. Renner and Abd El-Salam (1991) noted that the maximum ratio of protein to total solids attainable by diafiltration with water is 0.9 to 1, because the low ionic strength will cause protein to precipitate. The use of 0.9% NaCl for diafiltration by Kothe et al. (1987) probably prevented protein precipitation.

The Ig solution Kothe et al. (1987) obtained had 77.4% Ig with about 85% of the Ig being IgG (65.8% IgG of total protein). Jenness (1988) reported the IgG concentration was 50 mg/ml and the protein concentration was 10.1% in colostrum during the first week of lactation. Assuming that the colostrum used by Kothe et al. (1987) had a similar composition, approximately 25% of the low MW proteins were lost during diafiltration. Even though large volumes of diafiltration solution were used, a significant amount of low MW proteins remained in the concentrate. If a larger MWCO membrane was used, more low MW proteins might be lost. Different UF membranes also have different retentions for proteins, so it may be possible to improve the separation of IgG from other proteins by UF membrane selection. For example, Hvid (1990) reported that Hekla UF membranes (Dow Danmark A/S, Separation Systems) are made with reduced hydrophobicity to reduce protein affinity.

If whey is used as the starting material, the IgG concentration would remain low even after diafiltration, since the IgG concentration in whey is low compared to the protein concentration. The Ig concentration in whey is 0.06% compared to the protein concentration of 0.7% (Glover, 1985). Stott and Lucas (1989) reported that an Ig solution with 12% Ig could be obtained by concentrating and diafiltering whey using a 100 kDa MWCO

membrane. UF and diafiltration can only purify IgG in whey to a limited extent. Therefore, other methods to purify Ig from concentrated whey were investigated.

In one trial, permeate from the UF of skim milk through a 500 kDa MWCO membrane was concentrated and diafiltered using a 100 kDa MWCO membrane. The pH of the concentrate was adjusted to 4.65. The concentrate was then centrifuged at 10 000 × g for 15 min at room temperature to precipitate β-lactoglobulin according to the method of Amundson et al. (1982). Amundson et al. (1982) demineralized both whey and whey concentrated by UF using electrodialysis before precipitating β-lactoglobulin by adjusting the pH. SDS-PAGE of the concentrate before and after centrifugation showed a decrease in the intensity of the β-lactoglobulin band similar to the results of Amundson et al. (1982). Although some β-lactoglobulin was eliminated, the purity of IgG was still low.

The β-lactoglobulin content was also reduced using polyphosphate precipitation. The permeate from the concentration of skim milk through a 500 kDa MWCO membrane was concentrated with a 50 kDa MWCO membrane. Fifty ml of this concentrate was adjusted to pH 4.10 with 1.0 N HCl, and then 1.33 mg/ml of sodium hexametaphosphate was added. The solution was allowed to set at room temperature for 1 hr followed by centrifugation at 10 000 × g for 15 min at room temperature to precipitate β-lactoglobulin. This method was developed by Al-Mashikhi and Nakai (1987) to precipitate β-lactoglobulin from cheese whey. SDS-PAGE of the permeate before and after centrifugation revealed that most of the β-lactoglobulin was removed by polyphosphate precipitation.

Diafiltration, precipitation of β-lactoglobulin by demineralization and pH adjustment, and precipitation of β-lactoglobulin with polyphosphate resulted in the separation of other

more numerous whey proteins from IgG to varying degrees. If the purity of IgG required is not high such as for animal feeding, any of these methods could be used. However, these methods have other disadvantages. The precipitation methods are not suited for large-scale isolation of IgG due to the many steps involved. In addition, adding polyphosphate or any chemical is undesirable since it must be removed at some stage. Although, diafiltration can be run continuously on a large-scale; most UF membranes have a fairly high retention coefficient for proteins and so require large volumes of diafiltration solution. This can lead to disposal problems. Since a fast, simple, nontoxic, relatively inexpensive and efficient method to obtain IgG with high purity and yield from whey or milk was an objective of this research, a method using UF in conjunction with IMAC was investigated.

B. EFFECT OF IMAC MATRIX SUPPORT AND CONCENTRATION FACTOR ON THE ISOLATION OF IgG FROM CHEDDAR CHEESE WHEY (CCW)

Li-Chan et al. (1990) developed a method of isolating IgG from Cheddar cheese whey using immobilized metal affinity chromatography (IMAC) also referred to as metal chelate interaction chromatography (MCIC). They recovered about 100 mg of IgG with 75-95% purity per ml of Cu-loaded bed volume. Although recovery was high using IMAC, the method was time-consuming since large volumes of whey had to be applied due to the low IgG concentration of whey. To reduce the time required to apply whey to the column, it was proposed that whey concentrated by UF be used. However, the effects of using concentrated whey with IMAC had to be determined.

In this study, CCW was concentrated 10, 20 and 30 times using a 50 kDa MWCO membrane. These concentration factors were chosen since the maximum economical concentration obtainable by direct UF is 20-30 times (Glover, 1985). Columns were filled with 10 ml of Chelating Sepharose Fast Flow (CSFF) or Chelating Sepharose 6B (CS6B) and were loaded with 3.0 ml of 50 mM $CuCl_2$ to give 1/2 Cu saturation. Sufficient unconcentrated or concentrated whey was applied to the columns to pass the breakthrough point for IgG. The columns were eluted with 1.0 M NH_4Cl in buffer. Table II shows the composition of the whey after concentration. pH and conductivity decreased with concentration probably due to the loss of ions during UF. Lactose concentration remained relatively constant with concentration, but dropped at 30 times concentration. Protein was lost during UF due to the passage of low MW proteins through the membrane. These changes were similar to those noted by Renner and Abd El-Salam (1991) during a 95% volume reduction of whey using UF. The values for IgG concentration indicate that there was no loss or very little loss of IgG activity during UF. IgG activity should not have been

Table II. Change in the composition of Cheddar cheese whey following ultrafiltration.

Concentration Factor		pH	Conductivity (μMho)[a]	Lactose cconcentration (mg/ml)[b]	IgG concentration (mg/ml)[c]	Protein concentration (mg/ml)[d]
Unconc.	whey	5.98	6667	47.8±1.3	0.35	5.6±0.1
	concentrate	----	----	----	----	----
10 ×	whey	5.94	6491	50.7±2.0	0.44	5.3±0.4
	concentrate	5.68	6250	51.3±1.1	4.08	37.8±3.5
20 ×	whey	6.05	6542	50.5±1.0	0.38	6.8±0.5
	concentrate	5.63	5818	51.3±0.4	7.06	77.4±4.3
30 ×	whey	5.99	6786	53.9±1.2	0.50	6.8±0.3
	concentrate	5.37	5714	47.4±0.5	14.96	100.2±3.1

[a]Corrected for 18°C.
[b]Measured using the sulfuric acid and phenol colourimetric method of Lawrence (1968). Means ± SD; n=4.
[c]Measured by radial immunodiffusion. Values are the average of duplicates. The differences between duplicate measurements were less than 10%.
[d]Measured using the biuret method. Means ± SD; n=3.

affected since studies have found UF does not denature proteins. Matthews et al. (1976) found there was no significant protein denaturation during UF of whey using 2 pilot plant-scale, tubular UF units. Denaturation was measured using whey protein nitrogen and PAGE. Chojnowski and Dziuba (1982) found UF of whey caused no detrimental changes in the functional properties of whey proteins.

Tables III and IV show the recovery of IgG from unconcentrated and concentrated CCW using either CSFF or CS6B as the matrix support. For unconcentrated whey, the recovery of IgG was 190 mg for the 10 ml CSFF column, and was 224 mg for the 10 ml CS6B column. Using these values as controls, the capacity of CSFF for IgG was 38 mg/ml of Cu-loaded gel, and the capacity of CS6B was 42 mg/ml of Cu-loaded gel. CS6B appeared to have a 10% greater capacity for IgG than CSFF. Li-Chan et al. (1988) noted that CSFF had a 40% lower capacity for IgG than CS6B for an equivalent volume of 50 mM $CuCl_2$ applied.

The difference in capacity between the two supports may be related to the degree of Cu-loading associated with each support. In this study, it was noted that when 3.0 ml of 50 mM $CuCl_2$ was applied to both supports, the volume of Cu-loaded gel for the CS6B column was 5.4 ml compared to 5.0 ml for the CSFF column. Li-Chan et al. (1988) noted that 1.5 ml of 50 mM $CuCl_2$ caused 1/4 to 1/3 Cu-loading for CSFF, and 1/3 to 1/2 Cu-loading for CS6B. The large difference in Cu-loading between CSFF and CS6B noted by Li-Chan et al. (1988) could explain the 40% difference in capacity between the two supports that they observed. Since less difference in Cu-loading between the two supports was noted in this study, the difference in capacity was lower. Based on observations, different batches of matrix supports also differed in Cu-loading which may explain the discrepancies between the studies.

Table III. IgG recovery and purity of fractions obtained by IMAC of unconcentrated and concentrated Cheddar cheese whey using a 10 ml Chelating Sepharose Fast Flow column loaded with 3.0 ml of 50 mM $CuCl_2$.

Concentration Factor	Fraction	IgG Recovered[a] (mg)	Purity of IgG[b] (g/100 g protein)
Unconcentrated	Applied whey (922 ml)	320	6.4
	Unbound (0-400 ml)	30	1.4
	Unbound (401-897 ml)	68	2.2
	Wash (44 ml)	5	25.0
	Elution (NH_4Cl) (79 ml)	160	57.1
	Elution (dH_2O) (33 ml)	30	55.6
	EDTA wash (9 ml)	4	88.9
10 ×	Applied 10 × concentrate (93 ml)	380	10.8
	Unbound (0-46 ml)	40	3.8
	Unbound (47-97 ml)	80	4.7
	Wash (68 ml)	10	6.3
	Elution (NH_4Cl) (94 ml)	120	54.5
	Elution (dH_2O) (34 ml)	21	52.5
	EDTA wash (10 ml)	10	~100
20 ×	Applied 20 × concentrate (47 ml)	330	9.1
	Unbound (46 ml)	70	2.9
	Wash (85 ml)	22	5.1
	Elution (NH_4Cl) (88 ml)	130	68.4
	Elution (dH_2O) (37 ml)	43	84.3
	EDTA wash (20 ml)	20	~100

Table III (cont'd)

Concentration Factor	Fraction	IgG Recovered[a] (mg)	Purity of IgG[b] (g/100 g protein)
30 ×	Applied 30 × concentrate (32 ml)	480	15.0
	Unbound (31 ml)	170	8.2
	Wash (93 ml)	44	6.7
	Elution (NH_4Cl) (87 ml)	110	64.7
	Elution (dH_2O) (59 ml)	40	80.0
	EDTA wash (14 ml)	14	77.8

[a]Recovered IgG calculated as IgG activity (mg/ml) by radial immunodiffusion x volume (ml) of fraction. Values are the average of duplicates. The differences between duplicate measurements were less than 10%.

[b]Purity of IgG (g/100 g) calculated as IgG activity by radial immunodiffusion/total protein in fraction x 100. Protein content was calculated as means ± SD; n=3. Values are the average of duplicates. The differences between duplicate measurements were less than 10%.

Table IV. IgG recovery and purity of fractions obtained by IMAC of unconcentrated and concentrated Cheddar cheese whey using a 10 ml Chelating Sepharose 6B column loaded with 3.0 ml of 50 mM $CuCl_2$.

Concentration Factor	Fraction	IgG Recovered[a] (mg)	Purity of IgG[b] (g/100 g protein)
Unconcentrated	Applied whey (1510 ml)	530	6.3
	Unbound (0-600 ml)	30	0.9
	Unbound (601-1000 ml)	50	2.0
	Unbound (1001-1510 ml)	120	3.8
	Wash (52 ml)	8	13.3
	Elution (NH_4Cl) (90 ml)	210	63.4
	Elution (dH_2O) (47 ml)	14	70.0
	EDTA wash (14 ml)	18	18.2
10 ×	Applied 10 × concentrate (152 ml)	620	10.8
	Unbound (0-53 ml)	50	4.3
	Unbound (54-107 ml)	110	5.9
	Unbound (108-154 ml)	140	7.9
	Wash (111 ml)	2	0.9
	Elution (NH_4Cl) (99 ml)	110	45.8
	Elution (dH_2O) (29 ml)	11	36.7
	EDTA wash (16 ml)	33	25.4
20 ×	Applied 20 × concentrate (77 ml)	540	9.1
	Unbound (74 ml)	180	3.9
	Wash (109 ml)	21	7.2
	Elution (NH_4Cl) (100ml)	150	62.5
	Elution (dH_2O) (31 ml)	10	50.0
	EDTA wash (18 ml)	58	29.0

Table IV. (cont'd)

Concentration Factor	Fraction	IgG Recovered[a] (mg)	Purity of IgG[b] (g/100 g protein)
30 ×	Applied 30 × concentrate (52 ml)	780	15.0
	Unbound (52 ml)	340	8.7
	Wash (130 ml)	34	6.4
	Elution (NH_4Cl) (110 ml)	110	55.0
	Elution (dH_2O) (101 ml)	20	50.0
	EDTA wash (14 ml)	83	~100

[a]Recovered IgG calculated as IgG activity (mg/ml) by radial immunodiffusion x volume (ml) of fraction. Values are the average of duplicates. The differences between duplicate measurements were less than 10%.

[b]Purity of IgG (g/100 g) calculated as IgG activity by radial immunodiffusion/total protein in fraction x 100. Protein content was calculated as means ± SD; n=3. Values are the average of duplicates. The differences between duplicate measurements were less than 10%.

The results of this study do not conclusively indicate that CS6B had a higher capacity than CSFF. The capacity for IgG may have been influenced by the amount of IgG applied. As mentioned in the "Materials and Methods", enough sample was applied to the IMAC column to lie between the breakthrough and saturation points for IgG. Li-Chan et al. (1990) plotted the breakthrough profile of IgG during the application of whey to a 10 ml CS6B column. They noted that at the breakthrough point about 200 mg of IgG was bound, and at the saturation point about 250 mg of IgG was bound. The purity was not affected, though. Since more whey was applied to the CS6B column, the amount of IgG applied may have been closer to the saturation point than for the CSFF column. Slightly more IgG, therefore, may have bound to the CS6B column. As a crude estimate, the breakthrough point could be reached by applying sufficient whey to exceed the capacity of the column. More whey was, therefore, applied to the CS6B column, since Li-Chan et al. (1988) found a higher capacity for IgG with CS6B than CSFF. Ideally, the columns should have been loaded to the saturation point for IgG, but there was no simple method to determine this point.

Li-Chan et al. (1990) recovered about 100 mg of IgG/ml of Cu-loaded bed volume for a CS6B column. Li-Chan et al. (1988) recovered about 60 mg of IgG/ml of Cu-loaded bed volume for a CSFF column. These values were higher than those found in this study. The large discrepancy in capacity between this study and those of Li-Chan et al. (1988, 1990) could be partly accounted for by the measurement of Cu-loaded gel volume, which is measured by determining the volume of the column which is blue due to Cu binding. The volume can be calculated by measuring the height of the column which is blue and multiplying by the cross-sectional area of the column. The Cu front is not usually even, though, making measurements difficult to accurately determine. The Cu front also moves slightly during equilibration of the column with buffer and can move significantly during

application of the sample. Therefore, the Cu-loaded gel volume will vary depending on when and how it is measured.

The volume of 50 mM $CuCl_2$ applied to the column could also be used to determine capacity. However, the molarity of the $CuCl_2$ solution would have to be measured accurately. The molarity of $CuCl_2$ used by Li-Chan et al. (1988) seemed to differ from the molarity of $CuCl_2$ used in this study. Li-Chan et al. (1988) stated that applying 1.25 ml of 50 mM $CuCl_2$ to a 10 ml column resulted in the upper 2.5 ml of the column being loaded with Cu. The manufacturer gave the capacity of CSFF for Zn^{2+} as 22-30 µmol Zn^{2+}/ml gel. Hutchens et al. (1988) gave the capacity as 31-40 µmol Cu^{2+}/ml gel. Based on these values, the Cu^{2+} capacity of the column used by Li-Chan et al. (1988) was 25 µmol/ml, which probably indicated that the molarity of $CuCl_2$ was greater than 50 mM. In this study, $CuCl_2 \cdot 2H_2O$ was purchased and a 50 mM solution of $CuCl_2$ was prepared as accurately as possible. For a 50 mM solution, 8.524 g of $CuCl_2 \cdot 2H_2O$/liter was required. Two batches of 50 mM $CuCl_2$ were prepared and in both cases, when 3.0 ml of the solution was applied to a 10 ml CSFF column, the column was 1/2 Cu-loaded. The Cu^{2+} capacity was calculated as 30 µmol/ml which indicated the molarity of $CuCl_2$ was probably close to 50 mM. Measuring the capacity with respect to the volume of 50 mM $CuCl_2$ applied would be more accurate than measuring the Cu-loaded gel volume provided the molarity was accurate.

Another method to measure capacity would be to measure the amount of IgG that can be isolated from a column of a certain volume. This would be a better method to use since a column can only be loaded with a limited amount of Cu. If too much Cu is loaded on a column, Cu passes through the column during sample application or elution. The column becomes ineffective since the capacity will decrease due to the loss of Cu, and Cu will contaminate the sample and eluted fractions. The amount of Cu that can be applied will

depend on the particular batch of matrix support, on the nature of the sample applied and on elution conditions. Therefore, even though the capacity may be stated as 100 mg/ml of Cu-loaded gel for a 10 ml column; if only 2.5 ml of the column can be saturated with Cu, the actual capacity is only 250 mg for a 10 ml column, not 1000 mg. Using this definition of capacity for an IMAC column, the amounts of IgG recovered in this study were similar to the studies of Li-Chan et al. (1988, 1990) for a 10 ml column.

In this study, the amount of Cu passing out of the column was not determined. The movement of the Cu front was observed, and as long as the front did not reach the bottom of the column, no Cu leakage was assumed. To ensure no Cu escapes from the column, Cu in the effluent would have to be monitored, possibly using atomic absorption spectrophotometry. In this manner, the maximum amount of Cu that can be loaded onto a column without Cu escaping could be determined. Problems with Cu leaking out of the column would have to be investigated before IMAC is used commercially.

The recovery of IgG using concentrated whey rather than unconcentrated whey was up to 25% lower using CSFF and up to 48% lower using CS6B as noted in Tables III and IV. In subsequent experiments noted in the section titled "F. Effect of Elution Buffer on IgG Recovery Using IMAC", it was found that IgG was not lost, but was recoverable by changing elution conditions. This observation indicated that UF may be altering the conformation of IgG so that it binds stronger to the IMAC column. Chojnowski and Dziuba (1982) noted that UF resulted in a positive differential spectrum of protein solutions obtained from whey ultrafiltrates. The positive differential spectrum indicated the number of chromophobic groups such as hydrophobic groups of aromatic amino acids or bisulfide groups increased during UF. There was no change in the functional properties of proteins, though. These types of changes might explain why IgG from concentrated whey appeared to bind stronger

to the IMAC column. The concentration factor, therefore, does not seem to affect the recovery of IgG provided that an efficient eluting buffer is used. However, using a 30 times concentrate caused pressure to buildup in the column due to the slight increase in viscosity of the concentrate. Whey concentrated less than 30 times would be more suitable to use for IMAC.

The purity of IgG recovered from whey using CSFF was around 60%. For CS6B, the purity of IgG in the fraction eluted with NH_4Cl was 69% and in the fraction eluted with water was 91%. Li-Chan et al. (1988) noted that the purity of IgG in the fraction eluted with NH_4Cl was 55% and in the fraction eluted with water was 75% using CSFF. This was similar to the results obtained in this study. The purity of IgG using CS6B was not as high as the 75-95% purity found by Li-Chan et al. (1990). The difference may be related to the method of measuring protein content, to the nature of the whey used, or to the batch of matrix support used. Li-Chan et al. (1990) used the Kjeldahl method to measure protein whereas the biuret method was used in this study. The section titled "G. Comparison of the Biuret Method and Kjeldahl Method to Measure Protein Content" indicates there were some differences in values between the two methods. IgG purity also tended to vary depending on the particular batch of whey used and the particular batch of matrix support used.

