SEPARATION OF ANTIMICROBIAL PROTEIN FRACTIONS FROM ANIMAL RESOURCES FOR POTENTIAL USE IN INFANT FEEDING

by

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ABSTRACT

In the first part of this study, a non-ferric method for selective elimination of β-lactoglobulin from cheese whey was investigated. A new method was developed based on hexametaphosphate treatment of cheese whey. When Cheddar cheese whey was treated under the optimized conditions, i.e., 1.33 mg/mL sodium hexametaphosphate at 22°C and pH 4.07 for 1 hr, more than 80% of β-lactoglobulin was removed by precipitation. Almost all of the immunoglobulins and the major portion of α-lactalbumin were retained in the supernatant as indicated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and immunochemical assays. By dialysis against distilled water 72.2% of the phosphorus was removed from the supernatant.

In the second and the third part of the thesis, chromatographic methods were used for isolation of immunoglobulins and lactoferrin from whey proteins. By using gel filtration on Sephacryl S-300, 99, 83.3 and 92.1% biologically active immunoglobulin G were obtained for colostral whey, acid and Cheddar cheese whey, respectively. Lactoferrin, selectively adsorbed to the heparin-attached Sepharose, was eluted with 5 mM Veronal-HCl containing 0.5M NaCl, at pH 7.2. 1,4-Butanediol diglycidyl ether-iminodiacetic acid on Sepharose 6B, or so-called metal chelate-interaction chromatography (MCIC), was loaded with copper ion and used for the same purpose. Of the two peaks obtained, the first yellowish peak was rich in lactoferrin, while the second peak was rich in immunoglobulins. Some of the physical and chemical properties of the proteins in these peaks, including immunochemical properties, isoelectric points, binding to bacterial lipopolysaccharides, and the mechanism of protein-metal interaction via histidine modification, and the capacity of the

method were studied. The possibility of isolating immunoglobulins and lactoferrin from electrodialyzed whey was also investigated.

In the fourth, fifth and sixth parts of the thesis, the method developed for isolation of immunoglobulins and lactoferrin from whey protein was applied to isolate these biologically important proteins directly from skimmilk, blood and egg white. The casein in skimmilk was found to compete with immunoglobulins for binding to copper ion in MCIC column when skimmilk was loaded in presence of 0.05 M Tris-acetate buffer containing 0.5 M NaCl, pH 8.2; however, this problem was solved by changing the equilibrating buffer to 0.02 M phosphate buffer containing 0.5 M NaCl, pH 7.0. When blood was directly applied to MCIC column, the yield of biologically active IgG was more than 95%. Ovotransferrin, strongly adsorbed to the MCIC column, was eluted with two-step elution protocols which suggests it exists in two forms. The histidine residues in immunoglobulins, caseins, transferrin and ovotransferrin were found to be involved in the mechanism of the interaction with the MCIC column.

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INTRODUCTION

In developed societies, many infants are fed infant formula rather than human milk. National and international organizations such as the American Academy of Pediatrics (Committee on Nutrition, 1980), the European Society for Pediatric Gastroenterology and Nutrition (ESPGAN, 1982) and the World Health Organization (1981), recommend breast feeding of infants whenever possible. There are many cases, however, where it is difficult for women to nurse infants due to short supply of breast milk, insufficient nutrition and health conditions of nursing mothers, and the necessity of some mothers having to work. Thus, continuing effort has been made to decrease nutritional and protective ability differences between human milk and cows' milk (Kuwata et al., 1985).

Although, there are many different infant formulas on the market today, all of them have not reached the required standard, i.e. human milk (Wilkinson, 1981). Nutrition and prevention of disease both have to be considered when dealing with infant formulae. It is believed that the premature infant can benefit from breast milk to an even greater extent than can the full-term infant (Workshop Participants, 1976). The most compelling argument in favor of breast feeding is, however, the contention that breast milk contains factors that protect the infant against both systemic and gastrointestinal infections. The most dramatic testimonials in this regard are provided by the results of nursery epidemics where otherwise normal infants quickly succumb to a variety of infections unless they are fed raw human milk (Dortmann, 1967; Gerrard, 1974). The protection accorded the newborn by breast milk is also critical for premature infants where a relatively common fatal syndrome called necrotizing enterocolitis can be prevented by breast feeding (Mizrawi et al., 1965; Touloukian et al., 1967; Barlow et al., 1974). It also has been reported that

the incidence of sudden death syndrome in infants is lower in breast fed infants than it is in bottle-fed infants (Mobbs, 1972).

The resistance of breast-fed infants against infection is summarized in several review articles (Hanson and Winberg, 1972; Goldman and Smith, 1974; Bezkorovainy, 1977; Rieter, 1978; Gurr, 1981; Packard, 1982; Friend et al., 1983; Lönnerdal, 1985; Rieter, 1985a) which ascribe the beneficial effects of human milk to factors that are therein but are absent from bovine milk. These factors are, for the most part, proteins in nature, the most important of which are immunoglobulin A, lactoferrin, lysozyme, lactoperoxidase, leucocytes, the bifidus factor and interferon. Manufacturers of infant formulae must consider the factors mentioned above and not just the nutrient content, i.e., carbohydrate, protein, fat, mineral and vitamins. Therefore, one possible way to humanize cows' milk is to enrich cow's milk with these bioactive components.