The purity of IgG was similar whether concentrated or unconcentrated whey was used. Figures 3 and 4 show the SDS-PAGE profiles of eluted fractions from the different concentrated wheys. The profiles of the eluted fractions were similar in all cases. However, values for purity in Tables III and IV varied significantly in some cases. The discrepancies may be a result of using the biuret method to measure protein concentrations. Although the biuret method was sensitive between 0.0-10.0 mg of protein/ml, the method was not as accurate at concentrations below 2.0 mg of protein/ml. Therefore, purity values for fractions

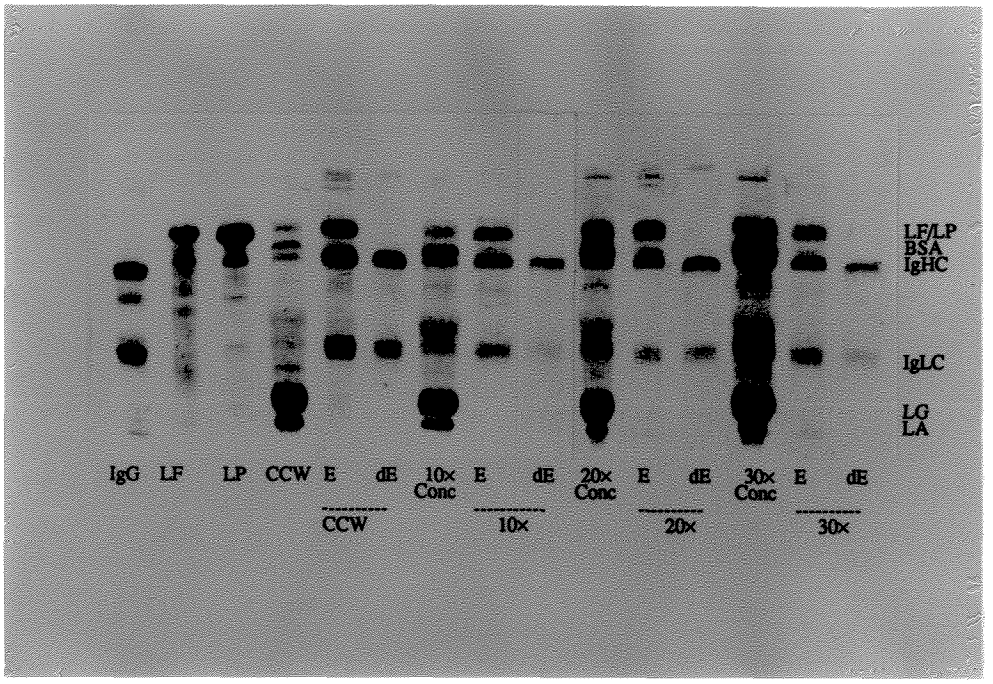

Figure 3. Reduced SDS-PAGE profiles of fractions eluted with 1.0 M NH_4Cl and distilled H_2O after Cu-IMAC treatment of unconcentrated and concentrated whey with Chelating Sepharose Fast Flow as the matrix support. Abbreviations for fractions: IgG = IgG standard, LF = lactoferrin, LP = lactoperoxidase, CCW = Cheddar cheese whey, Conc = concentrate (retentate), E = peak eluted with 1.0 M NH_4Cl, and dE = peak eluted with distilled H_2O. Abbreviations for bands: LP = lactoperoxidase; LF = lactoferrin; BSA = bovine serum albumin; IgHC, IgLC = IgG heavy and light chains; LG = β-lactoglobulin; and LA = α-lactalbumin.

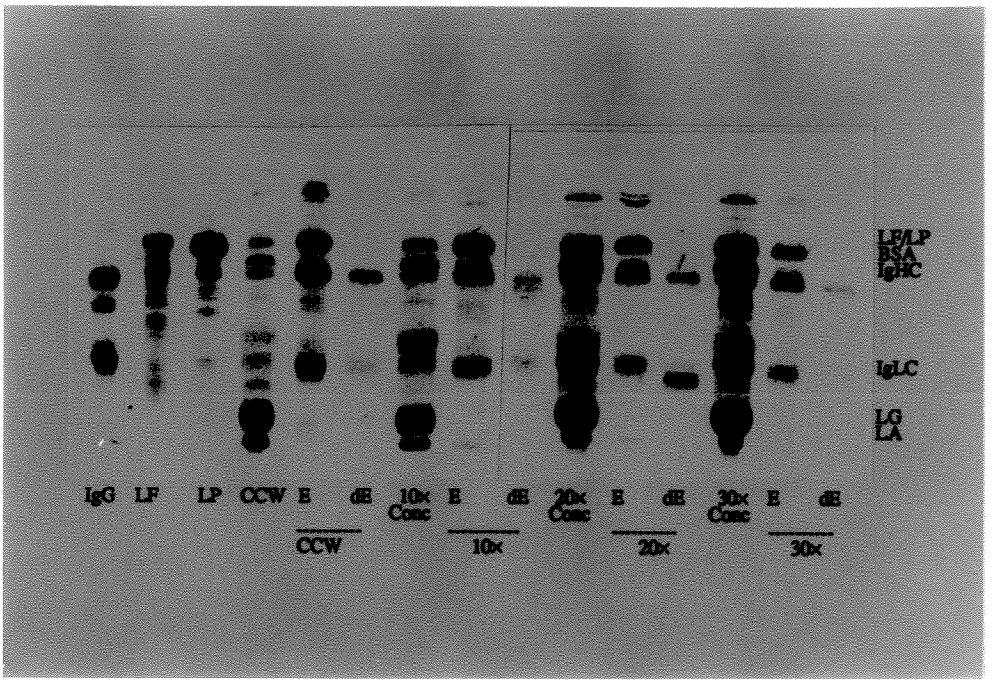

Figure 4. Reduced SDS-PAGE profiles of fractions eluted with 1.0 M NH_4Cl and distilled H_2O after Cu-IMAC treatment of unconcentrated and concentrated whey with Chelating Sepharose 6B as the matrix support. Abbreviations for fractions: IgG = IgG standard, LF = lactoferrin, LP = lactoperoxidase, CCW = Cheddar cheese whey, Conc = concentrate (retentate), E = peak eluted with 1.0 M NH_4Cl, and dE = peak eluted with distilled H_2O. Abbreviations for bands: LP = lactoperoxidase; LF = lactoferrin; BSA = bovine serum albumin; IgHC, IgLC = IgG heavy and light chains; LG = β-lactoglobulin; and LA = α-lactalbumin.

with low protein may not be accurate. The SDS-PAGE profiles show lactoferrin and lactoperoxidase were recovered with IgG. A high molecular weight protein also appears in the SDS-PAGE profiles which was suspected to be IgM or IgA.

The recovery and purity of IgG from unconcentrated or concentrated whey can be similar provided a good elution buffer is used. The main advantage in using concentrated whey was the application time being reduced proportionally to the concentration factor as shown in Tables V and VI. Since the time of application was reduced, there was less wear on the column and pump, and microbial growth was decreased. The time required for UF increased with concentration as expected. Considering that large-scale UF systems can process several hundred liters/hour, the time required for UF would be negligible if whey concentrated commercially was used. The time required for washing and the wash volume increased with the concentration factor of the whey used. Since protein concentration was higher at a greater concentration, more washing was probably required to remove unbound or weakly bound proteins. Elution time and elution volume also tended to increase with concentration. This was probably an indication that the elution buffer was not as effective as it could have been.

Another advantage of using concentrated whey rather than unconcentrated whey was the decrease in migration of the Cu front. Since Cu can contaminate eluted fractions if the Cu front migrates too far, decreased movement of Cu is an advantage. Table VII summarizes some of the changes noted in the column following application of sample. Using concentrated whey, movement of the Cu front was 20% of the Cu front movement when unconcentrated whey was used.

It was found that the movement of the Cu front was partly due to ions in the whey. This conclusion was reached after applying either equilibrating buffer or a salt solution that

Table V. Comparison of the time required to isolate IgG from unconcentrated and concentrated Cheddar cheese whey using IMAC with Chelating Sepharose Fast Flow as the matrix support.

Stage	Time required[a] (minutes)			
	Unconc.	10 ×	20 ×	30 ×
Ultrafiltration	----	53	61	71
Application	2004	202	102	70
Wash	96	148	185	202
Elution (NH_4Cl)	172	204	191	189
Elution (dH_2O)	72	74	80	128
Total Time	2344	681	619	660
% of unconc. time	100	29	26	28

[a] Time required for stages other than ultrafiltration were calculated based on volume at stage/flow rate of 0.46 ml/min.

Table VI. Comparison of the time required to isolate IgG from unconcentrated and concentrated Cheddar cheese whey using IMAC with Chelating Sepharose 6B as the matrix support.

Stage	Time required[a] (minutes)			
	Unconc.	10 ×	20 ×	30 ×
Ultrafiltration	----	90	100	107
Application	3356	338	171	116
Wash	116	247	242	289
Elution (NH_4Cl)	200	220	222	244
Elution (dH_2O)	104	64	69	224
Total Time	3776	959	804	980
% of unconc. time	100	25	21	26

[a] Time required for stages other than ultrafiltration were calculated based on volume at stage/flow rate of 0.45 ml/min.

Table VII. Changes in IMAC columns following application of samples.

Sample	Distance Cu front moved (cm)		Compression of column (cm)	
	CSFF	CS6B	CSFF	CS6B
Whey	2.0	2.5	<0.1	<0.1
10 × conc.	0.4	0.4	<0.1	0.2
20 × conc.	0.3	0.3	<0.1	0.4
30 × conc.	0.4	0.4	<0.1	0.6

simulates milk ultrafiltrate to the column. The salt solution was made according to Jenness and Koops (1962). The conductivity of the salt solution was 4900 μMhos and the conductivity of the buffer was 3200 μMhos corrected for 18°C. After applying 1 liter of equilibrating buffer (50 mM sodium phosphate buffer at pH 6.0) to the column, there was only slight movement of the Cu front (<0.1 cm). When applying salt solution adjusted to pH 6.0, the Cu front moved downwards as the amount of salt solution applied increased. After applying 1 liter of salt solution, the Cu front had moved approximately 1.0 cm. These observations could explain why the Cu front moved more using unconcentrated whey than concentrated whey. The concentrated whey and unconcentrated whey had similar conductivities, so probably had similar salt concentrations. Since the volume of unconcentrated whey applied was greater than the volume of concentrated whey applied, though; more ions were applied using unconcentrated whey which could have caused the Cu front to migrate more.

Table VII indicates that CS6B compressed when concentrated whey was used while CSFF showed no compression. Extra cross-linking of the 6% agarose in CSFF probably provided extra stability. It was also observed that CS6B tended to discolour after repeated use even after cleaning with 0.05 N NaOH. CSFF did not suffer the same problems. Since the recovery and purity of IgG were similar with CSFF and CS6B, but CSFF was more stable than CS6B, CSFF was used in subsequent experiments. Pharmacia Inc. (Uppsala, Sweden) also no longer manufactures CS6B.

C. EFFECT OF UF CONDITIONS ON THE ISOLATION OF IgG FROM CONCENTRATED WHEY USING IMAC

To determine if UF conditions would affect the isolation of IgG from concentrated whey using IMAC, 2700 ml of CCW was concentrated 14 times under 3 different UF conditions. The UF conditions used were 50 kDa MWCO membrane at 4°C, 50 kDa MWCO membrane at 45°C, and 100 kDa MWCO membrane at 4°C. UF was done in a coldroom to keep the temperature at 4°C. Microbial growth is minimized below 10°C or above 50°C (Glover, 1985). Temperatures of 4° and 45°C were therefore chosen. Although, the higher temperature should have been above 50°C, Glover (1985) stated temperatures around 50°C were used for UF of whey. Horton et al. (1972) also mentioned that UF of whey at 43°-49°C for several hours did not lead to bacteriological growth. Since Ig is temperature sensitive and the time for UF was quite lengthy using the small-scale UF system, 45°C was used instead of 50°C to prevent any unnecessary denaturation of Ig. Commercially, UF is usually done at a higher temperature since flux is greater making the process more economical. A lower temperature is often used for batch UF since the time of processing is much longer than for continuous UF. For long processing times, bacterial growth is minimized to a greater extent at a lower temperature than a higher temperature.

The composition of the concentrates after UF is shown in Table VIII. The concentrates were prepared from the same batch of CCW. Conductivity decreased during UF as ions were filtered out. Lactose concentration remained similar to whey when the 50 kDa MWCO membrane was used, but decreased when the 100 kDa MWCO membrane was used. The larger pore size of the 100 kDa MWCO membrane may have facilitated the passage of lactose molecules. Protein concentration was lower when the 100 kDa MWCO membrane was used. Again the larger pore size probably resulted in a lower retention of protein than

Table VIII. Composition of CCW concentrates following UF under different conditions.

UF conditions	pH	Conductivity[a] (µMho)	Lactose conc.[b] (mg/ml)	IgG conc.[c] (mg/ml)	Protein conc.[d] (mg/ml)
Whey	6.00	6500	48.4±1.4	0.49	6.4±0.5
50 kDa MWCO coldroom	5.96	6083	47.3±0.7	5.74	48.2±1.4
50 kDa MWCO 45°C	5.99	6158	49.0±0.9	7.07	53.4±2.1
100 kDa MWCO coldroom	5.98	6186	45.1±1.2	5.25	40.2±1.5

[a]Corrected for 18°C.
[b]Measured using the sulfuric acid and phenol colourimetric method of Lawrence (1968). Means ± SD; n=4.
[c]Measured by radial immunodiffusion. Values are the average of duplicates. The differences between duplicate measurements were less than 10%.
[d]Measured using the biuret method. Means ± SD; n=3.

for the 50 kDa MWCO membrane. The IgG concentrations of CCW processed at 4°C were lower than expected. The loss in IgG may have been due to IgG fouling the membrane due to the increased viscosity at the lower temperature, or IgG interacting with other proteins at the lower temperature causing loss of IgG. The exact cause of IgG activity loss at 4°C should be investigated further.

The time required for UF was greater at 4°C than at 45°C. It took 165 min to concentrate 2700 ml of CCW with the 50 kDa MWCO membrane at 45°C, 240 min with the 100 kDa MWCO membrane at 4°C, and 270 min with the 50 kDa MWCO membrane at 4°C. UF was faster at 45°C than at 4°C since viscosity was lower resulting in greater flux. The larger pore size of the 100 kDa MWCO membrane was expected to make UF faster than for the 50 kDa MWCO membrane. This study indicated UF at 45°C may be more advantageous than at 4°C since UF was faster and little IgG activity was lost.

The whey concentrated under the 3 UF conditions was applied to a 10 ml CSFF column loaded with 3.0 ml of 50 mM $CuCl_2$. Table IX shows the IgG recovery and purity for the 3 concentrates. The results indicate the purity of recovered fractions were similar for all 3 concentrates. The purity of fractions eluted with water may have varied due to the limitations of the biuret method to measure protein content at low concentrations. IgG recovery was similar for all 3 concentrates, but slightly higher for the concentrate processed at 45°C. Since less IgG was applied using concentrate processed at 4°C than 45°C, this may account for the slight difference in recovery. From these results, it appears UF conditions do not seem to affect the recovery of IgG from concentrated whey using IMAC, except some loss of IgG can occur if UF is conducted at a low temperature (4°C).

Table IX. IgG recovery and purity of fractions obtained by IMAC of concentrated CCW under different UF conditions using a 10 ml Chelating Sepharose Fast Flow column loaded with 3.0 ml of 50 mM $CuCl_2$.

UF condition	Fraction	IgG Recovered[a] (mg)	Purity of IgG[b] (g/100 g protein)
50 kDa MWCO	Applied concentrate (65 ml)	370	11.8
coldroom	Unbound (66 ml)	50	2.0
	Wash (59 ml)	20	10.0
	Elution(NH_4Cl) (92 ml)	160	69.6
	Elution (dH_2O) (37 ml)	47	81.0
	EDTA wash (15 ml)	8	88.9
50 kDa MWCO	Applied concentrate (63 ml)	440	13.1
45°C	Unbound (63 ml)	50	2.3
	Wash (64 ml)	20	8.7
	Elution(NH_4Cl) (95 ml)	170	68.0
	Elution (dH_2O) (33 ml)	61	91.0
	EDTA wash (15 ml)	8	~100
100 kDa MWCO	Applied concentrate (64 ml)	340	13.2
coldroom	Unbound (63 ml)	30	1.6
	Wash (87 ml)	10	5.9
	Elution(NH_4Cl) (96 ml)	140	60.9
	Elution(dH_2O) (36 ml)	66	~100
	EDTA wash (15 ml)	7	~100

[a]Recovered IgG calculated as IgG activity (mg/ml) by radial immunodiffusion x volume (ml) of fraction. Values are the average of duplicates. The differences between duplicate measurements were less than 10%.
[b]Purity of IgG (g/100 g) calculated as IgG activity by radial immunodiffusion /total protein in fraction x 100. Protein content was calculated as means ± SD; n=3. Values are the average of duplicates. The differences between duplicate measurements were less than 10%.

D. EFFECT OF DIAFILTRATION OF CCW CONCENTRATE ON THE ISOLATION OF IgG USING IMAC

To determine if diafiltration affects the isolation of IgG from whey concentrate using IMAC, 3000 ml of white CCW was concentrated 10 times and diafiltered with 2235 ml of distilled deionized water. Table X shows the change in protein concentration of the permeate and concentrate during UF. The results indicated that the protein content increased in both concentrate and permeate as UF proceeded. These results were similar to those reported by Glover (1985).

The composition of the white CCW following diafiltration is shown in Table XI. Conductivity and lactose concentration dropped to near 0 after diafiltration indicating substantial loss of low MW components, namely minerals and lactose, in the whey. Protein content decreased about 25% indicating protein retention by the UF membrane was much higher than for lactose or ions. Glover (1985) mentioned that the protein retention of UF membranes is much higher than lactose or salt retention. Lactose retention is around 10% whereas protein retention is usually around 80%. Retention of ions is usually close to 0%.

IgG from the diafiltered whey was isolated using a 10 ml column filled with CSFF and loaded with 3.0 ml of 50 mM $CuCl_2$. The recovery of IgG in the fraction eluted with 1.0 M NH_4Cl was 130 mg and in the fraction eluted with distilled water was 34 mg. These values were the average of duplicates. The differences between duplicate measurements were less than 10%. In previous experiments, 150-200 mg of IgG were isolated from the same column. The recovery may have been on the low side due to the use of white CCW. In other experiments, it was found that IgG recovery from white CCW was lower than from regular CCW. The section titled "H. Effect of Whey Used on the Isolation of IgG using UF and IMAC" should be noted. The purity of IgG remained similar to previous results.

Table X. Change in protein content of permeate and concentrate during concentration of white CCW using a 50 kDa MWCO membrane.

Concentration Factor	Protein content (mg/ml)[a]	
	Concentrate	Permeate
0	5.7±0.3	----
1.3 ×	9.6±0.4	3.0±0.1
1.72 ×	11.4±0.3	2.8±0.1
2.3 ×	13.7±0.3	2.6±0.2
3.4 ×	29.7±1.5	2.9±0.1
5.3 ×	39.3±3.1	3.3±0.1
10 ×	60.8±2.5	3.8±0.2

[a] Protein content measured using the biuret method. Means ± SD; n=3.

Table XI. Change in the composition of white CCW following concentration and diafiltration using a 50 kDa MWCO membrane.

	pH	Conductivity[a] (μMho)	Lactose conc.[b] (mg/ml)	Protein conc.[c] (mg/ml)
Whey	5.95	8036	49.9±5.2	5.7±0.3
10X conc.	----	----	43.5±1.4	60.8±0.2
Diafilt. conc.	5.27	500	~0	45.7±0.3

[a]Corrected for 18°C.

[b]Measured using the sulfuric acid and phenol colourimetric method of Lawrence (1968). Means ± SD; n=4.

[c]Measured using the biuret method. Means ± SD; n=3.

Diafiltered whey, therefore, could be used to isolate IgG from without any problems. Considering it requires more time and large volumes of water for diafiltration, it would be better to use plain concentrated whey.

E. ISOLATING IgG FROM SKIM MILK USING UF AND IMAC

UF together with IMAC was found to be a good method to isolate IgG from CCW. Whey would be an ideal source of IgG since whey is a waste product of cheese and casein manufacture. Zall (1984) stated that 44% of whey is unutilized. Eight percent of whey is used for whey protein concentrate (WPC) manufacture, which is processed mainly by UF. Therefore, using whey concentrated by UF to isolate IgG could be economical. However, if whey is not available, such as in countries with no large cheese or casein manufacture, milk would be the preferred source of IgG. A method to isolate IgG from skim milk was therefore investigated. Figure 5 shows the scheme proposed. First, skim milk is concentrated 5 times with a 500 kDa MWCO membrane. Glover (1985) stated that the maximum concentration which is economically feasible for skim milk is 5 times. The permeate containing IgG is then passed through a 50 kDa MWCO membrane to concentrate IgG. The resulting concentrate is applied to an IMAC column.

The concentrate from the 500 kDa MWCO membrane which contains mostly casein, and the permeate from the 50 kDa MWCO membrane could be recombined. This solution would have the same composition as skim milk minus some proteins, lactose and minerals, and could be used to make skim milk powder. The unbound fraction from the IMAC column could also be combined with the solution to add back more of the lost components except IgG.

Five liters of skim milk was processed using the scheme in Figure 5. The compositions of the various permeates and concentrates are summarized in Table XII. Conductivity remained relatively constant, but dropped in the concentrate from the 500 kDa

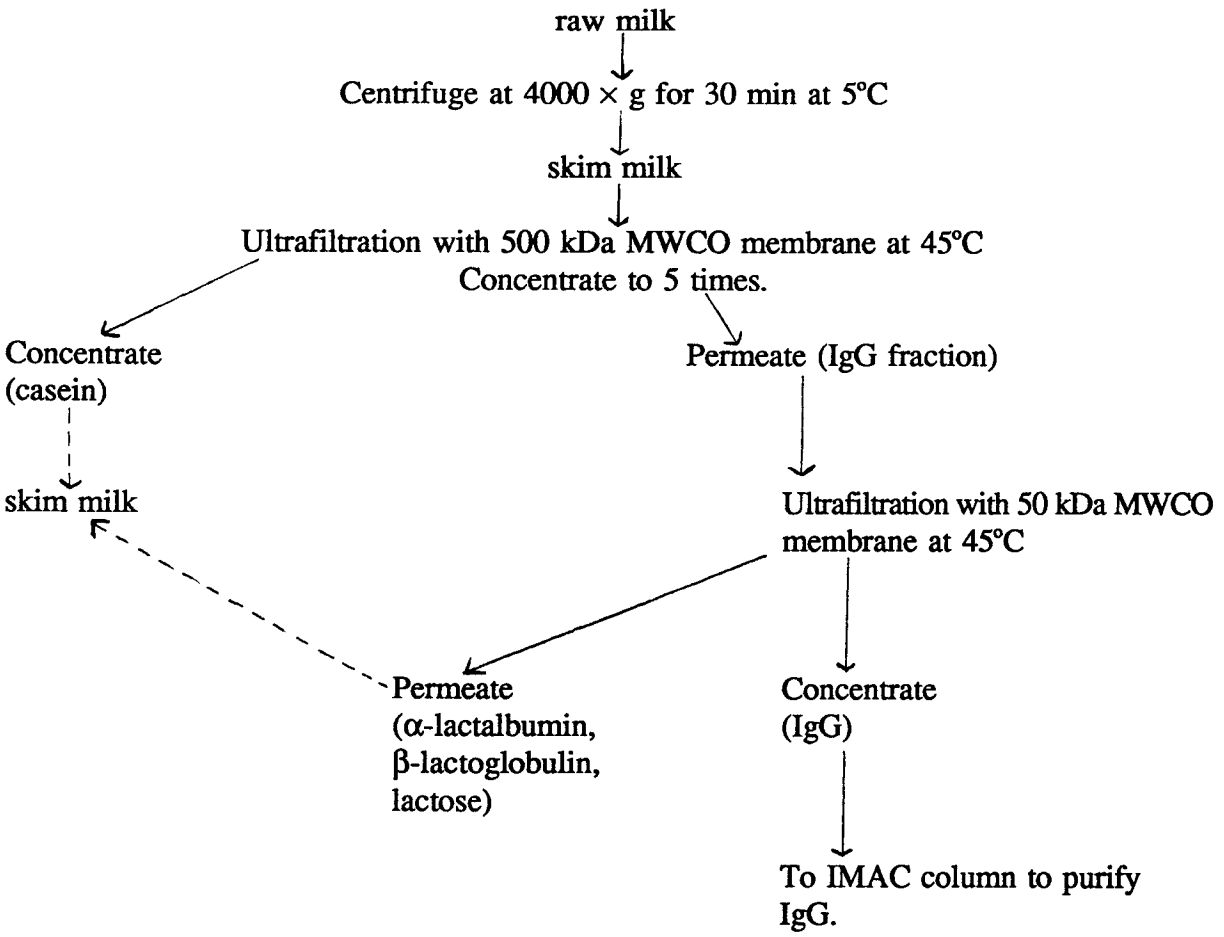

Figure 5. Method to isolate IgG from raw skim milk using ultrafiltration and immobilized metal affinity chromatography.

Table XII. Composition of permeates and concentrates obtained from the ultrafiltration of skim milk to isolate IgG.

Sample	pH	Conductivity[a] (μMho)	Lactose conc.[b] (mg/ml)	IgG conc.[c] (mg/ml) (IgG(mg))	Protein conc.[d] (mg/ml) (Protein (mg))
Skim milk (5000 ml)	6.67	4630	55.9±4.7	0.79 (3950)	21.2±0.4 (106000± 2000)
5 × conc. 500 kDa MWCO (1000 ml)	6.44	3704	45.5±1.5	2.94 (2940)	89.7±6.2 (90000± 6200)
Perm. 500 kDa MWCO (4000 ml)	6.52	5000	53.4±1.4	0.17 (680)	1.1±0.1 (4400±400)
31 × conc. 50 kDa MWCO (129 ml)	5.54	5182	57.9±1.8	5.14 (663)	18.5±1.2 (2400±160)
Perm. 50 kDa MWCO (3871 ml)	6.60	4907	53.5±1.3	not detected	0.6±0.2 (2300±800)

[a]Corrected for 18°C.

[b]Measured using the sulfuric acid and phenol colourimetric method of Lawrence (1968). Means ± SD; n=4.

[c]Measured by radial immunodiffusion. Values are the average of duplicates. The differences between duplicate measurements were less than 10%.

[d]Measured using the biuret method. Means ± SD; n=3.

MWCO membrane. A similar trend was noted for lactose. The decrease was probably caused by the large pore size of the membrane allowing low MW substances to pass through easily. pH remained relatively constant, but decreased significantly in the concentrate from the 50 kDa MWCO membrane, which may be due to changes in the ionic balance due to extensive UF. The protein content of the fractions indicated that only 15% of protein passed through the 500 kDa MWCO membrane, and about 50% of protein passed through the 50 kDa MWCO membrane. About 20% of the IgG in the skim milk was recovered in the permeate from the 500 kDa MWCO membrane. Most of the IgG did not pass through the membrane, but remained in the concentrate.

In other experiments, it was found that the recovery of IgG in the permeate from the 500 kDa MWCO membrane was higher with increased concentration or with diafiltration. 1600 ml of skim milk was processed by four different methods: whey was made by acid precipitation, milk was concentrated 7.27 times using the 500 kDa MWCO membrane, milk was concentrated 2 times and diafiltered with 2400 ml of distilled deionized water using the 500 kDa MWCO membrane, and milk was concentrated 2 times and diafiltered with 3100 ml of water using a 50 kDa MWCO membrane and then concentrated 8.1 times with a 500 kDa MWCO membrane. The IgG concentrations in the final fractions of interest were measured using the PhastImage Gel Analyser to scan the intensity of the IgG heavy band in the SDS-PAGE gels of the fractions. The results are shown in Table XIII.

Table XIII shows skim milk concentrated to 7.27 times had a similar recovery of IgG as diafiltering the 2 times concentrate. Diafiltering skim milk with a 50 kDa MWCO membrane prior to ultrafiltration with a 500 kDa MWCO membrane resulted in poor recovery

Table XIII. The recovery of IgG from 1600 ml of skim milk following various processing methods.

Processing Method	IgG Content (mg)[a]	% recovery of IgG from skim milk[b]
Whey (1600 ml) from acid precipitation of skim milk	461	100
7.27 x concentrate (220 ml) from concentration of skim milk with a 500 kDa MWCO membrane	189	41
Concentrate (1400 ml) from 50 kDa MWCO membrane of 800 ml of permeate and 2400 ml of diafiltrate from 500 kDa MWCO membrane	185	40
Permeate (1314 ml) from 500 kDal MWCO membrane of concentrate from a 50 kDa MWCO membrane diafiltered with 3100 ml of water	24	5

[a]IgG content measured using the PhastImage Gel Analyser of the IgG heavy chain band from SDS-PAGE.

[b]% recovery = IgG content of sample/ IgG content of whey produced from skim milk used as a control × 100%.

of IgG. The concentration factor of IgG in this case was 3.3 times. It therefore appeared that IgG recovery in the permeate from the UF of skim milk with a 500 kDa MWCO membrane increased as concentration increased. The IgG recovery and concentration factor may be logarithmically or exponentially related. Further studies could be done to determine if there is some type of relationship between IgG recovery and concentration factor. Although IgG recovery from skim milk can be improved by concentrating more than 5 times or by diafiltration, diafiltration would be the method of choice since concentrating more than 5 times is not economically feasible due to poor flux beyond 5 times concentration (Glover, 1985). However, diafiltration requires adding water into the system. The composition of the recombined skim milk would therefore change, which may be undesirable. It may be possible to use the permeate from the 50 kDa MWCO membrane as diafiltration medium to prevent volume changes. If large volumes of skim milk are processed, the poor recovery of IgG may be irrelevant.

The poor recovery of IgG may be due to IgG interacting with casein and other proteins restricting passage of IgG through the membrane, or due to the properties of the membrane restricting IgG passage. To determine if casein restricted IgG passage, whey was passed through a 500 kDa MWCO membrane and concentrated 5 times. Approximately 12% of the IgG was recovered in the permeate as measured by the PhastImage Gel Analyser. This experiment indicated that casein in skim milk did not restrict passage of IgG. The membrane was, therefore, probably largely responsible for restricting the passage of IgG. It may be possible to improve recovery of IgG by changing the membrane. The hollow fiber membrane used in this experiment was made of polysulfone which is hydrophobic and so restricts

protein passage. Hvid (1990) mentioned that there are new hydrophilic membranes on the market which are more suitable for use with protein solutions.

The concentrate from the 50 kDa MWCO membrane was applied to a 10 ml CSFF column loaded with 2.2 ml of 50 mM $CuCl_2$. The recovery and purity of IgG obtained from the IMAC column are found in Table XIV and the SDS-PAGE profiles of the fractions are shown in Figure 6. Based on previous results, the recovery of IgG from the column was expected to be around 153 mg. The IgG yield of 101 mg of IgG was slightly lower than expected. In addition, only 48% of the IgG bound to the column was recovered. This indicated that most of the IgG was probably still bound to the column even after elution. Extensive UF of the sample may have caused changes in the conformation of IgG causing it to bind stronger. Changing the elution buffer to 50 mM glycine may have improved recovery as noted in the section titled "F. Effect of Elution Buffer on IgG Recovery Using IMAC". The purity of IgG in the fraction eluted with water was similar to previous results. However, the purity of the fraction eluted with NH_4Cl was about 10% lower than expected which may be partly a reflection of the ineffectiveness of the elution buffer. From the SDS-PAGE profiles, BSA and a low molecular weight protein, possibly lysozyme or α-lactalbumin, were eluted along with IgG and lactoferrin/lactoperoxidase using NH_4Cl. These proteins caused a decrease in the purity of the IgG obtained. Extensive UF may have caused conformational changes in the BSA and the low molecular weight protein causing them to bind stronger to the IMAC column. This experiment showed IgG can be recovered from skim milk using UF and IMAC, but modifications are needed to improve IgG recovery and purity.

Table XIV. IgG recovery and purity of fractions obtained from a 10 ml IMAC column filled with Chelating Sepharose Fast Flow and loaded with 2.2 ml of 50 mM $CuCl_2$ using the concentrate from a 50 kDa MWCO membrane of permeate from the UF of skim milk with a 500 kDa MWCO membrane.

Fraction	IgG recovered (mg)[a]	Purity of IgG (g/100 g protein)[b]
Applied concentrate (51 ml)	260	27.6
Unbound (49 ml)	11	2.3
Wash (144 ml)	not detectable	----
Elution (NH_4Cl) (57 ml)	40	54.1
Elution (dH_2O) (40 ml)	67	~100
EDTA wash (17 ml)	13	86.7

[a]Recovered IgG calculated as IgG activity (mg/ml) by radial immunodiffusion x volume (ml) of fraction. Values are the average of duplicates. The differences between duplicate measurements were less than 10%.

[b]Purity of IgG (g/100 g) calculated as IgG activity by radial immunodiffusion/ total protein x 100. Protein content measured by the biuret method (Means ± SD; n=3.). Values are the average of duplicates. The differences between duplicate measurements were less than 10%.

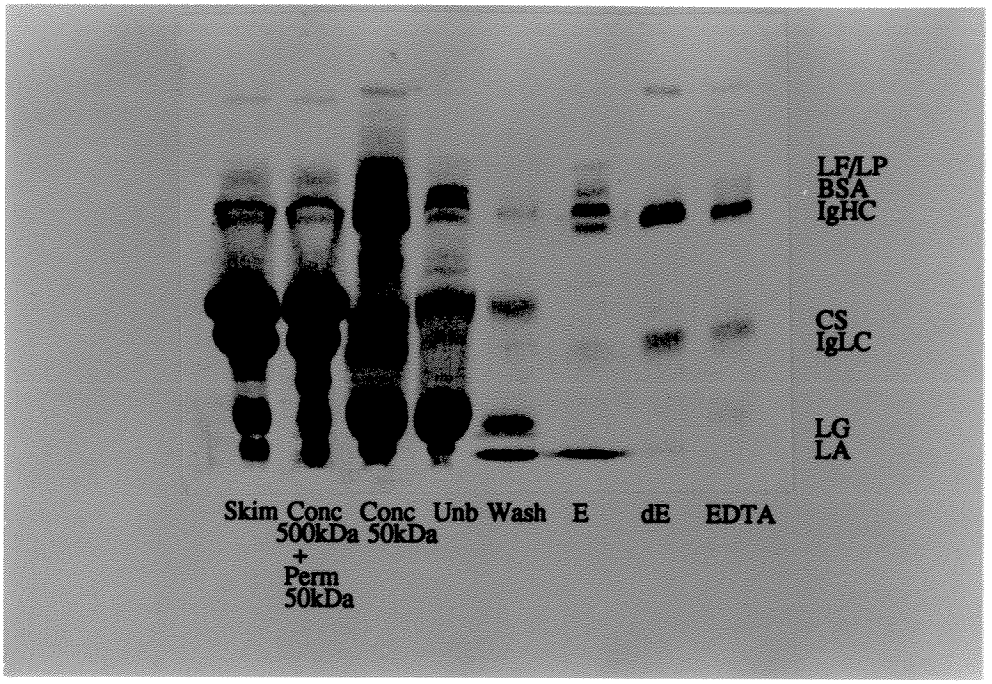

Figure 6. Reduced SDS-PAGE profiles of fractions eluted with 1.0 M NH_4Cl and distilled H_2O after Cu-IMAC treatment of concentrate from a 50 kDa MWCO membrane of permeate from the UF of skim milk with a 500 kDa MWCO membrane using a 10 ml CSFF column loaded with 2.2 ml of 50 mM $CuCl_2$. Abbreviations for fractions: Skim = skim milk, Conc = concentrate (retentate), Perm = permeate, Unb = unbound, Wash = wash, E = peak eluted with 1.0 M NH_4Cl, dE = peak eluted with distilled H_2O, and EDTA = 0.033 M EDTA wash. Abbreviations for bands: LP = lactoperoxidase; LF = lactoferrin; BSA = bovine serum albumin; IgHC, IgLC = IgG heavy and light chains; CS = casein; LG = β-lactoglobulin; and LA = α-lactalbumin.

F. EFFECT OF ELUTION BUFFER ON IgG RECOVERY USING IMAC

A 10 ml IMAC column filled with CSFF was scaled up to a 50 ml column, and was loaded with 17.3 ml of 50 mM $CuCl_2$ to get 2/3 Cu saturation of the column. Sufficient 15 times concentrated CCW was applied to the column to pass the breakthrough point for IgG binding. The column was eluted with 1.0 M NH_4Cl in 50 mM sodium phosphate buffer at pH 8.0. Approximately 55% of the IgG bound to the column was recovered as seen in Table XV. The recovery was found to be similar even if flow rates varied from 1.0-3.3 ml/min. A higher flow rate might also be used. A higher flow rate was not tested, though, since this was beyond the capacity of the pump used.

0.05 N NaOH was used to clean the column. The cleaning effluent from the column was collected and dialyzed. RID for IgG activity was done on the effluent. Surprisingly, the effluent contained IgG, which accounted for the missing 45% of the IgG bound. Since a large portion of the IgG was removed by the cleaning solution, it was concluded that a more effective eluting buffer was needed. Li-Chan et al. (1990) suggested using 0.5-1.0 M NH_4Cl or 50-100 mM glycine or 50-100 mM imidazole in 50 mM sodium phosphate buffer at pH 8.0 to act as competing ligands. Both NH_4Cl and glycine are food grade, but imidazole is not. Glycine was therefore chosen to replace NH_4Cl in the eluting buffer.

Table XVI shows the recovery of IgG obtained using 50 mM glycine in 50 mM sodium phosphate buffer at pH 8.0 instead of 1.0 M NH_4Cl. Most of the bound IgG was recovered during elution with glycine, and very little was recovered during elution with distilled water. When NH_4Cl was used, similar amounts of IgG were eluted with NH_4Cl and with water. This indicated glycine was a better eluting agent. The elution profiles for the

Table XV. IgG recovery and purity of fractions obtained by IMAC of CCW concentrated by UF using a 50 ml Chelating Sepharose Fast Flow column loaded with 17.3 ml of 50 mM $CuCl_2$ and eluted with 1.0 M NH_4Cl in buffer.

Fraction	IgG Recovered [a] (mg)	Purity of IgG[b] (g/100g protein)	Lactoferrin Recovered[c] (mg)	Purity of Lactoferrin[d] (g/100g protein)	Lactoperoxidase Recovered[e] (mg)	Purity of Lactoperoxidase[f] (g/100g protein)
Applied 15.3 × concentrate (248 ml)	1500	8.3	370	2.0	87±0	0.5±0.03
Unbound (250 ml)	260	1.8	130	0.9	ND	----
Wash (480 ml)	40	2.1	<48	~2.5	ND	----
Elution (NH_4Cl) (0-84 ml)	90	17.3	130	25.0	9±0.4	1.7±0.1
Elution (NH_4Cl) (85-403 ml)	190	51.4	<32	~8.6	34±5	9.1±1.4
Elution (dH_2O) (188 ml)	390	72.2	ND	----	2±0.4	0.4±0.1
EDTA wash (90 ml)	50	71.4	ND	----	<1	<0.1
0.05 N NaOH wash (115 ml)	580	52.7	ND	----	12±1	1.1±0.1
Equivalent CCW (3794 ml)	1500	5.6	380	1.4	29±0	<0.1
Permeate (3546 ml)	ND[g]	----	ND	----	1±0.2	<0.1

[a]Recovered IgG calculated as IgG activity (mg/ml) by radial immunodiffusion x volume (ml) of fraction. Values are the average of duplicates. The differences between duplicate measurements were less than 10%.