Prevention of pathogenic infections is only part of the problem. One must consider the prevention of immune response reaction in infants. The newborn is particularly vulnerable. For a few days the stomach is porous, even to whole proteins. The milk of any non-human species is more likely to induce an allergy in sensitive infants than mother's milk. In general, the occurrence of allergenic reactions due to infant formulae is much more common than human milk (Packard, 1982). Animal experiments suggest that β-lactoglobulin is more antigenic than either casein or α-Lactalbumin found in cow's milk. Compiled data from five studies of cows' milk protein intolerance in infancy showed sensitivity to β-lactoglobulin in 82% of the cases (Wilkinson, 1981). β-Lactoglobulin has been regarded as a major allergen for bottle-fed infant in several outstanding papers (Lebenthal, 1975; Wharton, 1981; Moneret-Voutrin et al., 1982; Heppell et al., 1984; Kurisaki et al., 1985; Otani et al., 1985;

Pahud et al., 1985). Allergenicity of β-lactoglobulin may be attributed to the fact that human milk, based on immunological reactions, contains only a trace of β-lactoglobulin (Brignon et al., 1985).

Therefore, the elimination of β-lactoglobulin is one way to humanize the protein composition of cow's milk for infant feeding. Several attempts have been made for separating β-lactoglobulin from bovine whey (Forsum, 1974; Mathur and Shahani, 1979; Amundson and Watanawamchakorn, 1982). In our laboratory, a ferric chloride method was established for selectively precipitating β-lactoglobulin from bovine whey (Kaneko et al., 1985; Kuwata et al., 1985). However, because of the potential loss of the antimicrobial activity of lactoferrin when it is saturated with iron, non-ferric methods have been recently investigated.

The objectives of this study were (1) to establish a new non-ferric method for eliminating β-lactoglobulin from cheese whey; (2) to investigate different chromatographic methods for the isolation of immunoglobulins and lactoferrin from cheese whey; (3) to investigate the possibility of using skimmilk directly for isolation of immunoglobulins; (4) to utilize the developed technique for isolation of immunoglobulins and transferrin from blood and (5) to apply the same technique for isolation of ovotransferrin from egg white. The biological activity and the possibility of using these proteins in fortification of infant formula were investigated.

LITERATURE REVIEW

A. HISTORY

Infant formula is required for infants whose mothers do not breast feed for socioeconomic, physical or psychological reasons. Before the industrial revolution, the only alternatives to mothers breast-feeding were starvation of the infant or the services of a wet-nurse (directly supplied donor breast milk). However by the end of the 18th century, a conical baby bottle was invented. Thus modern technological developments for the first time made possible a widespread shift away from breast-feeding. The modern infant formula industry found its origins in the mid-19th century when Nestle in Switzerland and Borden in the U.S. began producing sweetened condensed milk. In the 1880's, canned evaporated milk made its appearance (Miller, 1983).

Until the 20th century, however, there was little understanding of the nutritional needs of infants or of sanitary requirements. But medical scientists recognized the need for safe and effective alternatives to breast-feeding in order to deal with problems of childhood disease and malnutrition resulting from the failure of many mothers to breast-feed adequately. Most present-day infant formulas in the United States market are adaptations of the product designed by H.J. Gerstenberger and co-workers (1915). On a fluid basis this early formula consisted of 4.6% fat (a homogenized mixture of both plant and animal fat), 6.5% carbohydrate and 0.9% protein and was given the name Synthetic Milk Adapted (SMA). Over the years the composition of infant formulas has been altered and adjusted, mostly in response to scientific evidence of need (Packard, 1982).

B. HUMAN MILK VS. COW'S MILK

Milk, whether from the human or other mammals, is an exceptionally complex mixture of more than 200 fat-soluble and water-soluble components. The milk components may originate from direct transfer from the blood, from biosynthesis from blood precursors or from a combination of both. Consequently, the composition of milk is affected by biochemical, physiological and hormonal factors which influence the composition of blood, factors which influence the rate of transfer of nutrient from blood to milk and factors which influence the rate of biosynthesis of compounds in the mammary gland (Blanc, 1981).

Human and cow's milk consist mainly of water, fat, carbohydrate, protein and minerals; however, this is where the similarities end. Table 1 gives the gross composition of human and cow's milk (Gurr, 1981). This is not very surprising if one looks at mammalian animals in general. Milk from all mammalian species consists of the constituents listed above but the relative proportions of each vary as much as the species (Jenness, 1982). The proteins, fats, etc. also vary in their biochemical and physical properties as well as their detailed composition.

The fat concentration in human and bovine milk is not comparable i.e. 4.4 and 3.7% respectively (Packard, 1982). It should be noted that fat is the most variable constituent of milk both in absolute quantity and in composition. Human milk has a lower level of short chain fatty acids along with a higher concentration of polyunsaturated fatty acids than cow's milk. Another notable difference is that human milk has approximately a 50:50 ratio of saturated to unsaturated fatty acids while in bovine milk that ratio is 65:35. Unsaturated vegetable oils may be used to adjust the ratio in cow's milk.

Table 1. Gross Composition of Human and Cow's Milk (Grams per 100 Grams of Fluid Product)[a].

Component	Human milk	Cow's milk
Fat	4.4	3.7
Protein	1.0	3.3
Milk sugar (lactose)	6.9	4.7
Mineral matter (as ash)	0.2	0.7
Water	87.5	87.6
Energy, KJ/100mL[b]	290	273

[a] Packard, 1982.
[b] Gurr, 1981.

The principal carbohydrate in milk is lactose, a disaccharide specific to this secretion. It contributes about 40 and 29% of the total energy of human and cow's milk, respectively. A carbohydrate is generally added to infant formula to increase the concentration, so that it is in the range of human milk. There are some problems in deciding which carbohydrate to add, since the gut of some babies would be unable to handle this amount of lactose, resulting in lactose malabsorption and fermentative diarrhea. Other sources of carbohydrates should be added, i.e., maltodextrin.

The major differences in the mineral content between human milk and cow's milk is not so much in kind as amount. Human milk contains, on the average, about 0.2% ash, the mineral matter remaining after incineration. Cow's milk averages about 0.70%. The higher percentage of ash, or rather minerals, is due primarily to much higher concentrations of calcium and phosphorus in cow's milk. High mineral content should be reduced, if cow's milk is to be considered for use in infant feeding (Wharton, 1981).

Cow's milk contains well over three times as much protein as human milk i.e. 3.3 vs. 1.00, respectively. Table 2 gives a breakdown of the different protein fractions in cow's milk and human milk (Gurr, 1981). Cows' milk contains over eight times as much casein as human milk. Casein is a complex protein and can be broken down into three major fractions: α_s-, β- and κ-caseins. Cow's milk consists of approximately 45% α_s-casein while human milk consists of very little of this component. Human milk on the other hand, has mainly β-casein. The coagulation properties of α_s- and β-casein are quite different. While acidified β-casein forms a soft curd, α_s-casein is thought to be more digestible for the infant (Packard, 1982).

Other protein fractions that differ between human and cow's milk are α-lactalbumin, β-lactoglobulin, lactoferrin and the immunoglobulins fraction.

Table 2. Protein Composition of Human and Cow's Milk[a]

Protein	Human Milk		Cow's milk	
	g/100mL	Total, %	g/100mL	Total,%
Total	0.88	100	3.30	100
Caseins	0.31	35	2.6	79
Total whey:	0.57	65	0.7	21
α-lactalbumin	0.15	17	0.12	3.5
β-lactoglobulin	tr	--	0.30	9.0
lactoferrin	0.15	17	tr	--
serum albumin	0.05	6	0.03	1.0
lysozyme	0.05	6	tr	--
immunoglobulins	0.10	11	0.10	3.0
others	0.07	8	0.15	4.5

[a] Gurr, 1981.
tr=trace.

β-lactoglobulin (β-Lg) makes up a high percentage of the protein in cow's milk while human milk contains very little. The concentration of β-Lg in human milk is so low that for years it was thought that β-Lg was exclusive to cow's milk (Packard, 1982; Brignon et al., 1985). Lactoferrin (LF) is found in human milk but only trace amounts are found in cow's milk. The immunoglobulin fraction is relatively similar in concentration in the two milk sources but human milk contains mostly IgA while in cow's milk IgG is the predominant immunoglobulin.

From the previous discussion on compositional differences, it can be seen that it is important to look beyond the gross make-up and investigate human and cow's milk on a more definitive basis. The differences in composition between the two milk sources cause currently available infant formulae to be less than optimal substitutes for human milk.

C. HUMANIZING INFANT FORMULA

Due to many compositional differences between human and cow's milk, many attempts have been made to humanize infant formula. There have been three major ways in which cow's milk has been modified to bring its composition closer to human milk: added carbohydrate, substitute fat and using a combination of whey and skimmilk (Gurr, 1981; Wharton, 1981).

The simplest change involves the addition of carbohydrates. The purpose for adding carbohydrates is to increase the overall carbohydrate content and to dilute the concentration of protein, fat and mineral per unit of energy intake. Maltodextrin or sucrose are examples of two carbohydrates that have commonly been used in the infant formula industry (Wharton, 1981).

In some cases the fat from cow's milk has been removed and substituted with a mixture of plant and animal fats. The purpose for the combination of fats from plant and animal sources is to obtain a fatty acid composition similar to

that found in human milk. Studies have indicated that absorption is much more efficient in the substituted fat formulas and becomes close to that found in breast milk (Wharton, 1981).

Formulas based on demineralized whey have been used increasingly in recent years. Demineralized whey, containing whey protein and low concentrations of minerals, is used as the base and to this is added a small amount of skimmilk. The range of casein/whey protein ratios of human milk and cow's milk are 0.4 - 0.7 and 3.0 - 4.7, respectively. To humanize this ratio, some of the commercial infant formulae are fortified with whey proteins. For instance, mixing skimmilk with 4 volumes of whey changes the ratio to 0.75. However simple mixing of bovine whey with skimmilk does not minimize the compositional differences between the two milks, i.e. higher contents of α-lactalbumin, lactoferrin, lysozyme and immunogobulins (Ig) in human milk than in cow's milk (Hambraeus, 1977).

While β-lactoglobulin is the dominant whey protein in cow's milk (approximately 60% of the total whey proteins), it is completely lacking or very low in human milk (Liberatori and Napolitano, 1980; Brignon et al., 1985).