[b]Purity of IgG (g/100g) calculated as IgG activity by radial immunodiffusion/total protein in fraction x 100. Protein content measured by the biuret method (Means ± SD; n=3.). Values are the average of duplicates. The differences between duplicate measurements were less than 10%.

[c]Recovered lactoferrin calculated as lactoferrin activity (mg/ml) by radial immunodiffusion x volume (ml) of fraction. Values are the average of duplicates. The differences between duplicate measurements were less than 10%.

[d]Purity of lactoferrin (g/100g) calculated as lactoferrin activity by radial immunodiffusion/total protein in fraction x 100. Protein content measured by the biuret method (Means ± SD; n=3.). Values are the average of duplicates. The differences between duplicate measurements were less than 10%.

[e]Recovered lactoperoxidase calculated as lactoperoxidase activity (mg/ml) by ABTS assay for lactoperoxidase activity x volume of fraction. Means ± SD; n=4.

[f]Purity of lactoperoxidase (g/100g) calculated as lactoperoxidase activity by ABTS assay for lactoperoxidase/total protein in fraction x 100. Protein content measured by the biuret method (Means ± SD; n=3.). Values are means ± SD.

[g]Not detectable.

Table XVI. IgG recovery and purity of fractions obtained by IMAC of CCW concentrated by UF using a 50 ml Chelating Sepharose Fast Flow column loaded with 17.3 ml of 50 mM $CuCl_2$ and eluted with 50 mM glycine in buffer.

Fraction	IgG Recovered[a] (mg)	Purity of IgG[b] (g/100g protein)	Lactoferrin Recovered[c] (mg)	Purity of Lactoferrin[d] (g/100g protein)	Lactoperoxidase Recovered[e] (mg)	Purity of Lactoperoxidase[f] (g/100g protein)
Applied 13.6 × concentrate (280 ml)	1600	8.6	410	2.2	81±9	0.4±0.1
Unbound (285 ml)	250	1.6	150	1.0	ND	----
Wash (580 ml)	90	5.3	ND	----	ND	----
Elution (gly) (0-49 ml)	170	60.7	13	4.6	0.5±0.1	0.2±0.02
Elution (gly) (50-78 ml)	740	49.3	79	5.3	17±2	1.1±0.1
Elution (gly) (79-251 ml)	330	38.4	60	7.0	70±5	8.1±1
Elution (dH_2O) (90 ml)	30	60.0	ND	----	1±0.1	2.0±0.3
EDTA wash (80 ml)	ND[g]	----	ND	----	<1	<0.1
0.05 N NaOH wash (92 ml)	40	40.0	60	60.0	1±0.1	1.0±0.1
Equivalent CCW (3808 ml)	1600	5.5	400	1.4	6±1	<0.1
Permeate (3528 ml)	ND	----	ND	----	1±0.1	<0.1

[a]Recovered IgG calculated as IgG activity (mg/ml) by radial immunodiffusion x volume (ml) of fraction. Values are the average of duplicates. The differences between duplicate measurements were less than 10%.

[b]Purity of IgG (g/100g) calculated as IgG activity by radial immunodiffusion/total protein in fraction x 100. Protein content measured by the biuret method (Means ± SD; n=3.). Values are the average of duplicates. The differences between duplicate measurements were less than 10%.

[c]Recovered lactoferrin calculated as lactoferrin activity (mg/ml) by radial immunodiffusion x volume (ml) of fraction. Values are the average of duplicates. The differences between duplicate measurements were less than 10%.

[d]Purity of lactoferrin (g/100g) calculated as lactoferrin activity by radial immunodiffusion/total protein in fraction x 100. Protein content measured by the biuret method (Means ± SD; n=3.). Values are the average of duplicates. The differences between duplicate measurements were less than 10%.

[e]Recovered lactoperoxidase calculated as lactoperoxidase activity (mg/ml) by ABTS assay for lactoperoxidase activity x volumne of fraction. Means ± SD; n=4.

[f]Purity of lactoperoxidase (g/100g) calculated as lactoperoxidase activity by ABTS assay for lactoperoxidase/total protein in fraction x 100. Protein content measured by the biuret method (Means ± SD; n=3.). Values are means ± SD.

[g]Not detectable.

two buffers are seen in Figures 7 and 8. The elution profile for NH_4Cl did not form a sharp peak and tailed off. The elution profile for glycine showed a sharp peak with little tailing. These figures indicate glycine was a better eluting agent than NH_4Cl.

As a control, CCW was applied directly to a 50 ml CSFF column loaded with 13.5 ml of 50 mM $CuCl_2$ and eluted with glycine. The column was loaded with less $CuCl_2$ than when using concentrated whey since the Cu front migrated more when applying whey than concentrated whey. The recovery and purity of IgG in fractions obtained from the IMAC column are shown in Table XVII. The recovery of 1090 mg of IgG in the fraction eluted with glycine was close to that expected for concentrated whey. It was expected that IgG recovery would be about 938 mg (13.5 ml x 1202 mg/17.3 ml). The value of 1202 mg was based on the average recovery of IgG eluted with glycine in 5 trials using concentrated whey and a 50 ml IMAC column loaded with 17.3 ml of 50 mM $CuCl_2$. The standard deviation was 85 mg. The purities were also similar. These results indicated that eluting with glycine gave similar IgG recovery for both concentrated CCW or unconcentrated CCW. By using glycine instead of NH_4Cl, elution is more efficient and so saves time. There may not be any need to dialyze or diafilter the eluted fraction if glycine is used too, since its molarity is low. Glycine, being an amino acid, would not drastically affect the flavour of the IgG solution, and could also add to the nutritional value of the IgG solution. If NH_4Cl is used, the solution would have to be dialyzed or diafiltered since the high salt concentration would affect the flavour.

Tables XV and XVI indicate the purity of IgG was similar whether glycine or NH_4Cl was used. Figures 9 and 10 show the SDS-PAGE profiles of fractions obtained using NH_4Cl

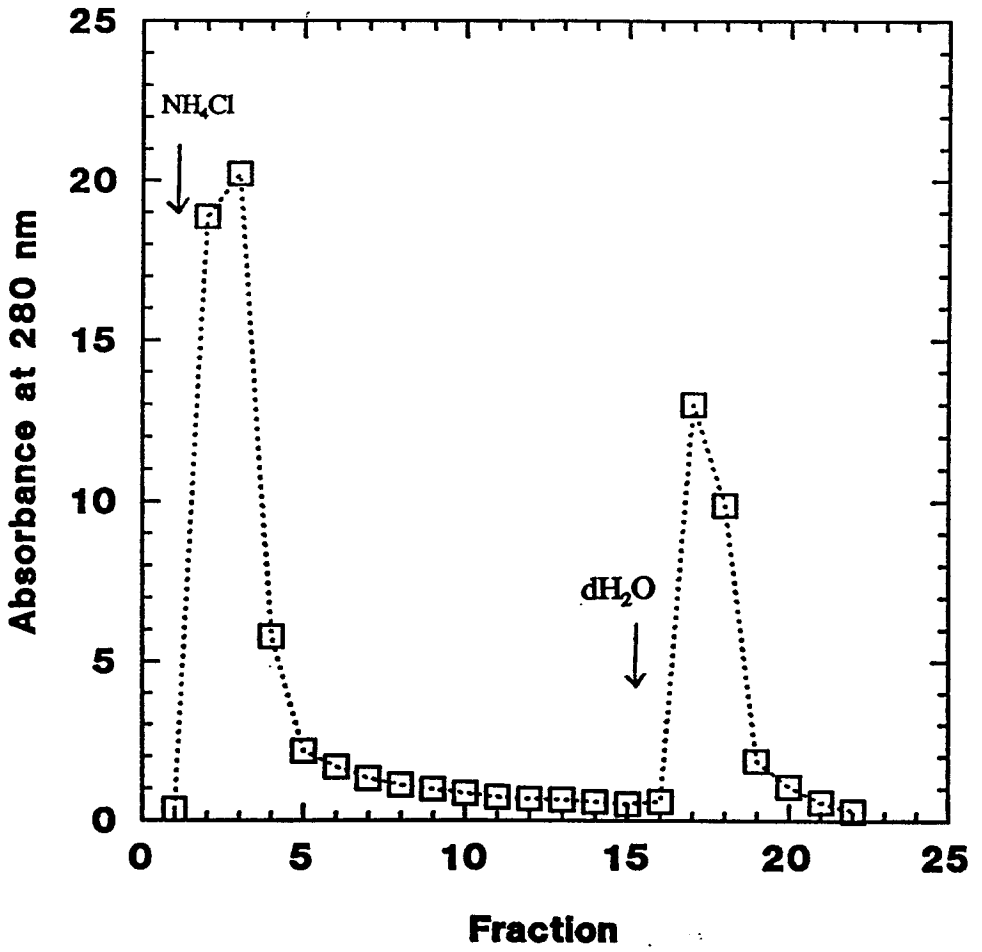

Figure 7. Elution profile of adsorbed proteins from a 50 ml CSFF column loaded with 17.3 ml of 50 mM $CuCl_2$ using 1.0 M NH_4Cl in buffer and distilled water. The flow rate was 2.2 ml/min. Fraction volumes were 25 ml.

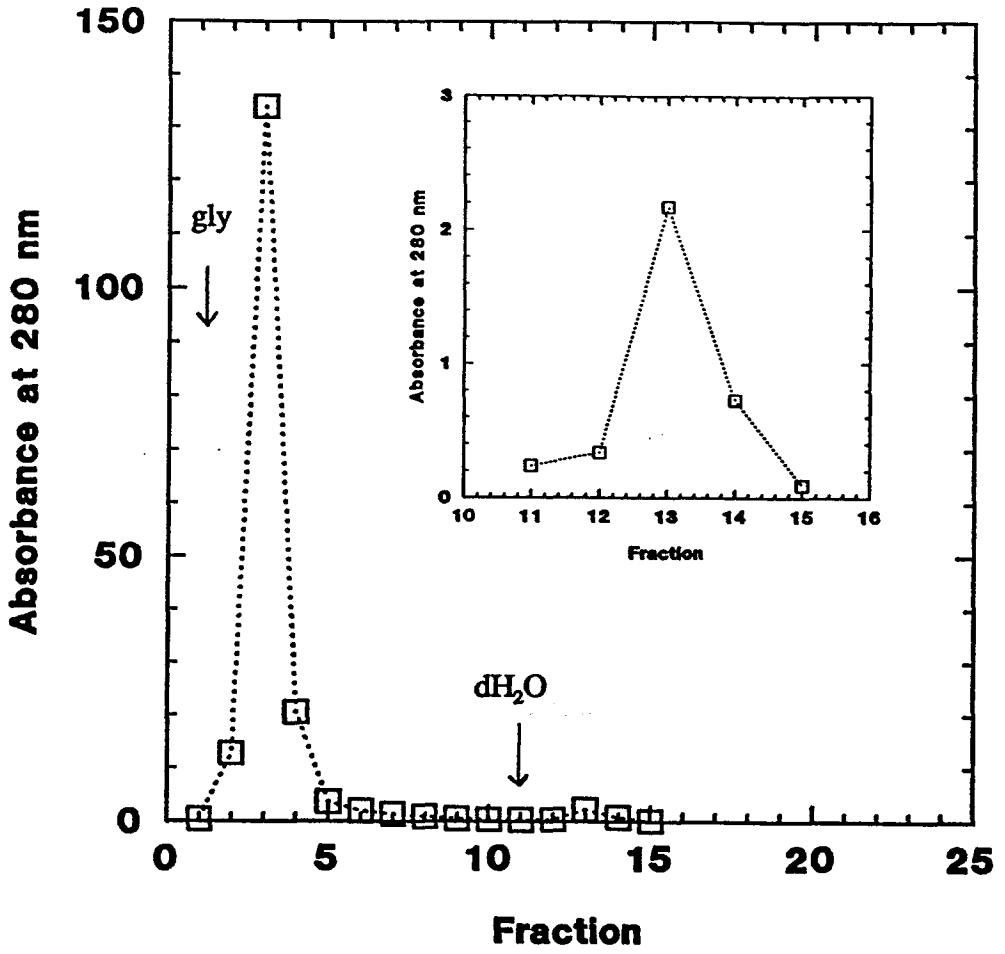

Figure 8. Elution profile of adsorbed proteins from a 50 ml CSFF column loaded with 17.3 ml of 50 mM $CuCl_2$ using 50 mM glycine in buffer and distilled water. The flow rate was 2.5 ml/min. Fraction volumes were 25 ml.

Table XVII. IgG recovery and purity of fractions obtained by IMAC of Cheddar cheese whey using a 50 ml Chelating Sepharose Fast Flow column loaded with 13.5 ml of 50 mM $CuCl_2$ and eluted with 50 mM glycine in buffer.

Fraction	IgG Recovered [a] (mg)	Purity of IgG[b] (g/100 g protein)
Applied whey (3200 ml)	1600	7.0
Unbound (3160 ml)	570	3.0
Wash (320 ml)	<40	<26.7
Elution (glycine) (0-49 ml)	200	54.1
Elution (glycine) (50-78 ml)	650	59.1
Elution (glycine) (79-179 ml)	240	41.4
Elution (dH_2O) (0-101 ml)	34	69.4
EDTA wash (70 ml)	not detectable	----
0.05 N NaOH wash (90 ml)	26	41.2

[a]Recovered IgG calculated as IgG activity (mg/ml) by radial immunodiffusion x volume (ml) of fraction. Values are the average of duplicates. The differences between duplicate measurements were less than 10%.

[b]Purity of IgG (g/100 g) calculated as IgG activity by radial immunodiffusion/total protein in fraction x 100. Protein content measured by the biuret method (Means ± SD; n=3.). Values are the average of duplicates. The differences between duplicate measurements were less than 10%.

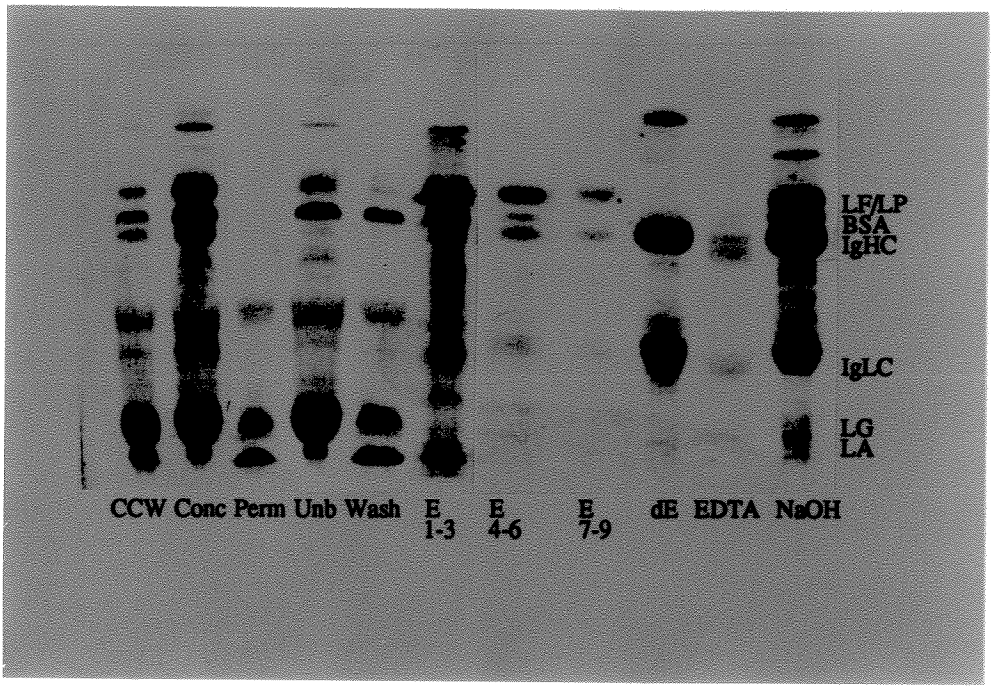

Figure 9. Reduced SDS-PAGE profiles of fractions eluted with 1.0 M NH_4Cl and distilled H_2O after Cu-IMAC treatment of concentrated whey using a 50 ml CSFF column loaded with 17.3 ml of 50 mM $CuCl_2$. Abbreviations for fractions: CCW = Cheddar cheese whey, Conc = concentrate (retentate), Perm = permeate, Unb = unbound, Wash = wash, E = peak eluted with 1.0 M NH_4Cl (numbers are fraction numbers, where fraction volume = 25 ml), and dE = peak eluted with distilled H_2O, EDTA = 0.033 M EDTA wash, and NaOH = 0.05 N NaOH cleaning. Abbreviations for bands: LP = lactoperoxidase; LF = lactoferrin; BSA = bovine serum albumin; IgHC, IgLC = IgG heavy and light chains; LG = β-lactoglobulin; and LA = α-lactalbumin.

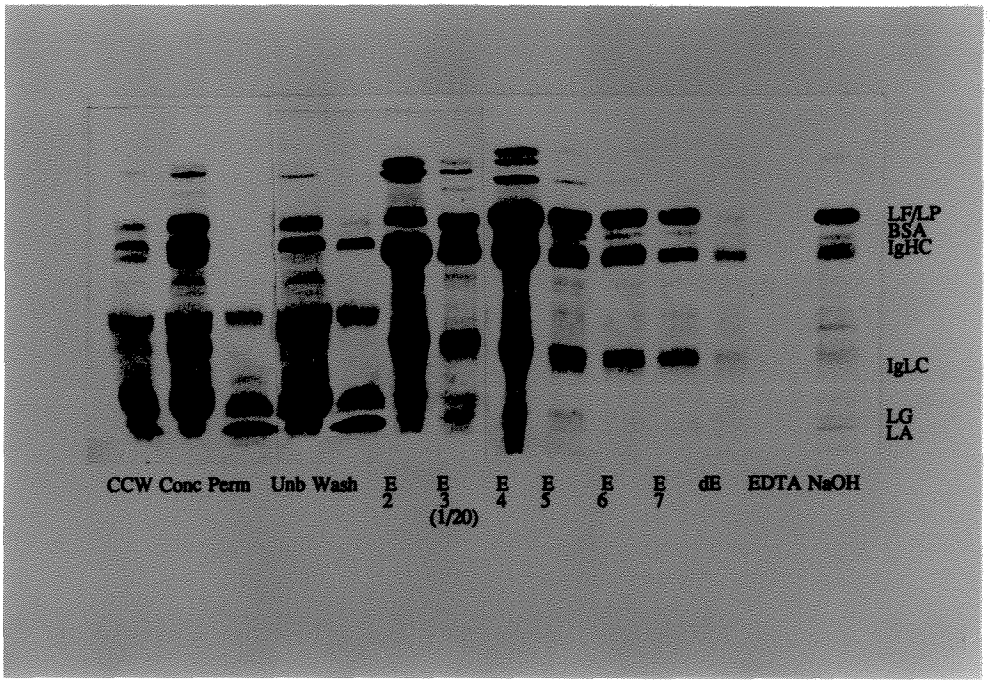

Figure 10. Reduced SDS-PAGE profiles of fractions eluted with 50 mM glycine and distilled H_2O after Cu-IMAC treatment of concentrated whey using a 50 ml CSFF column loaded with 17.3 ml of 50 mM $CuCl_2$. Abbreviations for fractions: CCW = Cheddar cheese whey, Conc = concentrate (retentate), Perm = permeate, Unb = unbound, Wash = wash, E = peak eluted with 50 mM glycine (numbers are fraction numbers, where fraction volume = 25 ml), and dE = peak eluted with distilled H_2O, EDTA = 0.033 M EDTA wash, and NaOH = 0.05 N NaOH cleaning. Abbreviations for bands: LP = lactoperoxidase; LF = lactoferrin; BSA = bovine serum albumin; IgHC, IgLC = IgG heavy and light chains; LG = β-lactoglobulin; and LA = α-lactalbumin.

or glycine in the eluting buffer. The SDS-PAGE profiles also indicate that the purity of IgG in the various fractions was similar. The SDS-PAGE profiles show most of the β-lactoglobulin, α-lactalbumin and bovine serum albumin (BSA) were eliminated leaving mostly IgG, lactoferrin and lactoperoxidase. Some high molecular weight proteins were also eluted with the IgG. These proteins may be IgM and IgA. Further studies would be needed to identify these proteins, though. The bands for IgG were more intense when glycine was used instead of NH_4Cl, even though the samples had been prepared identically. This supported the findings using RID, which indicated IgG recovery was higher using glycine than NH_4Cl. If gradient elution is used, the purity of IgG might be improved if increased purity is desired. Gel filtration could also be used to further purify IgG.