D. ALLERGENICITY OF WHEY PROTEINS

In adapted formulas, the mass balance of casein versus whey proteins of bovine origin can be corrected to a 40:60 ratio in favor of the whey proteins (Anderson et al., 1982; Theuer, 1983). This adjustment adapts the cow's milk formula closer to the protein composition of human milk. It also increases the content of some essential amino acids (threonine, tryptophan, and lysine) as well as cystine in comparison with normal cow's milk; thus, adaptation by addition of whey protein corresponds to nutritional improvement (Forsum, 1974).

Presumably any of the individual proteins in cow's milk may induce specific antibody and provoke allergy in a susceptible child, but some proteins seem more antigenic than others (Wharton, 1981). The newborn is particularly vulnerable.

For a few days the stomach is porous, even to whole proteins. The milk of any non-human species is more likely to induce an allergy in sensitive infants than mother's milk (Savilahti, 1981). A protein, or an antigenic fragment of protein will simply be absorbed whole. The body reacts to the offending agent by simply producing IgE. The IgE is specially designed to recognize and bind to this specific antigen. There are, however, two ends to an antibody: the Fc, or the base of the Y-shaped antibody, and the Fab, the two protruding fingers of the Y. The fingers are designed to bind to the antigen. The base, the F_c segment, binds in this instance to mast cells or basophils (Packard, 1982). When an infant has been exposed to an allergen, IgE is produced. It attaches itself to mast cells of body tissue and at the same time issues a chemical command to the mast cell. In essence, the command calls for release of histamines which in turn cause the various disorders that accompany an allergic reaction.

Animal experiments suggest that β-lactoglobulin is more antigenic than either casein or the small amount of α-lactalbumin found in cow's milk (Ratner et al., 1958; Goldman et al., 1963 a,b; Moneret-Vautrin and Grilliat, 1979). Compiled data from five studies of cow's milk protein intolerance in infancy showed sensitivity to β-lactoglobulin in 82%, casein in 43%, α-lactalbumin in 41%, bovine serum globulin in 27%, bovine serum albumin in 18% of the patients (Lebenthal, 1975).

In view of the above results, the well-known allergencity of β-lactoglobulin has to be considered when manufacturing infant formula. Different approaches have been considered to decrease β-Lg allergencity: heat denaturation, enzymatic hydrolysis or selective elimination of β-Lg. Heat denaturation of the protein was suggested by Ratner et al. (1958), Anderson et al. (1979), McLaughlan et al. (1981), and more recently by Kilshaw et al. (1982)

and Heppell et al. (1984). However, heat treatment of whey proteins may destroy the biological activity of bioactive proteins present in the whey i.e. immunoglobulins, lactoferrin and lactoperoxidase. Tryptic and chymotryptic hydrolysis of whey proteins suggested by Pahud et al. (1985) and Asselin et al. (1986) are not recommended for the same reasons.

Selective elimination of the allergic compound may therefore be the method of choice to humanize the protein composition of cow's milk for infant feeding. Several attempts have been made for separating β-Lg from bovine whey. Sephadex G-75 gel filtration was suggested as a method for whey protein fractionation to humanize infant formula (Forsum, 1974; Mathur and Shahani, 1979). Also, β-Lg was preferentially precipitated at pH 4.65 from cheese whey after concentration by ultrafiltration and demineralization by electrodialysis (Amundson and Watanawamchakorn, 1982; Slack et al., 1985). Pearce (1983) used heat treatment and pH treatment for separating β-Lg and an α-La rich fraction from bovine cheddar cheese whey. Recently, a ferric chloride method was established for selectively precipitating β-Lg from bovine whey (Kaneko et al., 1985; Kuwata et al., 1985). However, the use of ferric chloride may saturate lactoferrin and abolish its antimicrobial activity.

Antigenic reactivities of chemically modified β-Lg was studied by Otani et al. (1985). These researchers found that modification of arginine residues, tryptophan residues, or sulfhydryl groups had little effect on the antigenic reactivity. However, a significant decrease in the reactivity was noted when β-Lg was acetylated, succinylated or modified with diethyl pyrocarbonate or coupled with glycine amide. These results suggest that there is a possibility that the amino group, histidine residue and carboxyl group may play an important role in the antigencity of bovine β-Lg.

E. ANTIMICROBIAL SYSTEM IN HUMAN MILK AND MILK SUBSTITUTES

Several substances in human milk provide resistance to infant diseases, particularly diseases of the intestinal tract (Cunningham, 1977; Larsen, 1978; Cunningham, 1979; Fallot et al., 1980; France et al., 1980; Pullan et al., 1980). Cunningham (1979) noted that first year mortality increased with the extent of formula feeding and was two-fold higher in artificially fed infants; during the first four months the difference was 16-fold. Similarly, breast feeding reduced the incidence of infection by Salmonella (France et al., 1980), respiratory syncytial virus (Pullan et al., 1980), and the incidence of hospital admissions for infection in infants (Fallot et al., 1980).

The factors in human milk thought to be responsible for the breast fed infant's increased resistance to diseases have been reviewed extensively (Gothefors and Winberg, 1975; Bezkorovainy, 1977; Reddy et al., 1977; McClelland et al., 1978; Reiter, 1978; Pittard, 1979; Welsch and May, 1979; Blanc, 1981; Gurr, 1981; Hanson and Soderstrom, 1981; Packard, 1982). Some of the antimicrobial factors are associated with cellular components present in human milk and thus are not present in human milk substitutes. Other antimicrobial factors are present in cow's milk and other substitutes in low or trace amounts. Only lactoperoxidase is present in larger quantities in cow's milk compared to human milk. The function of the antimicrobial factors will be discussed.

1. Immunoglobulins

Immunoglobulins or antibodies are a class of proteins that are comprised of four polypeptide chains. Each immunoglobulin unit is formed from two identical heavy chains and two identical light chains. The peptides are linked by disulfide bridges (Butler, 1983). In humans, there are five classes of antibodies and they are named for their heavy chains. They consist of

immunoglobulin G (IgG), immunoglobulin M (IgM), immunoglobulin A (IgA), immunoglobulin D (IgD) and immunoglobulin E (IgE). The heavy chains for each class are designated by the appropriate Greek letter: γ, μ, α, δ and ε, respectively (Atassi et al., 1984). The light chains are either kappa (κ) or lambda (λ). Light chains have one constant region and one variable region. Heavy chains consist of three constant regions and one variable region. There are also hypervariable regions within the variable regions (Brock, 1979; Nisonoff, 1982).

The simplest immunoglobulin is IgG, as it is comprised of only one basic unit of four polypeptide chains and has a low molecular weight of 150,000 daltons. When the molecule is treated with a reducing agent, it dissociates into two heavy and two light chains. The molecule may also be split by proteolytic enzymes such as papain, to give two types of fragments: F_{ab} (antigen binding) and Fc (crystalline). Proteolysis by pepsin at pH 4 yields an $F(ab')_2$ (Fab-dimer) and various peptides derived from fragment Fc (Whitney et al., 1976; Butler, 1983).

In bovine colostrum, there are three major immunoglobulins (i.e., IgG, IgM and IgA) with IgG comprising about 80% in both milk and colostrum (Butler, 1983). From early investigations it is already known that different subclasses of IgG exist in bovine colostral IgG (Murphy et al., 1964). IgG_2 and IgG_1 have been identified by some groups of workers (Butler, 1969; Duncan et al., 1972). Concentration of Ig in colostrum is high at parturition and decreases with each successive postpartum milking (Butler, 1983). Table 3 summarizes the concentration of various species of bovine immunoglobulins in serum and secretions. However, these values may provide a rough guide for investigators, since there are different factors affecting the immunoglobulins concentration in

Table 3. Concentration of bovine immunoglobulins in serum and secretions (mg/mL)[a].

Body Fluid Samples	IgG_1	IgG_2	IgA	IgM
Serum	11.2	9.2	0.37	3.05
Colostrum (whey)	46.4	2.87	5.36	6.77
Milk	0.58	0.055	0.081	0.086
Nasal secretion	1.56	--	2.81	0.04
Saliva	0.034	0.016	0.34	0.006
Tears	0.32	0.01	2.72	.176
Urine	0.009	tr	0.0013	tr
Bile	0.10	0.09	0.08	0.05
Vaginal secretion	0.23	0.13	0.90	--

[a] Butler, 1983.

milk i.e. age, breed differences, differences in the techniques used for measurement and immunization.

The amino acid and carbohydrate composition of bovine IgG_1 and IgG_2 have been studied by several investigators (Groves and Gordon, 1967; Lisowski et al., 1975). The carbohydrate content, sulfhydryl content and other physicochemical characteristics of bovine Igs are shown in Table 4 (Butler, 1983).

The human fetus and infant receives antibodies in utero, where IgG is absorbed through the placenta, and from human milk. Packard (1982) noted that the milk antibodies function as specific host resistance factors by aggregating bacteria in the intestine to facilitate their removal, by interfering with bacterial colonization of the intestinal lining, by assisting other host resistance factors, fixing complement, by neutralizing toxins and by killing viruses. Thus they provide crucial immunological protection until the newborn infants' defence systems can be established (Ogra and Ogra, 1978; Hanson and Soderstrom, 1981).

Although classes of immunoglobulins (i.e., IgA, IgG and IgM) are present in human milk throughout the period of lactation, the highest concentrations are in colostrum (Reddy et al., 1977; Hambraeus et al., 1978; McClelland et al., 1978; Ogra and Ogra, 1978; Ones, 1979; Goldman et al., 1982). Small amounts of colostrum IgG are absorbed from the intestine during the first 18-24 hours after birth (Ogra et al., 1977), although the significance of this is not known. Intact secretory IgG, the major milk antibody, has been found in the intestine of breast-fed infants (Hambraeus et al., 1978), where it appears to function in the passive transfer of specific immunity to mucosal surfaces to prevent penetration by microorganisms, viruses and antigens (Walker and Isselbacher, 1977; Hanson and Soderstrom, 1981). Antibodies against enteropathogenic Escherichia coli and E. coli enterotoxin have been postulated to play a role in

Table 4. Biochemical characteristics of bovine immunoglobulins[a].

Characteristics	IgG_1	IgG_2	IgA	IgM	IgE	SC
Heavy chain	1	2				--
$S_{20,w}$	6.9	6.9	10.9	19.5	--	4.1
$E_{1\%}^{278}$	13.5	12.3	--	11.8	--	--
Carbohydrate (%) Total	2.8	2.6	8	11	--	5.9
Sulfhydryl groups						
Half-cysteine/100 residues	3.1	2.6	--	--	--	--
Total SH (mole/mole)	--	--	--	43.6	--	--
Free SH (mole/mole)	--	--	--	0.9	--	--
S-S linkages/mole	--	--	--	21	--	--
Molecular weight	162K	152K	408K	1,030K	--	74K
Heavy chain (mol. wt.)	57K	54K	62K	76K	--	--
Light chain (mol. wt.)	25K	23K	23K	22.5K	--	--

[a] Butler, 1983.