Tables XV and XVI show the recovery and purity of lactoferrin and lactoperoxidase obtained using the IMAC column with either NH_4Cl or glycine in the eluting buffer. Approximately 35% of the lactoferrin applied to the column did not bind. Of the lactoferrin applied to the column, 57% was recovered with NH_4Cl elution, and 37% was recovered with glycine elution. Most of the lactoperoxidase applied to the column was bound. About 52% of the lactoperoxidase applied to the column was recovered by eluting with NH_4Cl whereas almost all was recovered when eluting with glycine. Li-Chan et al. (1990) recovered 88% of the total lactoperoxidase activity, of the whey applied, in the wash. The amount of lactoperoxidase bound to the IMAC column was similar to the result of Li-Chan et al. (1990). It appeared the lactoperoxidase bound stronger to the column in this study, though. The whey used, UF, or the particular batch of IMAC support used may have affected the binding of lactoperoxidase. The differences in how much IgG, lactoferrin or lactoperoxidase were

eluted with either NH_4Cl or glycine were probably a reflection of how these competing ligands interact with the column.

The amount of lactoferrin and lactoperoxidase measured in the concentrate was comparable to literature values. Glover (1985) stated the Ig content in milk is 0.06%, lactoferrin is 0.02% and lactoperoxidase is 0.003%. Assuming 3800 ml of milk would produce an equivalent amount of whey, the Ig content of the whey would be 2280 mg, the lactoferrin content would be 760 mg and the lactoperoxidase content would be 114 mg. These values tend to be slightly higher than those found in this experiment. An exact comparison of values is difficult though, since milk composition varies with season, stage of lactation, age of cow, and processing conditions to name a few (Jenness, 1988).

Based on the purity values for lactoferrin and lactoperoxidase, these two proteins account for only a small fraction of the total protein in whey, but their concentration increased considerably in the eluted fractions. The lactoferrin and lactoperoxidase isolated along with the IgG could add to the immunologic activity of the IgG solution. Both have antimicrobial activity. However, the activity of lactoferrin is dependent on iron content. The lactoferrin should remain unsaturated to have good activity. Payne et al. (1990) found the activity of bovine lactoferrin against Listeria monocytogenes in UHT 2% fat milk was dependent on the concentration and the degree of iron saturation. Lactoferrin with 52% iron saturation resulted in minimal growth of L. monocytogenes at concentrations of 23 and 46 mg/ml. Lactoferrin with 18% iron saturation (apo-lactoferrin) resulted in minimal growth of L. monocytogenes at concentrations of 15 and 30 mg/ml.

The only disadvantage in using glycine was glycine caused the Cu front to migrate

when concentrated whey was used. After application of concentrated whey, the Cu front moved 0.4 cm, but after elution with glycine, the Cu front moved a total of 1.7 cm. After application of unconcentrated whey, the Cu front moved 2 cm, and after elution, the Cu front moved a total of 2.5 cm. The amount of $CuCl_2$ loaded onto the column could be adjusted to prevent Cu contamination of the effluent, though.

Based on this study, glycine should be used for elution instead of NH_4Cl when using concentrated whey with IMAC. Glycine appears to be a stronger competing ligand. Concentrated whey might require a stronger eluting buffer since UF may cause slight conformational changes in IgG causing it to bind better to the column. The change in conformation probably does not result in the loss of IgG activity since RID values remained high. Based on these results, a method to obtain IgG from concentrated whey is outlined in Figure 11. By centrifuging the concentrated whey before it was applied to the IMAC column, fat was removed. Fat can bind to the IMAC column soiling it. For large-scale removal of fat from whey, crossflow microfiltration could be used. Merlin and Daufin (1990) mention that crossflow microfiltration can be used to remove fat, calcium ions, casein fines and bacteria from whey. The flux during UF of microfiltered whey would also be improved. Considering fat can have adverse effects on whey protein concentrates (WPC), crossflow microfiltration of whey prior to UF could also improve WPC made by UF.

During the course of this study, evidence was also collected by Li-Chan et al. (1992) indicating the Cheddar cheese whey used had activity against certain pathogens. Li-Chan et al. (1992) tested the activity of IgG in milk products against lipopolysaccharide fractions of specific bacteria. Figure 12 shows the specific activity of IgG in various milk products. The

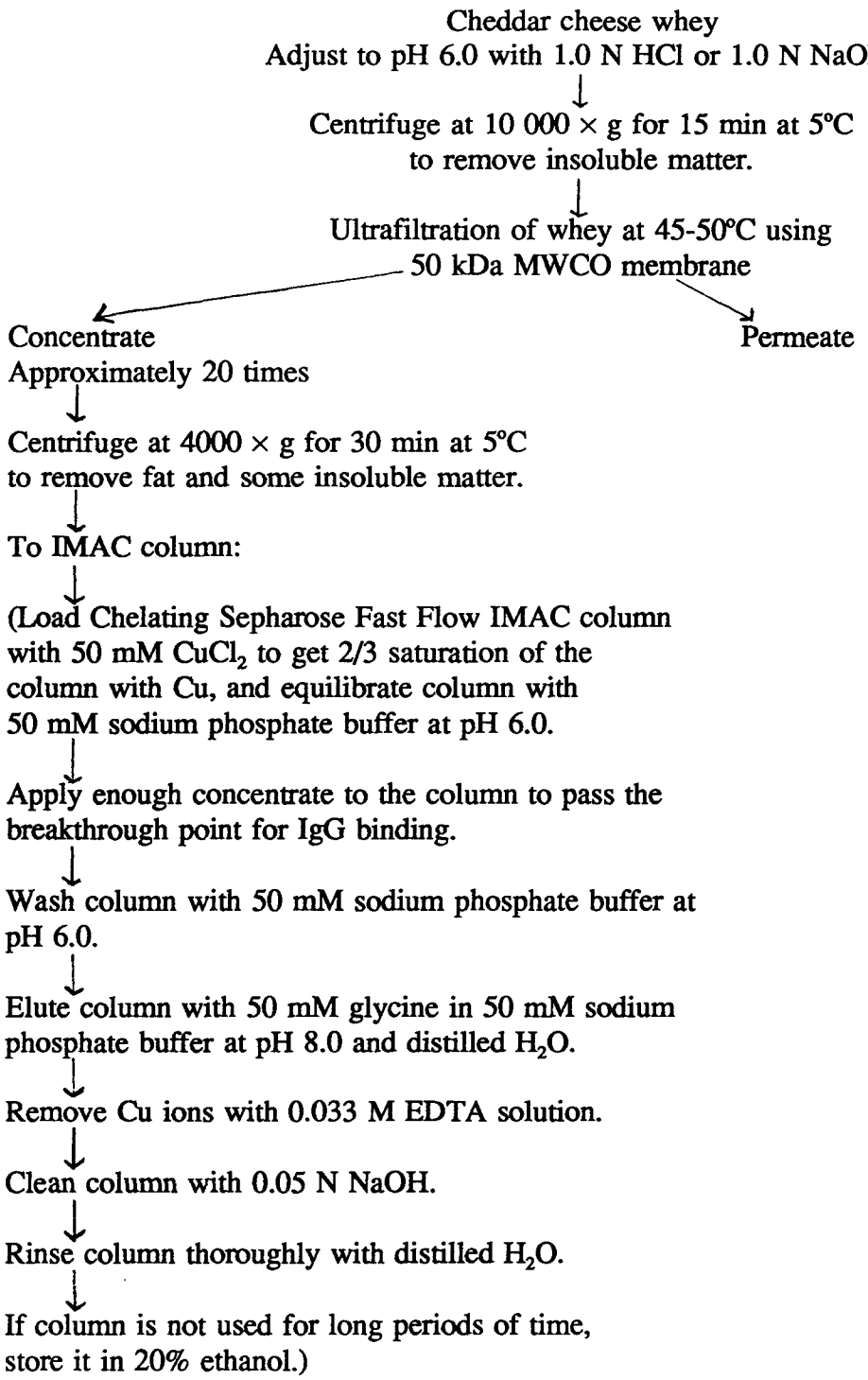

Figure 11. Final method to isolate IgG from Cheddar cheese whey using ultrafiltration and immobilized metal affinity chromatography.

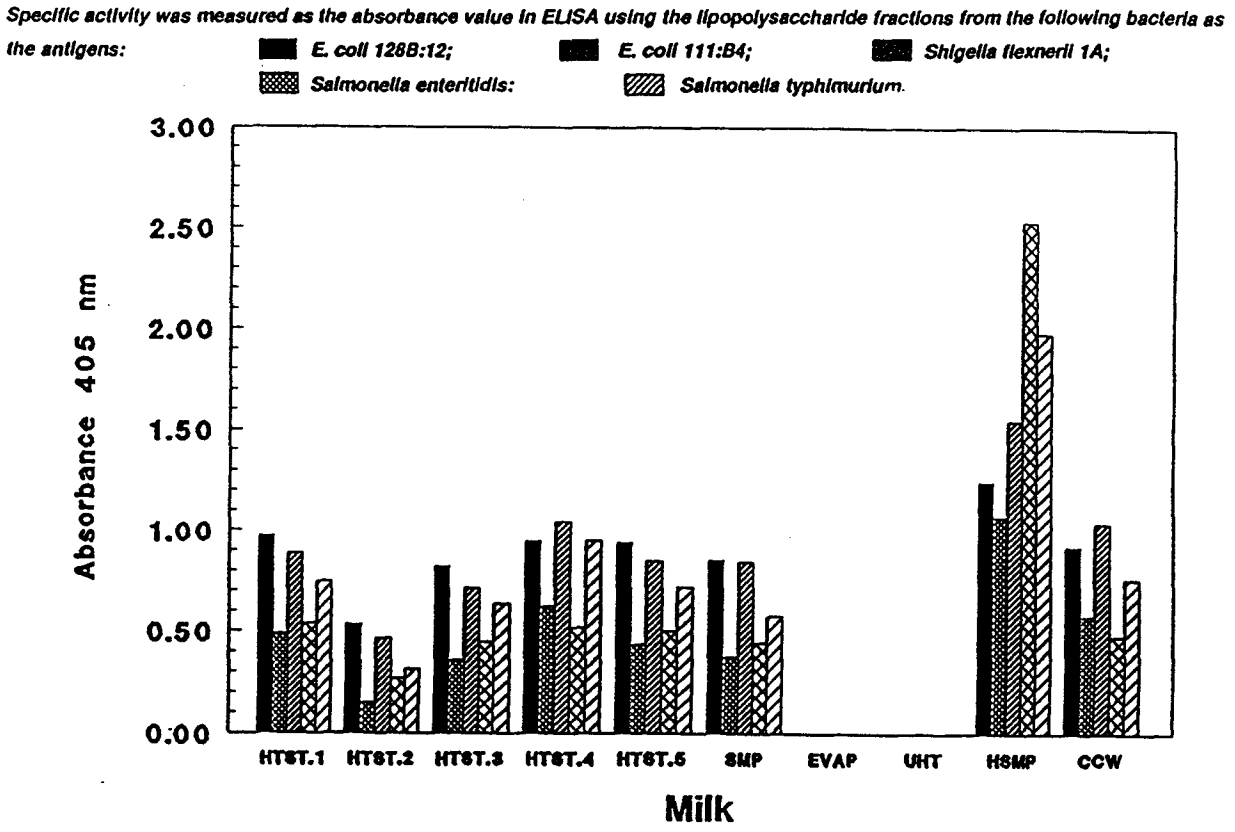

Figure 12. Specific activity of IgG in various milk products. Abbreviations for milk products: HTST=high temperature-short time pasteurized, SMP=skim milk powder, EVAP=canned evaporated milk, UHT=ultra-high-temperature sterilized milk, HSMP=skim milk powder from the milk of hyperimmunized cows, and CCW=Cheddar cheese whey. (Adapted from Li-Chan et al. (1992)).

specific bacteria tested against were E. coli 128B:12, E. coli 111:B4, Shigella flexnerii 1A, Salmonella enteritidis and Salmonella typhimurium. The results show that the CCW used in this experiment had similar activity to HTST pasteurized milk. Canned evaporated milk and UHT milk had no specific activity probably due to severe heat treatment of these samples. Skim milk powder from the milk of hyperimmunized cows showed the highest activity. These results indicate that IgG in Cheddar cheese whey may be active against some bacteria. The levels of IgG needed to prevent infection would have to be determined, though.

G. COMPARISON OF THE BIURET METHOD AND KJELDAHL METHOD TO MEASURE PROTEIN CONTENT

The protein content of fractions from the IMAC column was measured using a modified biuret method. Since some fractions tended to be cloudy, a correction factor which consisted of measuring the absorbance of the sample with 0.6 N NaOH was subtracted from the absorbance of the sample with reagent to find the protein concentration. The accuracy of the protein concentration was questionable even with the correction factor. Kjeldahl protein determination was run on some samples to see if the values obtained were comparable to those obtained using the biuret method. Table XVIII shows the comparison of values.

From Table XVIII, it appears that the biuret and Kjeldahl methods gave similar results in most cases. When there were large differences in values, the Kjeldahl values tended to be lower. It is difficult to determine which value was more accurate, though. The biuret method was subject to error depending on the cloudiness of the sample and the concentration of protein in the sample. In some samples, the protein content was less than 1.0 mg/ml. The biuret method was accurate between 0.0-10.0 mg of protein/ml, but was less sensitive when the concentration was around 1.0-2.0 mg/ml. The Kjeldahl method depended on the conversion factor of N x 6.38. The purity of some fractions therefore may not be accurate, but were probably reasonable judging from the SDS-PAGE profiles. SDS-PAGE bands are not necessarily a reflection of concentration, though, since different proteins stain differently.

Table XVIII. Comparison of the protein content of fractions obtained from IMAC using the biuret and Kjeldahl methods.

Fraction	Protein content (mg/ml)	
	Biuret Method[a]	Kjeldahl Method[b]
Cheddar cheese whey	7.2±0.1	7.49
Concentrate 15.4 ×	73.1±5.1	77.29
Permeate	3.2±0.1	2.99
Unbound	58.5±1.7	56.21
Wash	4.1±0.1	3.89
Elution (NH_4Cl) (0-84 ml)	6.3±0.2	5.59
Elution (NH_4Cl) (85-319 ml)	1.2±0.1	1.34
Elution (dH_2O)	2.9±0.1	2.58
EDTA wash	0.8±0.7	0.76
0.05 N NaOH wash	9.5±0.5	8.52
Cheddar cheese whey	7.6±0.3	7.39
Concentrate 13.6 ×	70.2±3.2	59.08
Permeate	2.8±0.1	2.85
Unbound	54.4±2.8	51.50
Wash	2.9±0.1	2.49
Elution (glycine) (0-49 ml)	5.7±0.1	4.15
Elution (glycine) (50-78 ml)	53.1±1.6	47.67
Elution (glycine) (79-251 ml)	5.0±0.1	4.64
Elution (dH_2O)	0.5±0.1	0.43
EDTA wash	3.2±0.1	0.43
0.05 N NaOH wash	1.1±0.1	1.54

[a] Means ± SD; n=3.

[b] Sufficient volumes of samples were placed in micro-Kjeldahl flasks to obtain a protein content of 6-10 mg, and then samples were oven-dried before Kjeldahl digestion was done.

H. EFFECT OF WHEY USED ON THE ISOLATION OF IgG USING UF AND IMAC

White CCW was obtained in addition to regular CCW. The processing of the white Cheddar cheese and regular Cheddar cheese differed only in the heating of the milk prior to cheesemaking and no colouring being added. The milk for white Cheddar cheese was pasteurized at 72°C for 15 s. The milk for regular Cheddar cheese was only heat-treated at 67°C for 15 s. The white CCW was ultrafiltered in the same manner as the regular CCW. 257 ml of a 14 times concentrate of white CCW was applied to a 50 ml CSFF column loaded with 17.3 ml of 50 mM $CuCl_2$. Table XIX summarizes the recovery and purity of IgG from the IMAC column. 1080 mg of IgG was recovered in the fraction eluted with glycine. An average recovery of 1202 mg of IgG was eluted with glycine using regular whey in 5 trials. The standard deviation was 85 mg. The purity of IgG was similar for both white and regular CCW. The lactoferrin recovery and purity for white CCW were similar to values obtained for regular CCW as seen in Table XVI.

Although the recovery of IgG from white CCW was not much lower than for regular CCW; the lower recovery was also noted when 1.0 M NH_4Cl was used in the eluting buffer. The D values obtained for IgG in the section titled "L. Thermal Resistance Curve for IgG" indicate that heating at 67° or 72°C for only 15 s should not affect IgG activity. The decrease in recovery was probably not a direct loss of IgG activity from heating. However, the exact heating conditions would have to be known. Perhaps the difference in heating temperatures caused conformational changes in IgG resulting in a slight decrease in IgG binding. The difference in heating temperatures may have also caused changes in other whey proteins resulting in a slight decrease in IgG binding.

Table XIX. IgG recovery and purity of fractions obtained by IMAC of white CCW concentrated by UF using a 50 ml Chelating Sepharose Fast column loaded with 17.3 ml of 50 mM $CuCl_2$ and eluted with 50 mM glycine in buffer.

Fraction	IgG recovered (mg)[a]	Purity of IgG (g/100 g protein)[b]	Lactoferrin recovery (mg)[c]	Purity of lactoferrin (g/100 g protein)[d]
Applied 14 × concentrate (257 ml)	1500	6.4	380	1.6
Unbound (263 ml)	210	1.1	210	1.1
Wash (643 ml)	150	8.3	ND	----
Elution (glycine) (0-51 ml)	130	39.4	ND	----
Elution (glycine) (52-78 ml)	660	50.8	70	5.4
Elution (glycine) (79-250 ml)	290	46.8	60	9.7
Elution (dH_2O) (78 ml)	30	50.0	ND	----
EDTA wash (80 ml)	ND[e]	----	ND	----
0.05 N NaOH (85 ml)	40	50.0	40	50.0
Equivalent volume of whey (3598 ml)	1700	6.5	not measured	----
Permeate (3341 ml)	ND	----	ND	----

[a]Recovered IgG calculated as IgG activity (mg/ml) by radial immunodiffusion x volume (ml) of fraction. Values are the average of duplicates. The differences between duplicate measurements were less than 10%.

[b]Purity of IgG (g/100 g) calculated as IgG activity by radial immunodiffusion/total protein in fraction x 100. Protein content measured by the biuret method (Mean ± SD; n=3). Values are the average of duplicates. The differences between duplicate measurements were less than 10%.

[c]Recovered lactoferrin calculated as lactoferrin activity (mg/ml) by radial immunodiffusion x volume (ml) of fraction. Values are the average of duplicates. The differences between duplicate measurements were less than 10%.

[d]Purity of lactoferrin (g/100 g) calculated as lactoferrin activity by radial immunodiffusion/total protein in fraction x 100. Protein content measured by the biuret method (Mean ± SD; n=3). Values are the average of duplicates. The differences between duplicate measurements were less than 10%.

[e]Not detectable.

Mozzarella whey was also used. Judging from the SDS-PAGE profiles, more β-lactoglobulin and BSA (bovine serum albumin) were bound decreasing the purity of IgG in this case. When whey made by the acid precipitation of casein in pasteurized skim milk was used, the results were similar to those obtained for mozzarella whey. CCW was therefore found to give the best results. Considering sweet whey is preferred over acid whey for many applications including the manufacture of whey protein concentrates (Hill, 1982), CCW would be ideal to use. A whey should first be tested with IMAC to see what results to expect.

I. EFFECT OF TEMPERATURE ON IMAC

The IMAC column was usually run at room temperature. To slow microbial growth, the column should be run a temperature less than 10°C (Glover, 1985). To see if a lower temperature affected the running of the IMAC column, the column was run in a coldroom. The temperature in the coldroom was 7°C. To a 50 ml CSFF column loaded with 17.3 ml of 50 mM $CuCl_2$, 258 ml of 13.5 times concentrated CCW was applied. The concentrate was applied at a rate of 2.0 ml/min. Fifty mM glycine in 50 mM sodium phosphate buffer at pH 8.0 and distilled water were used to elute the bound proteins.

The recovery and purity of IgG obtained from the IMAC column are shown in Table XX. The purity of IgG was similar whether IMAC was run at 7°C or room temperature. The recovery of IgG eluted with glycine was 1060 mg which was slightly lower than results obtained at room temperature. At room temperature, the average recovery of IgG eluted with glycine was 1202 mg with a standard deviation of 85 mg for 5 trials.

The overall recovery of IgG was similar at both 7°C and room temperature, but the distribution of IgG in the eluted fractions varied. At 7°C, the recovery of IgG eluted with distilled water was 200 mg, which was higher than results obtained at room temperature. At room temperature, the average recovery of IgG eluted with distilled water was 27 mg with a standard deviation of 4 mg for 5 trials. There was an increase of IgG in the fraction cleaned with NaOH at 7°C as well. These changes seem to support the idea that IgG binding was affected by temperature. A lower temperature may have caused changes in the conformation of IgG affecting the interaction of IgG with the column. The increase in viscosity with decreased temperature may also have affected the binding of IgG. The

Table XX. IgG recovery and purity of fractions obtained by IMAC of concentrated whey using a 50 ml Chelating Sepharose Fast Flow column loaded with 17.3 ml of 50 mM $CuCl_2$ and run at 7°C.

Fraction	IgG recovered (mg)[a]	Purity of Ig (g/100 g protein)[b]
Applied 13.5 times concentrate (258 ml)	1500	8.2
Unbound (270 ml)	280	1.7
Wash (660 ml)	150	9.4
Elution (glycine) (0-99 ml)	860	44.4
Elution (glycine) (100-288 ml)	200	57.1
Elution (dH_2O) (102 ml)	200	58.8
EDTA wash (80 ml)	ND[c]	----
0.05 N NaOH wash (100 ml)	190	51.4
Equivalent volume of whey (3483 ml)	1700	6.3
Permeate (3225 ml)	ND	----

[a]Recovered IgG calculated as IgG activity (mg/ml) by radial immunodiffusion x volume (ml) of fraction. Values are the average of duplicates. The differences between duplicate measurements were less than 10%.

[b]Purity of IgG (g/100 g) calculated as IgG activity by radial immunodiffusion/total protein in fraction x 100. Protein content measured by the biuret method (Means ± SD; n=3.). Values are the average of duplicates. The differences between duplicate measurements were less than 10%.

[c]Not detectable.

decrease in IgG during elution with glycine indicated that the eluting buffer was not as effective at the lower temperature. This may have possibly been due to stronger binding of IgG, or the lower temperature affecting the behaviour of glycine with the IMAC column.

Figure 13 shows the elution profile for the column. The tailing of the peak eluted with glycine suggests 50 mM glycine may not have been strong enough. This profile is similar to that seen when 1.0 M NH_4Cl was used instead of 50 mM glycine in the eluting buffer as shown in Figures 7 and 8. Increasing the concentration of glycine may improve the effectiveness of the eluting buffer, so less IgG comes off in the fraction eluted with water. The results indicated that IMAC can be run at a lower temperature. One disadvantage in running the column at a lower temperature was the difficulty in pumping the concentrated whey due to an increase in viscosity.

In the literature, most studies using IMAC run columns at room temperature. Porath and Olin (1983) suggested studying the effect of temperature on protein adsorption and chromatographic behaviour. No studies in the literature could be found on the effect of temperature on IMAC, though. Studies could be carried out at other temperatures to further investigate the effect of temperature on IgG isolation using IMAC.

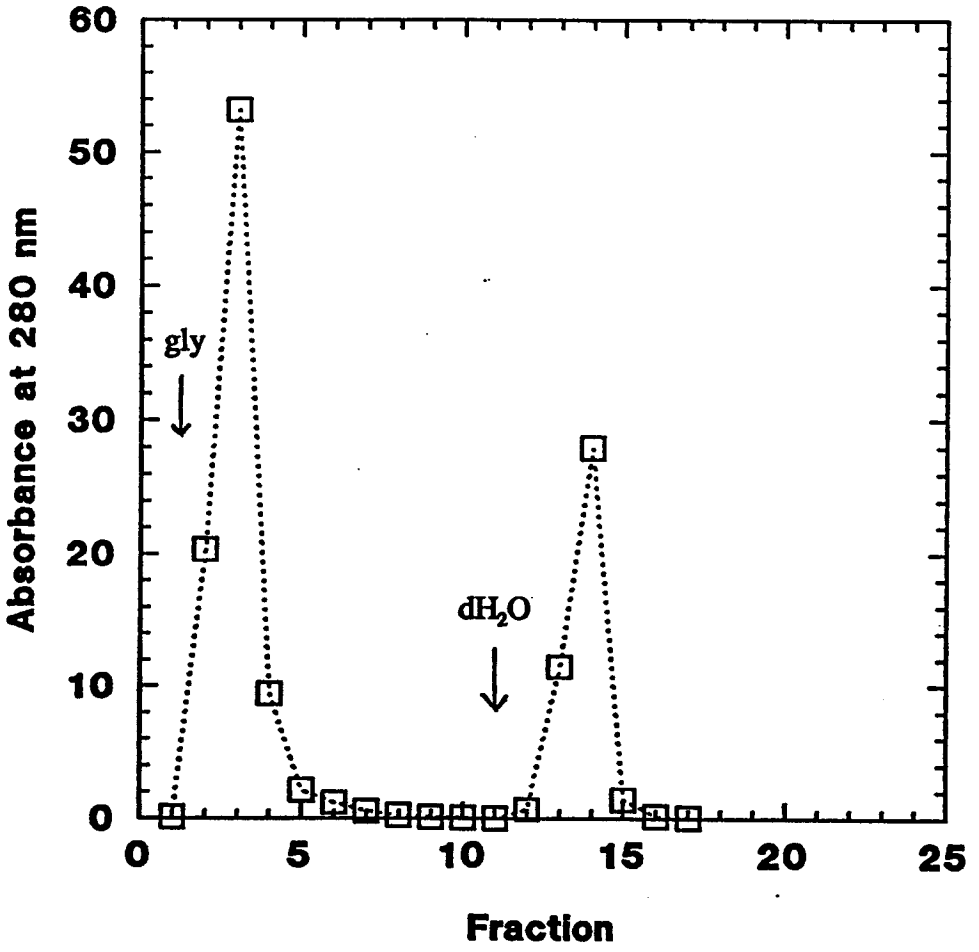

Figure 13. Elution profile of adsorbed proteins from a 50 ml CSFF column loaded with 17.3 ml of 50 mM $CuCl_2$ using 50 mM glycine in buffer and distilled water and run at 7°C. The flow rate was 2.5 ml/min. Fraction volumes were 25 ml.

J. ISOLATING IgG FROM WHEY CONCENTRATED COMMERCIALLY BY UF USING IMAC

Experiments showed that IgG could be isolated from Cheddar cheese whey concentrated in the lab using a small-scale UF system. If the process is to be carried out on a large-scale, whey concentrated with a commercial UF system would have to be used. Whey is concentrated commercially in the manufacture of whey protein concentrates (WPC). Most WPC is made using UF presently. Briefly, WPC is produced by concentrating whey by UF and then drying the whey to form a powder (Renner and Abd El-Salam, 1991). To see if whey concentrated commercially by UF could be used with the IMAC column, a commercially concentrated whey was obtained from Olympia Cheese (Olympia, WA). The composition of the whey and the UF processing conditions for the whey were not available.

The pH of the concentrated whey was adjusted to 6.0. The concentrated whey was then centrifuged at 4000 × g for 30 minutes at 5°C to remove any fat or insoluble matter. The concentrated whey was too viscous to pump easily and so, it was diluted 1.3 times with distilled deionized water. The IgG content of the concentrated whey was measured by RID and sufficient concentrated whey was applied to a 50 ml CSFF column loaded with 17.3 ml of 50 mM $CuCl_2$ to pass the breakthrough point for IgG binding. During application of the concentrated whey, a white precipitate formed on the top of the column and then started moving through the column. This precipitate tended to restrict flow and it was suspected that it might consist of $CaPO_4$ or other Ca salts. Using whey with a low Ca salt concentration would probably eliminate this problem.

The recovery and purity of IgG obtained from the IMAC column are shown in Table XXI. The recovery of 1040 mg of IgG eluted with glycine with a purity of IgG around

Table XXI. IgG recovery and purity of fractions obtained by IMAC of whey concentrated commercially by UF using a 50 ml Chelating Sepharose Fast Flow column loaded with 17.3 ml of 50 mM $CuCl_2$ and eluted with 50 mM glycine in buffer.

Fraction	IgG recovered (mg)[a]	Purity of IgG (g/100 g protein)[b]
Applied WPC (292 ml)	2600	5.2
Unbound (280 ml)	1670	3.7
Wash (473 ml)	370	9.5
Elution (glycine) (0-51 ml)	140	37.8
Elution (glycine) (52-81 ml)	570	47.5
Elution (glycine) (82-285 ml)	330	55.0
Elution (dH_2O) (74 ml)	45	62.5
EDTA wash (80 ml)	not detectable	----
0.05 N NaOH wash (90 ml)	50	100.0

[a]Recovered IgG calculated as IgG activity (mg/ml) by radial immunodiffusion x volume (ml) of fraction. Values are the average of duplicates. The differences between duplicate measurements were less than 10%.

[b]Purity of IgG (g/100 g) calculated as IgG activity by radial immunodiffusion/total protein in fraction x 100. Protein content measured by the biuret method (Means ± SD; n=3.). Values are the average of duplicates. The differences between duplicate measurements were less than 10%.

60% were similar to values obtained using concentrated white CCW as seen in Table XIX. The recovery was slightly lower than that obtained using regular CCW. The average recovery of IgG using regular CCW was 1202 mg with a standard deviation of 85 mg for 5 trials. Depending on the processing conditions of the concentrated whey, the IgG activity or the ability of IgG to bind to the IMAC column may have been affected. The loss of 62 mg (13%) was not substantial, though.

IgG could be isolated from whey commercially concentrated by UF in the manufacture of WPC using IMAC. The unbound fraction from IMAC could be combined with concentrated whey and be used for WPC manufacture. Li-Chan et al. (1990) found that the proteins in the unbound fraction from IMAC retained good emulsifying activity. WPC made from the unbound fraction should, therefore, have little loss in functional activity, but further studies are needed. Care should be taken to use whey with low Ca salt content to prevent problems with the IMAC column. Different concentrated wheys might also behave differently, so each should be tested to determine its suitability for IMAC. Since most commercial UF systems can produce several hundred liters of concentrate per hour, obtaining large volumes of concentrate for IMAC would not be difficult if the IgG isolation method was scaled up.

The size of the IMAC column would also have to be increased to handle large volumes. Based on the work done using IMAC, few difficulties are anticipated for scaling up the column. CSFF is quite stable and should be able to handle high pressures that might be experienced in a larger column. The manufacturer states the flow rate for CSFF can be greater than 300 cm/hr in a column 5 cm in diameter and 15 cm high. The running of an

IMAC column is straightforward and simple. In addition, the IMAC column can be reused. Columns were reused 15 times or more without any problems as long as they were maintained properly. CSFF can also be autoclaved at 121°C in 20% ethanol.

K. USING ION EXCHANGE CHROMATOGRAPHY TO ISOLATE IgG FROM CCW CONCENTRATED BY UF

Ion exchange chromatography was used to determine how IMAC compares to other chromatography methods. Ion exchange chromatography is based on the reversible adsorption of charged solute molecules to an immobilized ion exchange group of the opposite charge (Anonymous, 1991). Studies have found that ion exchange chromatography can be used to isolate IgG from whey (Butler & Maxwell, 1972; Stott & Lucas, 1989). A column with an inner diameter of 2.6 cm was filled with 60 ml of carboxymethyl (CM) cellulose (HC-2), a cation exchanger. The column was run at a pH of 5.4 or 5.0. pH 5.4 was chosen since it was below the isoelectric point of IgG and above the isoelectric point of more abundant whey proteins. Marshall and Harper (1988) give the isoelectric point of β-lactoglobulin as 5.35-5.49, α-lactalbumin as 4.2-4.5, Ig as 5.5-8.3, BSA as 5.1, proteose-peptone as 5.1-6.0, and β-caseins as 4.7. pH 5.0 was also used since it is recommended that ion exchange chromatography be run at least 1 pH unit away from the isoelectric point of the protein of interest (Anonymous, 1991). In addition, the pH had to be above or near the isoelectric point of β-lactoglobulin to prevent the binding of β-lactoglobulin, so pH 5.0 was chosen.

Since the ionic strength of the sample affects the binding of protein in ion exchange chromatography, the concentrated whey was electrodialyzed to decrease the ionic strength. The conductivity of the electrodialyzed concentrate was measured to ensure the ionic strength had decreased sufficiently. Stott and Lucas (1989) suggest the conductivity be between 2-3 mMhos for ion exchange chromatography. The conductivity was found to change from 6.1 mMhos to 2.2 mMhos after electrodialysis in one case, and from 6.1 mMhos to 2.6 mMhos in another case. The conductivity was corrected for 18°C.

The recovery and purity of IgG obtained by ion exchange chromatography at pH 5.4 and 5.0 are shown in Table XXII. The recovery and purity were much lower than for IMAC. At pH 5.4, the IgG recovery was 28 mg with 20% purity. At pH 5.0, the IgG recovery was 88 mg with 35% purity. Figure 14 shows the SDS-PAGE profiles of the fractions obtained by ion exchange chromatography at pH 5.4 and 5.0. A large amount of β-lactoglobulin and α-lactalbumin remained in the eluted fractions at pH 5.0, even though the recovery of IgG was high. Ion exchange chromatography may not give as good results as IMAC since the isoelectric point of many whey proteins are not distinct, but have a range making it difficult to selectively bind IgG.

Based on these results, at pH 5.0, the capacity of the ion exchange column was 1.47 mg of IgG/ml of gel (88 mg/60 ml) with 35% purity, and at pH 5.4, the capacity was 0.47 mg of IgG/ml of gel (28 mg/60 ml) with 20% purity. The capacity at pH 5.0 was similar to expected values for Ig recovery using ion exchange chromatography. Stott and Lucas (1989) state the purity of IgG they obtained from ion exchange chromatography was about 50%. The purity may be increased by changing the ion exchange gel or applying less concentrate. Different ion exchange gels will have different capacities and selectivities. For ion exchange chromatography, it is recommended not to apply more sample than 10-20% of the capacity of the column to get good resolution (Anonymous, 1991). Since concentrate was applied in excess of this amount, the purity may not have been as high as it could have been.

Although, the capacity of the ion exchange column was much lower than for IMAC; the cost of the ion exchange gel was much cheaper. In Table XXIII, a comparison of ion exchange and IMAC is shown based on capacity and price. Table XXIII shows that 16 times

Table XXII. IgG recovery and purity of fractions obtained by ion exchange chromatography of electrodialyzed concentrated CCW at pH 5.4 and pH 5.0.

Fraction	IgG recovered (mg)[a]	Purity of IgG (g/100 g protein)[b]
pH 5.4		
Applied concentrate (42 ml)	340	9.2
Unbound (76 ml)	310	9.4
Wash (200 ml)	not detectable	----
Elution (0.25 M NaCl) (110 ml)	30	21.4
Elution (1.0 M NaCl)	8	6.7
pH 5.0		
Applied concentrate (42 ml)	260	9.6
Unbound (50 ml)	200	13.3
Wash (465 ml)	<1	----
Elution (0.25 M NaCl) (158 ml)	90	36.0
Elution (1.0 M NaCl) (173 ml)	<1	----

[a]Recovered IgG calculated as IgG activity (mg/ml) by radial immunodiffusion x volume (ml) of fraction. Values are the average of duplicates. The differences between duplicate measurements were less than 10%.

[b]Purity of IgG (g/100 g) calculated as IgG activity by radial immunodiffusion/total protein in fraction x 100. Protein content measured by the biuret method (Means ± SD; n=3.). Values are the average of duplicates. The differences between duplicate measurements were less than 10%.

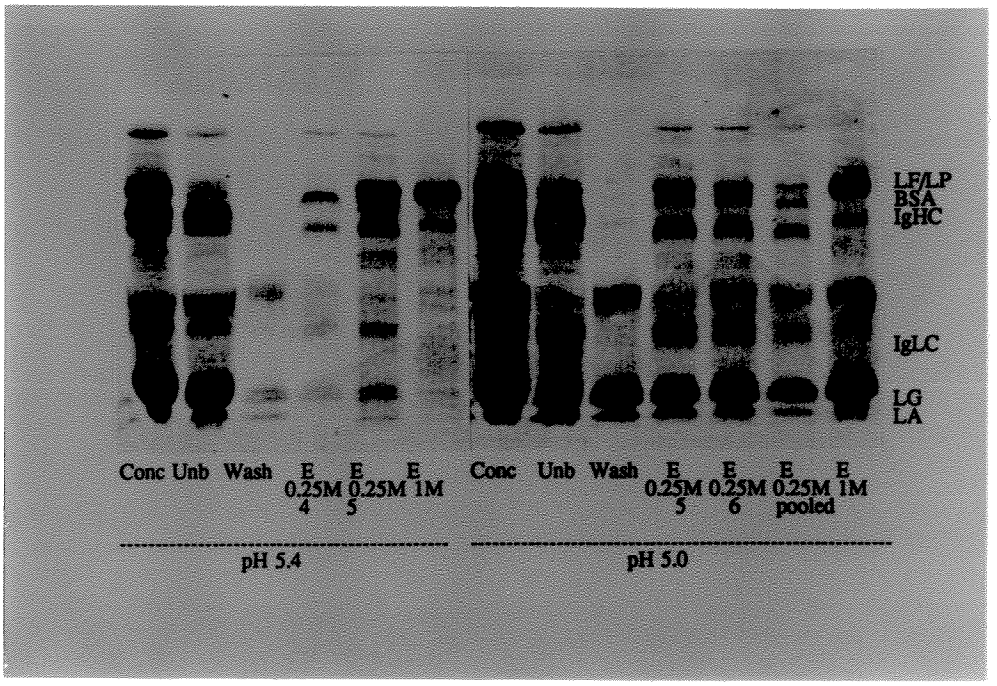

Figure 14. Reduced SDS-PAGE profiles of fractions eluted with 0.25 M and 1.0 M NaCl in buffer at pH 5.4 or pH 5.0 from an ion exchange chromatography column. Abbreviations for fractions: Conc = concentrate (retentate), Unb = unbound , Wash = wash, E 0.25M = peak eluted with 250 mM NaCl in buffer (numbers are fraction numbers where fraction volume = 12 ml), and E 1M = peak eluted with 1.0 M NaCl in buffer. Abbreviations for bands: LP = lactoperoxidase; LF = lactoferrin; BSA = bovine serum albumin; IgHC, IgLC = IgG heavy and light chains; LG = β-lactoglobulin; and LA = α-lactalbumin.

Table XXIII. Comparison of the cost and capacity of ion exchange and immobilized metal affinity chromatography gels.