$S_{20,W}$= Svedberg values

$E_{1\%}^{278}$= Absorbance of 1% protein solution at 278 nm.

preventing infant diarrhea (Gindrat et al., 1972; Stoliar et al., 1976; Rogers and Synge, 1978). IgM also provides protection against gram negative pathogens (Packard, 1982).

The importance of oral administration of immunoglobulins in both animals and human infants is well documented. In animals, supplementing a milk replacer with Ig separated from porcine blood maintained 86% survival of piglets compared to no survival of the control group fed with the milk replacer alone (Elliot, 1978). Similar survival rates are obtained by feeding piglets with bovine colostrum (McCallum et al., 1977). Improved survival and immunity of chicks by feeding whey containing Ig has also been reported from USSR (Kuznetsov and Rebrova, 1983).

In humans, the importance of Ig in infant feeding has been well demonstrated in clinical test results from India (Narayanan et al., 1983). Of sixty-six low birth weight infants split into two equal groups only 7 infants in the group fed with human colostrum developed infection compared to 18 in the infant formula-alone group, which is significantly different ($P<0.01$). Significant results were also shown by Hilpert et al. (1974/1975) where infants fed colostrum from sensitized cows had an infection rate of 24% compared to 67.4% for the control group who were not fed colostrum. Rotavirus infection was also prevented by orally administering cow's colostrum (Ebina et al., 1985). Diarrhea developed in only 1 of 6 infants given Rota colostrum, while 6 out of 7 infants given milk developed diarrhea.

2. Lactoferrin

Lactoferrin, an iron-binding protein of breast milk, is considered to be of great importance for the breast-fed infant. It has been shown (Kirkpatrick et al., 1971; Bullen et al., 1972, Reiter, 1983) that lactoferrin can bind iron in

vitro and *in vivo*, thereby preventing the growth of iron-requiring microorganisms. Lactoferrin is a single-chain glycoprotein with an approximate molecular weight of 75,000-85,000 (Blackberg and Hernell, 1980). The structure of lactoferrin consists of two largely independent domains, each carrying its own iron-binding site. This proposed structure has received strong support from studies demonstrating that cleavage of lactoferrin, usually by proteases, could under certain conditions yield half-molecules capable of binding just a single iron atom (Brock, 1985).

Human milk contains from 3 to 100 times as much iron-binding protein lactoferrin as cow's milk (Packard, 1982), and a trace amount of the serum iron-binding protein transferrin. Lactoferrin is active *in vitro* against enteropathogenic *E. coli* (Rogers and Snyge, 1978; Spik et al., 1978; Dolby and Honour, 1979; Samson et al., 1979; Samson et al., 1980;), *Vibrio cholerae* (Arnold et al., 1977), *Streptococcus mutans* (Arnold et al., 1977), and *Candida albicans* (Kirkpatrick et al., 1971), presumably by chelating iron and making it unavailable for microbial growth (Packard, 1982).

Lactoferrin, together with secretory IgA from human milk, have a considerable bacteriostatic effect against human enteropathogenic strains of *E. coli* (Stephens et al., 1980; Dolby and Stephens, 1983). Similarly, bovine colostral IgG, together with lactoferrin, is found to be active against a strain of *E. coli* pathogenic to calves (Stephens et al., 1980). Since the anti-*E. coli* activity of lactoferrin is not destroyed by proteolytic digestion, lactoferrin may also play an antibacterial role *in vivo* (Samson et al., 1980).

3. Lactoperoxidase and lysozyme

Lactoperoxidase (LP), which catalyzes the oxidation of the thiocyanate by

hydrogen peroxide to hypothiocyanate, serves as a major antimicrobial agent in cow's milk (Reiter et al., 1976; Reiter, 1978; Pruitt and Tenovuo, 1985). Lactoperoxidase is active against E. coli, Pseudomonas fluorescens, Salmonella typhimurium and strains of Klebsiella aerogenes (Pruitt and Tenovuo, 1985). Bovine LP behaves differently from other protective proteins. Its concentration is low in bovine colostrum and increases rapidly to reach a peak at 4-5 days post-partum. Human peroxidase is highest in colostrum and declines rapidly within 1 week (Gothefors and Marklund, 1975; Reiter, 1985b). Bjorck (1978) reported a concentration limit of 0.5 µg/mL human milk which is far below the average of 10-30 µg/mL present in bovine milk. It is important to note that lactoperoxidase is not inactivated by the gastric juice from an infant (pH 5) (Gothefors and Marklund, 1975), whereas pepsin at pH 2.5 inactivates lactoperoxidase (Paul and Ohlsson, 1985).

Lysozyme cleaves the cell wall peptoglycan of a number of gram positive and gram negative microorganisms, and appears to potentiate the activity of IgA against E. coli (Adinolfi et al., 1966; Hill and Porter, 1974), and with peroxide and with ascorbate to lyse E. coli and Salmonella (Miller, 1969). Human milk contains approximately 3000 times as much lysozyme as cow's milk (Chandan et al., 1964; Chandan et al., 1968). Human milk lysozyme possesses a molecular weight, amino acid composition, specific activity, thermal stability and antigenicity which are quite different from the lysozyme in cow's milk (Eitenmiller et al., 1974; Eitenmiller et al., 1976).

4. Bifidobacteria

The bifidobacteria (previously designated Lactobacillus bifidus) protect the infant against disease by producing volatile acids which inhibit the

proliferation of pathogenic microorganisms in the gut (Friend et al., 1983). Within 3-4 days after birth, the intestinal tract of breast fed infants contains up to 99% Bifidobacterium bifidum type IV (Gyllenberg and Raine, 1957; Haenel, 1970). The flora of formula fed infants does not contain type IV, but rather 30-40% B. bifidium Type II (Haenel, 1970). A number of substances or factors in human milk have been reported to stimulate the proliferation of the bifidobacteria. These factors include: buffer capacity, lactulose, lactoferrin, pantothenic acids, oligosaccharides and glycoproteins.

Infants fed either human milk or a test formula with low buffering capacity have a relatively low fecal pH (5.1-5.4) and a higher proportion of bifidobacteria than fecal coliforms or streptococci (Willis et al., 1973; Bullen et al., 1977). Those infants who were fed highly buffered cow's milk formula had a significantly higher fecal pH (5.9-8.0) and a mixed fecal flora (Willis et al., 1973; Bullen et al., 1977). It has been suggested (Bullen et al., 1976) that E. coli and S. faecium initially colonize the gut and produce acid. In breast fed infants the pH of the intestine drops and growth conditions become favorable for bifidobacteria and unfavorable for other organisms. The crucial drop in pH and thus the proliferation of the bifidobacteria is prevented when infants are fed highly buffered formulas (Friend et al., 1983).

Even though lactulose is not present in human milk or cow's milk, it has been reported that supplementation of prepared formulas with lactulose (formed during heating of sterilized formula) increases the proportion of intestinal bifidobacteria (Mendez and Olano, 1979; Shvedova, 1981).

Lactoferrin may indirectly promote the growth of the bifidobacteria by inhibiting the growth of competing E. coli (Spik et al., 1978). Pantothenic acid derivatives have also been shown to stimulate one strain of B. infantis (Tamura et al., 1972). A nitrogen-containing oligosaccharide (Bezkorovainy and

Topouzian, 1981) and glycoproteins (Hirano et al., 1968; Bezkorovainy and Nichols, 1976) have been shown to stimulate the growth of bifidobacteria (Gyorgy, 1953). The greatest stimulatory activity is found in human colostrum, followed by human milk, cow's colostrum and cow's milk. A recent report (Ashoor and Monte, 1983) noted that human milk contains two distinct B. bifidum var. Penn. bifidus stimulating factors, the activity of which varies from sample to sample.

5. Ovotransferrin

Ovotransferrin (OVT), also called conalbumin, is a glycoprotein with a molecular weight about 76,000 and contains no free sulfhydryl groups or phosphorus. The protein moieties of ovotransferrin of egg white and transferrin of chicken blood serum are identical, but the carbohydrate prosthetic groups are different (Powrie and Nakai, 1986).

Schade and Caroline (1944, 1946) first reported that ovotransferrin and serum transferrin inhibited the growth of E. coli and other bacterial species. This antibacterial effect was destroyed by the addition of Fe^{+3}which saturated the iron binding sites of the proteins (Brock, 1985).

Valenti et al. (1983) concluded that the antimicrobial activity of hen's ovotransferrin was quantitatively and qualitatively similar to that of human lactoferrin. These proteins demonstrated a similar protective effect on experimentally-induced bacterial infections in newborn guinea pigs. These observations have led to the concept of "nutritional immunity" (Weinberg, 1977).

A greater resistance against enterobacterial infection of human infants fed with breast milk than those fed with artificial formula is well documented (Packard, 1982). This has been attributed, to a great extent, to the presence of a large quantity of lactoferrin in human milk compared to cow's milk. The

similarities in structure and biological activity between ovotransferrin and lactoferrin justify the antimicrobial effect of ovotransferrin added to infant formula. In addition, ovotransferrin did not sensitize ovotransferrin fed infants (Giacco-Del et al., 1985).

F. ISOLATION OF BIOACTIVE PROTEINS

1. General methods

With a view to the isolation of immunoglobulins as well as of all other biopolymers, one must make use of those physicochemical properties or parameters that are peculiar to the polymer in question, and that are quantitatively different from the physicochemical properties of the other accompanying (but unwanted) polymers (Van Oss, 1982-83). There are five fundamentally different physicochemical parameters of biopolymers: solubility, electric change, surface tension, size and shape, and ligand specificity. Some of these parameters will be reviewed in isolation strategies of immunoglobulins, lactoferrin and ovotransferrin from different biological sources.

Methods currently available for the isolation of bovine immunoglobulins and their subclasses based on solubility, electric charge and size and shape are batch processes, which are difficult to mechanize (Butler and Maxwell, 1972; Fey et al., 1976; Kanamaru et al., 1977; Butler et al., 1980; Kanamaru et al., 1980; Kanamaru et al., 1981; Kanamaru et al., 1982a; Kanamaru et al., 1982b; Shimazaki and Sukegawa, 1982; Butler, 1983; Brooks and Stevens, 1985; Bokhout et al., 1986). Other methods based on affinity chromatography using protein A-Sepharose (Ey et al., 1978; Martin, 1982) or immuno-adsorbents (Bokhout et al., 1986) are quite expensive for large scale purification.