Matrix support	Cost ($Can)[a]	Capacity for IgG (mg/50 ml)[b]
Carboxymethyl cellulose (ion exchange)	72/kg	73
Chelating Sepharose Fast Flow (IMAC)	240/50 ml 1 500/500 ml 11 200/5 liters	1229

[a]Cost of Chelating Sepharose Fast Flow is based on the February 1991 Price List of Pharmacia (Canada) Inc. (Baie D'Urfé, PQ). Cost of CM cellulose is based on the 1991 price for grade 2 (150-250 μm) carboxymethyl cellulose (HC-2) from Phoenix Chemicals Ltd. (Christchurch, N.Z.).

[b]Capacity is based on the IgG recovered from a column by calculating the IgG activity (mg/ml) using RID x volume of eluted fraction (ml)/50 ml of gel. The value for IMAC was based on the average recovery of IgG from 5 trials using a 50 ml CSFF column loaded with 17.3 ml of 50 mM $CuCl_2$ and eluted with 50 mM glycine in buffer and distilled water.

more gel is required for ion exchange chromatography than IMAC based on capacity. The cost of IMAC was about 31 times more than for ion exchange chromatography based on the cost of 5 liters of gel or 5 kg of gel. For this calculation, it was assumed that 5 kg of CM cellulose makes up a volume of 5 liters. Taking the lower capacity of ion exchange chromatography and thus the need for more gel into account, IMAC still costs twice as much as ion exchange chromatography. Even though IMAC is slightly more expensive, there are still advantages to IMAC that may make up for the extra cost. The column for IMAC is much smaller for the same capacity, so smaller volumes of buffers are used, less wear on pumps occurs, and the columns take up less space. There is also no need to remove ions from concentrated wheys when using IMAC which decreases the cost of processing. During elution of the ion exchange chromatography column, the gel was observed to compress which may cause problems with larger columns. In addition, IMAC isolates IgG with higher purity so the end product may have higher value.

L. MEMBRANE STERILIZATION OF IgG SOLUTION

The small-scale membrane sterilization procedure consisted of reconstituting lyophilized IgG obtained from IMAC with distilled water to obtain a protein concentration of 60-70 mg/ml. The solution was then passed through 3 layers of GF/A as a prefiltration, then through 2 Acrodisc 37 GF syringe filters for sterilization. Microbial populations of the IgG solution following the various steps were determined. Table XXIV shows the microbial counts obtained from two sterilization trials. The microbial count of the IgG solution was initially quite high. Microbial counts in raw milk can range from 4 800 - 71 500 colony forming units/ml (Henderson, 1971). Assuming whey would have similar counts and bacteria would be concentrated by UF, the microbial counts for the IgG solution seem reasonable. It should be noted that the IgG solution was lyophilized, which may have affected bacterial growth. The microbial counts indicated that prefiltration with GF/A reduced microbial counts by a factor of 10 times. The Acrodisc 37 GF resulted in complete sterilization as measured by 0 counts.

Although there was no bacterial growth after final filtration, small numbers of microorganisms could be in the IgG solution due to inadequate sterilization or contamination during the sterilization procedure. The membrane sterilization procedure may have been inadequate if some bacteria passed through the 0.2 μm membrane. Spiral-shaped bacteria and small bacteria could pass through. Reid et al. (1990) mentioned that the pharmaceutical industry is using 0.1 μm membranes for sterilization since smaller and smaller bacteria are being discovered. In the beer industry, 0.45 μm membrane filters are accepted as removing all beer spoilage organisms, and the standard for microbiological stability set by most

Table XXIV. Microbial counts for IgG solution following membrane sterilization.

Step in membrane sterilization	Microbial count (CFU/ml)[a]
Trial 1	
IgG solution initially	2.70×10^5
IgG solution after prefiltration through GF/A	2.6×10^4
IgG solution after filtration through Acrodisc 37 GF	0
Trial 2	
IgG solution initially	5.09×10^5
IgG solution after prefiltration through GF/A	7.1×10^4
IgG solution after filtration through Acrodisc 37 GF	0

[a]Bacterial counts were measured by spread plating 0.1 ml of sample on standard plate count agar. Plates were incubated at 25°C for 48 hrs. Values are the average of duplicates. The differences between duplicate measurements were less than 10%.

manufacturers is 0 microorganisms per 250-500 ml (Reid et al., 1990). Since large volumes of IgG solution were not obtained, a reliable estimate of the effectiveness of the membrane sterilization procedure could not be made using membrane filtration to enumerate bacteria. If membrane sterilization of IgG solution was to be done on a large-scale, a microbiological standard should be established.

Contamination could have occurred if there were problems with membrane integrity. For large-scale membrane sterilization, membrane integrity can be tested using the pressure hold test (Reid et al., 1990). Such a test could not be used with the syringe filters. Two syringe filters were used, though, in case the first filter was defective. Another problem with the syringe filters was the applied pressure was not measurable. If the pressure used was close to the maximum operating pressure, bacteria could have been forced through the membrane. Membrane sterilization of the IgG solution could only be done in batches of 20-25 ml. Although measures were taken to prevent contamination during membrane sterilization, contamination could have occurred since there was some exposure to an unsterile environment.

The membrane sterilization procedure used seemed to be adequate and no contamination probably occurred, since no growth was detected in cartons of UHT milk injected with membrane sterilized IgG and stored over 5 months as noted in the section titled "N. Shelf Life Study of IgG in UHT Milk". The membrane sterilization procedure was only a rudimentary method developed for the small volumes of IgG solution available, though. If IgG is to be membrane sterilized on a large-scale, a better system could be developed. Large-scale membrane sterilization equipment for aseptically injecting sterilized solution into

UHT products is available. Dairyland Foods (Burnaby, B.C.) has such a system. In large-scale systems, the membrane integrity can be tested, the pressure on the membrane can be measured, and contamination is less likely to occur since the system is closed. A microbial standard would also have to be established for IgG solution depending on expected growth during storage. If large volumes of sterilized IgG solution are available, membrane filtration could be used to enumerate microorganisms in the solution to establish a standard.

The effect of membrane sterilization on IgG activity was also determined. RID was conducted on the IgG solution following the various stages of sterilization for the first trial. Initially, the IgG concentration was 38.8 mg/ml. After prefiltration with GF/A, the IgG concentration was 41.6 mg/ml. After sterilization, the IgG concentration was 40.0 mg/ml. These values are the averages of duplicates. The differences between duplicate measurements were less than 10%. During prefiltration and final filtration, there was little hold up volume in the filter, so the volume of the solution remained relatively constant. Based on these values, the membrane sterilization procedure did not seem to affect IgG activity or concentration.

M. THERMAL RESISTANCE CURVE FOR IgG

A shelf life study to test the stability of the membrane sterilized IgG in white 2% UHT milk was to be carried out. Morgan et al. (1986) determined the stability of IgA in human milk. They found the D values for IgA in seconds were 49 000, 1707, 319, 59.6 and 25.7 at 60°, 68°, 72°, 76° and 78°C, respectively. The z-value was found to be 5.5°C. Using these values, the extrapolated D value for IgA in human milk at 25°C would be 3528 years. It was expected that IgG in bovine milk would have similar D values and so, IgG fortified UHT milk stored at 4°, 25° and 35°C should theoretically be stable for years. Since a shelf life study could not be carried out for such a long time, D values for IgG were to be found at higher temperatures, so a thermal resistance curve could be plotted to extrapolate D values at lower temperatures.

Preliminary trials were done at 62°, 66°, 70°, 74°, 78° and 80°C to find appropriate time intervals to record a one log decrease in IgG concentration. Based on these results, another set of trials was carried out. First-order destruction curves for IgG were plotted from these trials. It was found that the curves tended to level off after long time intervals. Leveling off appeared to be a result of the limitations of the RID plates and not of the nature of the samples. A sandwich ELISA was done for some samples at 78°C as shown in Figure 15. The more sensitive ELISA method showed that the IgG concentration was actually decreasing and not leveling off. RID plates were made that could measure lower IgG concentrations. Samples with low IgG concentration were reapplied to these plates to get more accurate values for IgG concentration.

Values that were not affected by the RID plate limitations were used to plot first-order

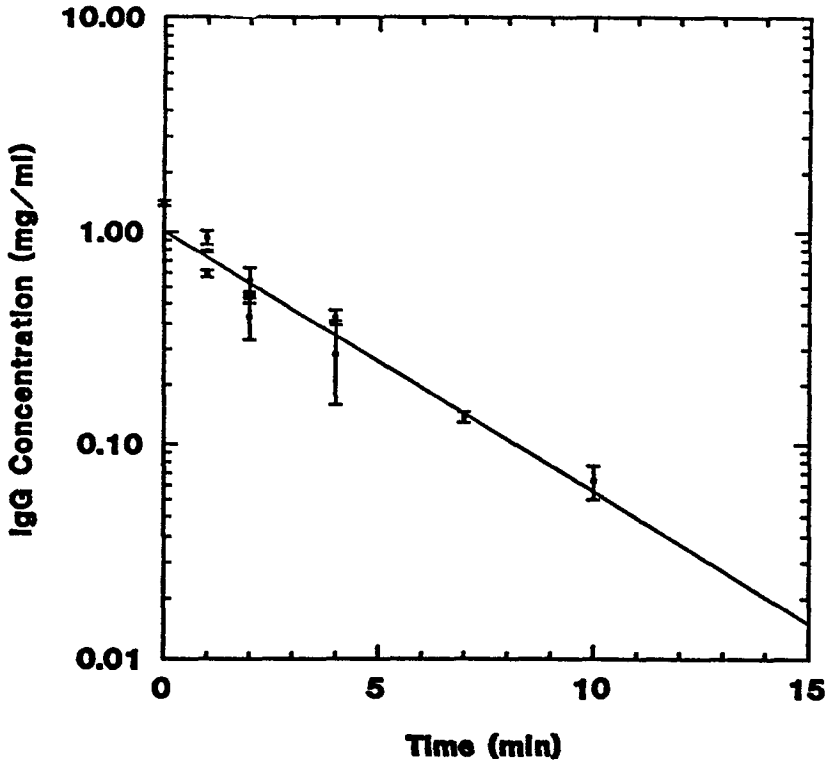

Figure 15. First-order destruction of IgG in UHT milk fortified with IgG at 78°C measured using a sandwich ELISA method.

destruction curves as seen in Figures 16, 17, 18, 19, 20, and 21. It should be noted that even though it was desired to plot a one log decrease in IgG concentration, this was not achieved at 66° and 62°C. The samples at these temperatures were incubated for long periods of time and this caused the samples to brown and thicken, so studies were not carried out any longer. Some of the plots indicated that the semi-logarithmic relationship of concentration and time may not be first-order, but variations could have resulted due to the limitations associated with RID. For the purposes of this study, it was assumed that destruction was first-order. More trials would be needed to establish if the relationship was first-order or not. Regression lines were calculated for the curves. Based on the regression lines, a D value was found for each temperature. The D values were 6.59 min at 80°C, 12.89 min at 78°C, 81.57 min at 74°C, 701.76 min at 70°C, 8542.92 min at 66°C, and 76 417.92 min at 62°C. These D values were much larger than for IgA in human milk. Differences in the structure of IgG and IgA, or differences in the composition of human and cow's milk may have resulted in the different thermal stabilities of these immunoglobulins.

The D values were then plotted on a thermal resistance curve as seen in Figure 22. From this curve, the D value extrapolated at 4°C was 9.33×10^{19} min or 1.77×10^{14} yr, at 25°C was 2.02×10^{13} min or 3.85×10^{7} yr, and at 35°C was 1.02×10^{11} min or 1.94×10^{5} yr. The z-value, which equals -1/slope, was calculated as 4.35°C. It was therefore estimated that IgG in white 2% UHT milk would be stable for the shelf life of the UHT milk and probably longer. The shelf life of white 2% UHT milk given by Dairyland Foods (Burnaby, B.C.) is 4 months.

In this study, first-order reaction kinetics were evaluated using the thermal death time

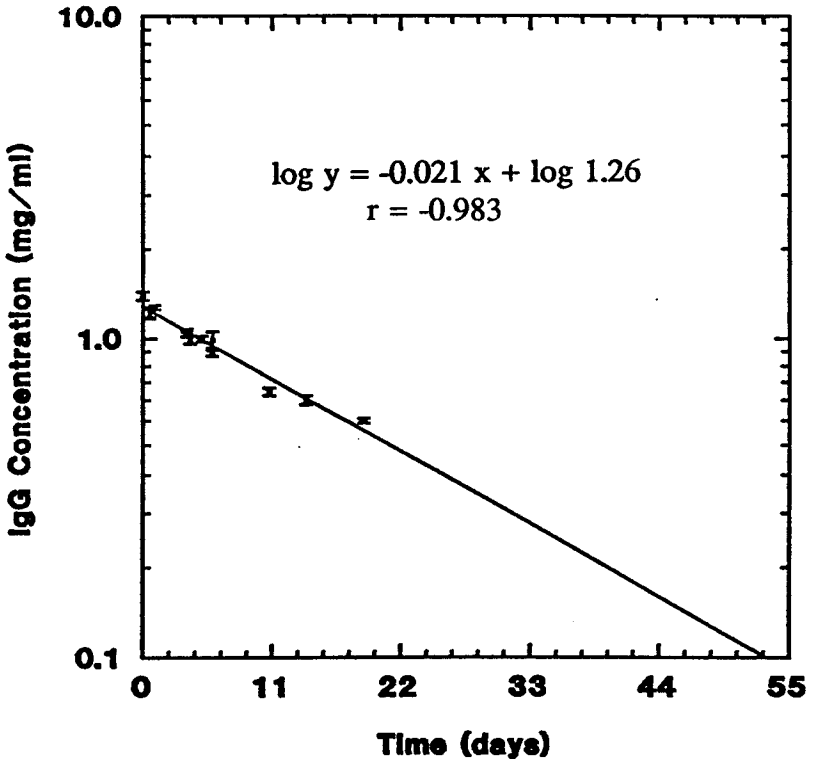

Figure 16. First-order destruction of IgG in UHT milk fortified with IgG at 62°C.

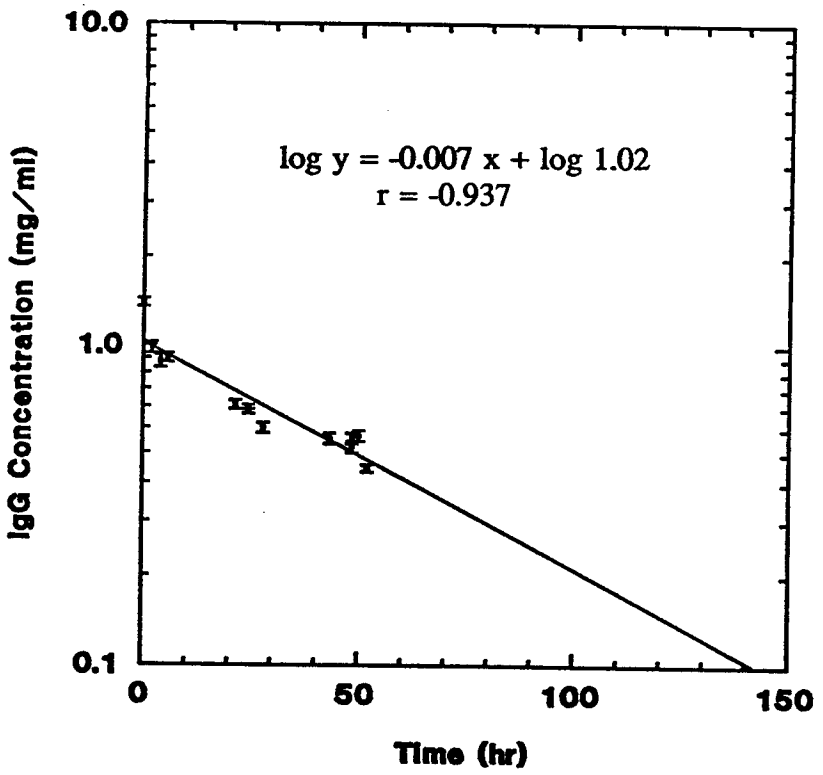

Figure 17. First-order destruction of IgG in UHT milk fortified with IgG at 66°C.

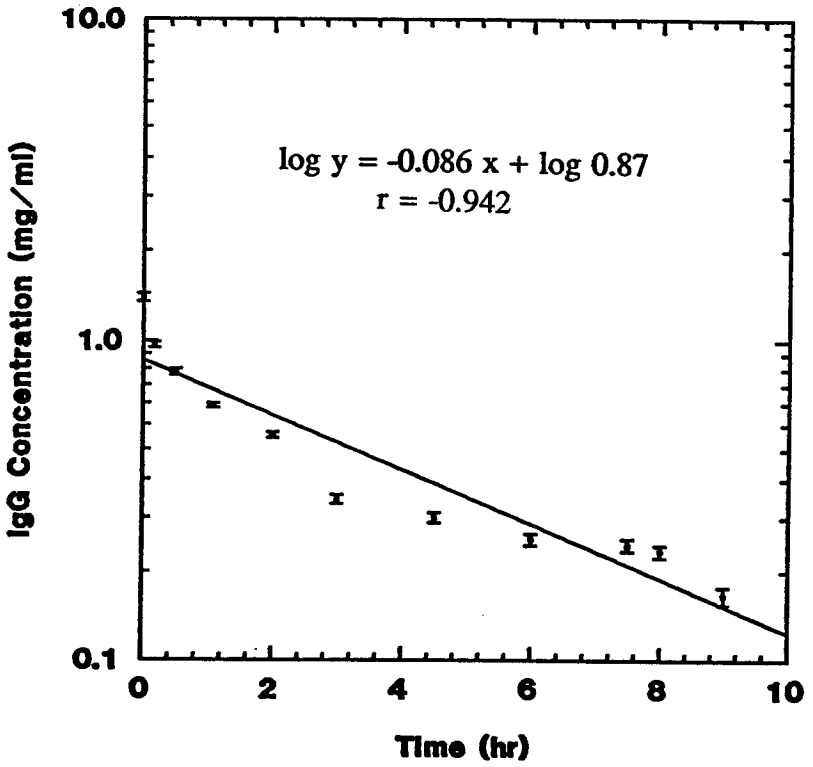

Figure 18. First-order destruction of IgG in UHT milk fortified with IgG at 70°C.

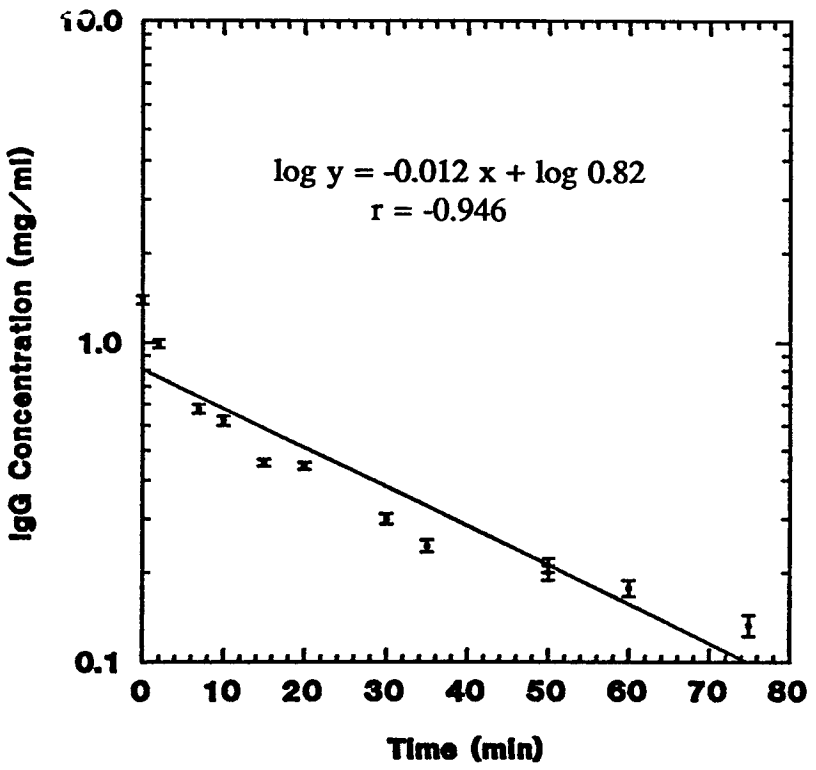

Figure 19. First-order destruction of IgG in UHT milk fortified with IgG at 74°C.

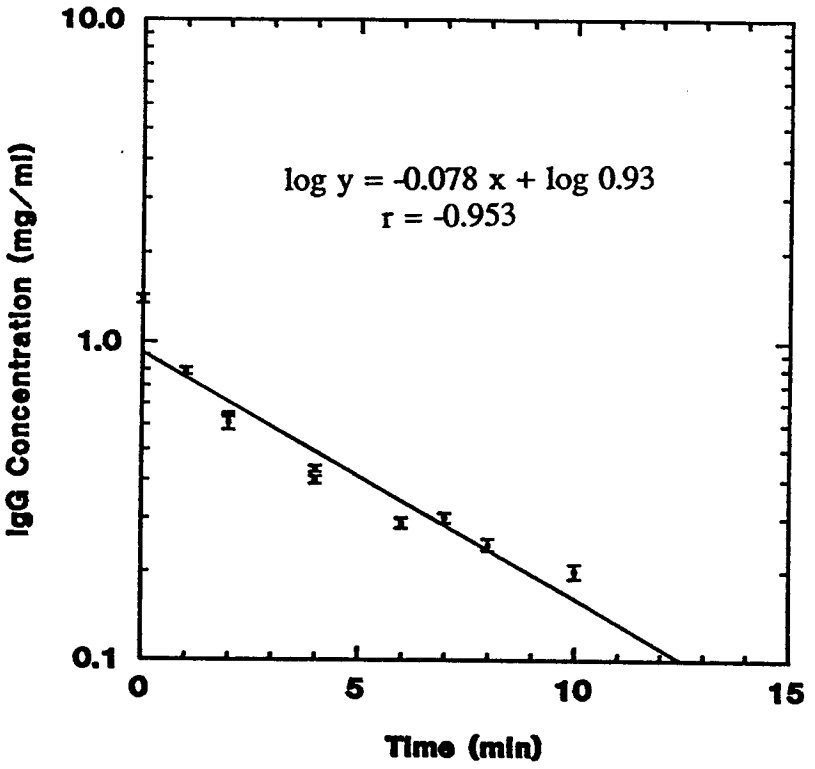

Figure 20. First-order destruction of IgG in UHT milk fortified with IgG at 78°C.

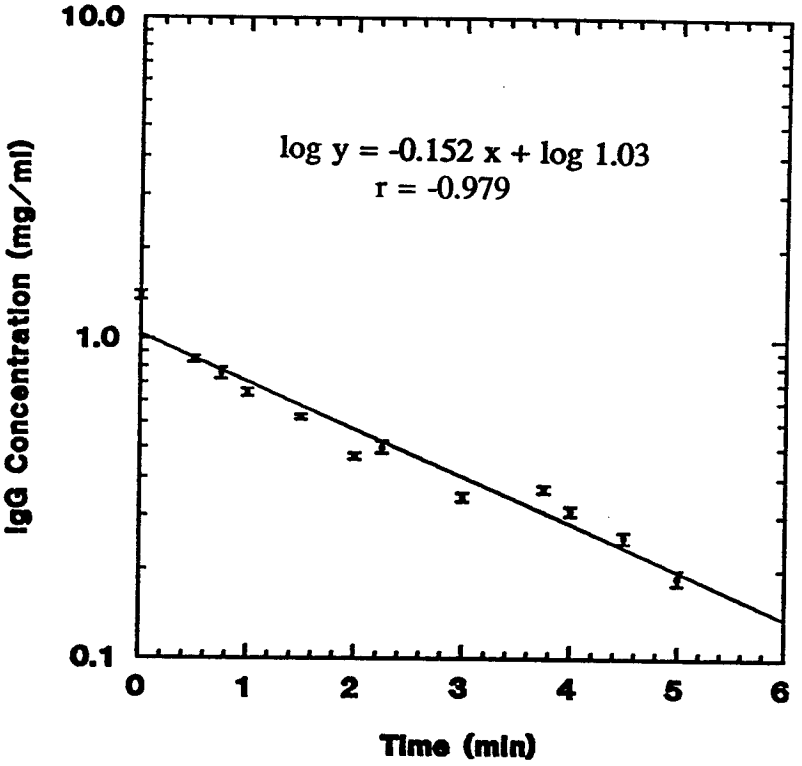

Figure 21. First-order destruction of IgG in UHT milk fortified with IgG at 80°C.

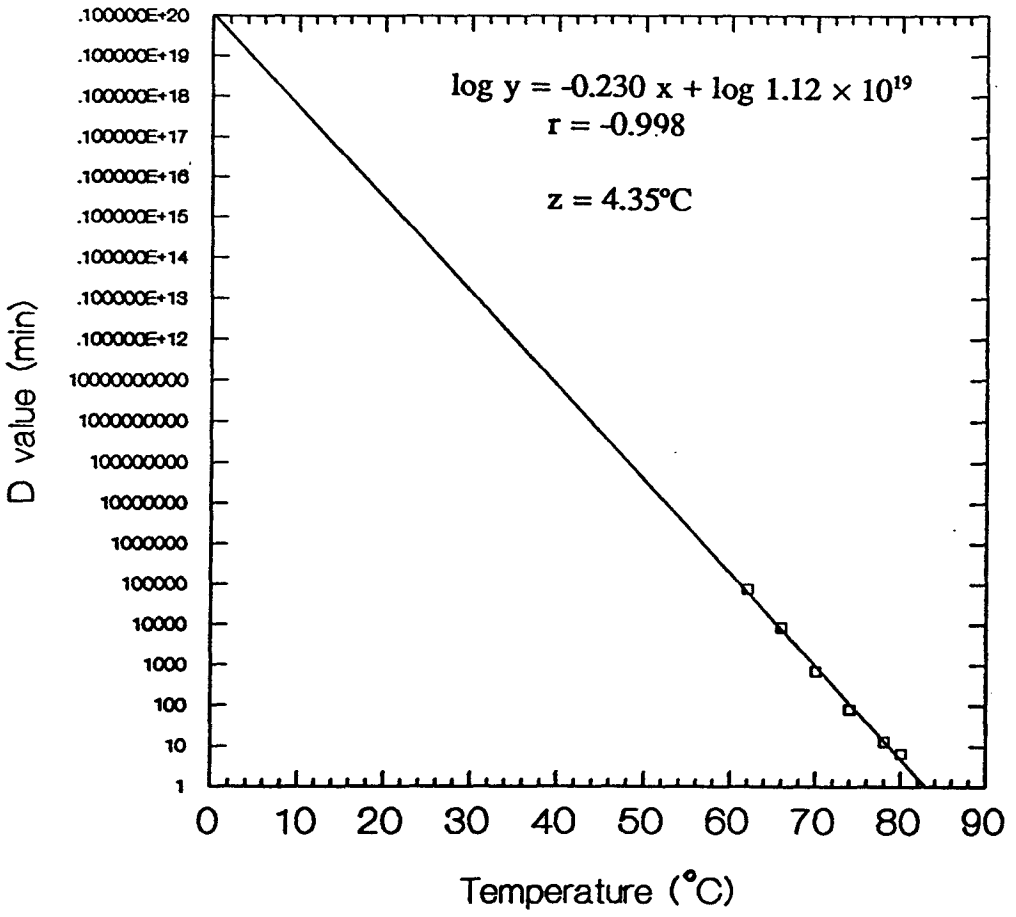

Figure 22. Thermal resistance curve for IgG in UHT milk fortified with IgG.

(TDT) method, but the conventional Arrhenius approach could also have been used. The two methods differ in their basic assumptions. The TDT approach assumes the decimal reduction time (D value) of microorganisms/nutrients follows a semi-logarithmic relationship with temperature (Ramaswamy et al., 1989). The Arrhenius equation relates the reaction rate constant, k, to the reciprocal of absolute temperature (Ramaswamy et al., 1989). Ramaswamy et al. (1989) stated that there is no proof as to which is the better approach. Care must be taken in converting factors from one system to the other outside the temperature limits of the original data since major discrepancies can result. Since the TDT method is commonly used in food science, this approach was chosen.

N. SHELF LIFE STUDY OF IgG IN UHT MILK

Lyophilized IgG from the IMAC column was reconstituted with water so the protein concentration was 60-70 mg/ml. IMAC using a 50 ml column and the procedure outlined in Figure 11 was used to obtain IgG. When the IgG was reconstituted, its composition was tested. The pH was 6.89, the protein concentration was 62.1±2.0 mg/ml as measured by the biuret method, and the IgG concentration was 34.8 mg/ml. The value for the protein concentration is the mean ± standard deviation, where n=3. The value for IgG concentration is the average of duplicates. The difference between duplicate measurements was less than 10%. The purity of IgG was approximately 56.0%. The pH was probably stabilized around 7.0 due to the buffering capacity of proteins in the IgG solution. The colour of the solution was dark green brown, probably due to lactoperoxidase. Lactoperoxidase standard has a dark green colour in solution. When the IgG solution was added to milk, the colour was masked by the colour of the milk. Therefore, no problems with the colour of the IgG solution are expected as long as the IgG solution is mixed with a solution that masks its colour. The solution was then membrane sterilized. The microbial counts from this procedure are shown in Trial 2 in Table XXIV.

Based on the IgG concentration of the solution, 8.0-9.0 ml of the IgG solution was aseptically injected into a 250 ml carton of white 2% UHT milk to obtain a final IgG concentration in the milk of about 1.0 mg/ml. One mg/ml was chosen since changes in this concentration were easy to detect using RID. Preliminary trials injecting sterilized water into prepared cartons indicated contamination was not likely to occur during injection or withdrawal of samples. It should be noted that if membrane sterilized IgG is fortified in

products at high levels, the product will be diluted. The maximum protein concentration of IgG solution that can be filtered efficiently is around 70 mg/ml. The IgG concentration in such a solution is about 39 mg/ml, so large volumes would have to be added to get high doses, or the IgG solution would have to be concentrated under sterile conditions. Concentrating solution under sterile conditions could be expensive. Hilpert (1984) reported a similar limitation with membrane sterilization. He produced a milk immunoglobulin concentrate (MIC) from cow's milk by removing fat and casein, and concentrating and diafiltering the resulting whey using UF. The MIC solution was membrane sterilized. Hilpert (1984) mentioned the protein concentration of the solution was 7-8%. To get dry MIC, Hilpert had to evaporate and then lyophilize the membrane sterilized MIC under sterile conditions.

Twelve cartons were injected with membrane sterilized IgG solution, and samples were withdrawn from each carton to determine the initial IgG concentrations. The cartons were stored at 4°, 25° and 35°C. Four injected cartons were stored at each temperature, and 4 cartons that were not injected were stored at each temperature as controls. At one month intervals, 2 injected and 2 control cartons at each temperature were tested for microbial growth and IgG concentration. Two cartons were saved as reserves in case of contamination.

The change in IgG concentration during storage is shown in Table XXV. The results indicate that there was no measurable change in IgG concentration over 5 months as was predicted from the thermal resistance curve for IgG. There were some fluctuations in IgG values from month to month which could be accounted for by experimental error. Dairyland Foods uses a shelf life of 4 months for 2% white UHT milk and 6 months for flavoured UHT

Table XXV. IgG concentration in white 2% UHT milk injected with membrane sterilized IgG solution and stored at 4°, 25° and 35°C over a 5 month interval.

Temp. (°C)	Package[a]	IgG concentration (mg/ml)[b]					
		0 months	1 month	2 months	3 months	4 months	5 months
4	1 IgG	1.22	1.29	1.25	1.19	1.23	1.19
	2 IgG	1.23	1.28	1.25	1.21	1.28	1.22
	1 no IgG	ND[c]	ND	ND	ND	ND	ND
	2 no IgG	ND	ND	ND	ND	ND	ND
25	1 IgG	1.25	1.34	1.39	1.26	1.32	1.42
	2 IgG	1.22	1.26	1.16	1.15	1.19	1.17
	1 no IgG	ND	ND	ND	ND	ND	ND
	2 no IgG	ND	ND	ND	ND	ND	ND
35	1 IgG	1.27	1.36	1.25	1.28	1.30	1.26
	2 IgG	1.23	1.34	1.30	1.25	1.24	1.36
	1 no IgG	ND	ND	ND	ND	ND	ND
	2 no IgG	ND	ND	ND	ND	ND	ND

[a]Packages consisted of 2 injected with IgG solution and 2 packages without any IgG solution as controls.
[b]IgG concentration was measured using radial immunodiffusion. Values are the average of duplicates. The differences between duplicate measurements were less than 10%.
[c]Not detectable.

milk based on physical/chemical changes. IgG would therefore be stable for the duration of UHT product shelf life.

No microbial growth was observed in any of the cartons tested using spread plates with 0.1 ml of sample up to 4 months. After 3 months, the milk in cartons injected with IgG and stored at 25° and 35°C had a slight change in colour. Changes were obvious in these cartons after 4 months of storage. The samples of milk withdrawn from these cartons were watery like whey and had some small white clumps. Some samples had a cheesy odour. Changes were also noted in injected cartons kept as reserves at both temperatures. None of the cartons stored at 4°C showed changes, and none of the control cartons stored at 25° or 35°C showed changes. After 4 months, milk in control cartons stored at 35°C were slightly brown in colour, though. Initially, microbial growth was assumed as causing the changes. Microbial counts were expected to be high if microorganisms were growing. However, spread plates of the samples indicated no growth. For one IgG injected carton stored at 25°C, mold was found growing on the spread plates. A microbial count of 280 colony forming units (CFU)/ml of mold was measured. The value was the average of duplicate plates with the difference between duplicate measurements being less than 10%. Upon closer inspection of this carton, mold was found growing on the surface of the carton under the tape and silicone plug. The tape had loosened upon prolonged storage. The mold was probably not growing in the milk since anaerobic conditions existed in the carton. The mold likely entered the sample during withdrawal of sample with a contaminated needle. This carton was disregarded due to the evidence of contamination.

Since no growth was observed using spread plates, it was suspected that either the spread plate method was not suitable to detect microorganisms in the UHT cartons, or there

was no growth. The spread plate method was suitable for detecting aerobic microorganisms, but anaerobic microorganisms may have caused spoilage in the UHT milk. The environment in UHT cartons would be favourable to anaerobic microorganisms. After 5 months of storage, further microbial analysis was carried out to determine if anaerobic microorganisms, or yeast and molds were causing spoilage. Pour plates using 1.0 ml of sample were prepared for each carton. Plates were incubated at 25° and 35°C for 48 hrs. Plates were also incubated anaerobically at 21°C for 72 hrs. To detect yeasts and molds, pour plates were prepared using potato dextrose agar. The plates were incubated at 25°C for 48 hrs. All plates had no growth which indicated the changes in the milk were not due to microbial spoilage. In addition, if microbial growth had occurred, it was expected that microbial counts would increase with storage, and should be detectable after 5 months.

Enzymes in the IgG solution probably caused the changes noted in the milk. The enzymes did not affect IgG activity, though. Trace amounts of enzyme could have been isolated along with the IgG during IMAC. Since the IgG solution was not heat-treated, the enzymes were not inactivated. During extensive storage, the enzymes could have been activated and caused the changes noted in the milk. Changes were probably noted only in cartons stored at 25° and 35°C, and not in cartons stored at 4°C due to the effect of temperature on enzyme activity. Most enzymes have an optimum activity near physiological temperatures. The enzyme or enzymes causing the changes could be any of several enzymes found in milk. Shahani et al. (1973) reviewed the enzymes found in bovine milk including proteases and lipases. Seitz (1990) lists several enzymes in milk or from microorganisms in milk which could cause the cheesy odour noted in some cartons. Testing could be carried out to determine the type of enzyme causing the changes. Free fatty acid content and

nonprotein nitrogen (NPN) could be measured in injected cartons and control cartons. If free fatty acid content is high, lipases could be present, whereas, if NPN is high, proteases could be present. The enzymes could also be isolated from the IgG solution.

This study indicated that membrane sterilized IgG is stable in UHT milk for several months. As long as the membrane sterilization of IgG solution is effective, microorganisms should not grow in UHT milk which has been sufficiently processed. Small amounts of enzymes were probably isolated with IgG during IMAC. In UHT milk aseptically injected with membrane sterilized IgG solution, these enzymes were activated after 4 months of storage at 25° and 35°C, and caused changes in the appearance of the UHT milk. A method may have to be found to inactivate or remove these enzymes depending on storage temperatures and times.

The results suggest aseptic injection of IgG solution into UHT sterilized infant formulas or other sterilized products is a feasible method to fortify products with IgG depending on the level of fortification. Large-scale aseptic injection systems are currently available. Johnson (1987) mentioned that large-scale aseptic injection systems can be used to filter sterilize fruit essences to add to juices preventing flavour losses, or to filter sterilize lactase solutions to add to aseptic products. Modifications to existing aseptic injection systems may have to be made, though, when using IgG solution. The aseptic injection systems are designed to add membrane sterilized solutions to the products prior to aseptic packaging. During aseptic packaging, H_2O_2 is usually used. Therefore, the effect of H_2O_2 on IgG activity should be examined if a large-scale system is used.

V. CONCLUSIONS

Ultrafiltration can be used to concentrate IgG from Cheddar cheese whey and milk. IgG can be isolated from the concentrated whey with high purity and recovery using IMAC. Other immunologic agents, namely lactoferrin and lactoperoxidase, can also be isolated along with the IgG. Concentration up to 30 times, IMAC matrix gel support, UF membrane size, UF temperature, and diafiltration did not affect the recovery or purity of IgG obtained from concentrated whey using IMAC. The recovery of IgG from the IMAC column was improved by using 50 mM glycine instead of 1.0 M NH_4Cl as a competing ligand in the eluting buffer. IMAC could be run at room temperature or 7°C. At 7°C, IMAC was more difficult due to increased viscosity of solutions. The elution profile also changed indicating IgG binding was altered. High IgG recovery and purity were also obtained using whey concentrated commercially by UF.

When ion exchange chromatography was used to isolate IgG from electrodialyzed concentrated whey, the recovery and purity of IgG were lower than when IMAC was used. The recovery was about 16 times lower and the purity decreased from 56% to 35%. The cost of ion exchange chromatography gel was about 31 times cheaper than IMAC gel. After taking the capacity into account, IMAC was only 2 times more expensive than ion exchange chromatography. Considering the advantages of IMAC such as higher purity of IgG obtained and smaller columns needed, IMAC may be more cost effective than ion exchange chromatography. IMAC gels like ion exchange gels can be reused.

A membrane sterilization procedure was developed to sterilize IgG solution on a small-scale. Prefiltration with 1.6 μm glass microfiber paper and final filtration through a

0.2 μm membrane was used. The IgG solution should have a protein content between 60-70 mg/ml to facilitate filtration. The membrane sterilized IgG could be aseptically injected into UHT milk.

First-order destruction curves were plotted for membrane sterilized IgG in 2% white UHT milk at 62°, 66°, 70°, 74°, 78° and 80°C. D values obtained from the first-order destruction curves indicated IgG was relatively stable at these temperatures. A thermal resistance curve for IgG was plotted based on the D values obtained. By extrapolating D values for lower temperatures from this curve, IgG was predicted to be stable for years in UHT milk at 4°, 25° and 35°C.

Over 5 months of a small-scale shelf life study, no changes were seen in IgG activity in 2% white UHT milk aseptically injected with membrane sterilized IgG solution and stored at 4°, 25° and 35°C as predicted. Aseptically injecting membrane sterilized IgG into UHT sterilized products could be a feasible method to fortify products with IgG depending on the level of fortification required. However, physical changes occurred in the milk in cartons injected with IgG solution and stored at 25° and 35°C for 3-4 months. The changes were probably caused by residual enzymes in the IgG solution. Depending on the storage temperatures and times for products injected with IgG, the enzymes may have to be inactivated.

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