Lactoferrin was first isolated from human milk by Groves (1962) and from cow's milk by Gordon et al. (1962) by using ammonium sulfate precipitation

and/or ion exchange column. Several methods for its isolation have been described but most of them are rather laborious (Johansson, 1969; Querinjean et al., 1971; Law, and Reiter, 1977; Kawakata, 1984). However, Lönnerdal et al. (1977) used metal chelate affinity chromatography and Blackberg and Hernell (1980) used heparin-Sepharose for lactoferrin isolation.

The most frequently used isolation methods for ovotransferrin are based on salt precipitation and ion exchange (Warner and Weber, 1951; Williams, 1962; Azari and Baugh, 1967; Antonini, 1977). These methods, however, are labor-intensive and difficult to mechanize.

2. Metal Chelate-Interaction Chromatography (MCIC)

A little more than a decade ago, a novel purification technique for proteins using "immobilized metal affinity chromatography" was introduced by Porath et al. (1975). This technique was later called metal chelate-interaction chromatography (MCIC) by Rassi and Horvath (1986). Since its introduction, the technique has gained wide acceptance and was recently reviewed (Lönnerdal and Keen, 1982; Sulkowski, 1985). The application of MCIC has been reported for separation of human serum proteins (Porath et al., 1983; Andersson, 1984; Ramadan and Porath, 1985), human lactoferrin (Lönnerdal et al., 1977), lysozyme (Torres et al., 1979), human fibroblast interferron (Edy et al., 1977) and human serum albumin (Hansson and Kagedal, 1981).

As explained by Lönnerdal and Keen (1982), the binding of proteins is believed to be the result of the ability of electron-rich ligands, such as histidine, cysteine and tryptophan, to substitute weakly bonded ligand, such as water or buffer ions, in the complexes. When a protein, with surface exposed amino acids having electron-donating capacity, is exposed to a metal, a strong multipoint attachment can result. This binding is stable even in 1M NaCl ruling

out the possibility of ionic interaction being the principal force in the binding. It is important to realize that this kind of interaction is independent of whether the protein is iron binding or not or, in the case of an iron-binding protein, whether the protein is in Fe-saturated form or in the apo-form. Fe-saturated lactoferrin can bind to a copper-loaded gel as strongly as the apo-form of lactoferrin (Lönnerdal et al., 1977; Lönnerdal and Keen, 1982).

MATERIALS AND METHODS

A. MATERIALS

Sodium hexametaphosphate (SHMP, purified grade) was purchased from Fisher Scientific Company (Fairlawn, NJ); β-lactoglobulin, α-lactalbumin, bovine immunoglobulins, rabbit anti-bovine IgG, bovine serum albumin, lactoferrin, alkaline phosphotase conjugated rabbit anti-bovine IgG, lactoperoxidase, ovalbumin, ovotransferrin, α-casein and diethyl pyrocarbonate were purchased from Sigma Chemical Company (St. Louis, MO). β-Casein was obtained from Chemalog (South Plainfield, NJ). κ-Casein was prepared according to the method of Zittle and Custer (1963). α_{s1}-Casein was a gift from Dr. R. Yada and sulfhydryl blocked κ-casein (sss-κ-casein) was a gift from Dr. S. Nakai.

Materials for column chromatography were: silica (sand) (fine granular type No. S-150 from Fisher Laboratory Chemical, Fairlawn, NJ), controlled pore glass 80-120 mesh, PG 1400-120 and fumed silica S-5055 (from Sigma Chemical Company, St. Louis, MO); alumina (neutral AG7, 100-200 mesh, No. 132-1140 from BioRad Laboratories, Mississauga, ON). Electrodialyzed and sweet whey powders were from Mead Johnson and Company (Evansville, IN). Bovine blood plasma was obtained from Intercontinental Packers Ltd., Vancouver, BC. Skimmilk was purchased from a local market. Cheddar cheese whey was obtained from Dairyland Foods (Burnaby, BC). Escherichia coli Serotype 0142:K86(B):H6 (ATCC No. 23985), Salmonella typhimurium (ATCC No. 13311) and Bordetella parapertussis (ATCC No. 15311) were supplied by American Type Culture Collection (Rockville, MD). All other chemicals were of analytical reagent grade.

B. ACID WHEY PREPARATION

Raw milk and colostrum were obtained from the University Animal Science Farm. Acid whey was prepared from raw milk and colostrum. The milk was centrifuged at 4,000 x g for 30 min at 5°C for cream separation. Acid whey was

prepared from the skimmilk by adding 50% acetic acid solution to pH 4.6 at 25°C and centrifuging at 10,000 x g for 15 min to remove casein precipitates.

C. SODIUM DODECYL SULFATE - POLYACRYLAMIDE GEL ELECTROPHORESIS

The method of Laemmli (1970) was used after modifications. Polyacrylamide gel electrophoresis in the presence of 0.2% sodium dodecyl sulfate (SDS-PAGE) was performed with a slab type vertical gel system using the Atto SJ 1060 DSH Electrophoresis unit (Atto Co., Tokyo, Japan).

1. Discontinous SDS-PAGE

A whole gel was composed of separating gel (lower gel) 0.2 cm thick, 11 cm long, and 13.5 cm wide, and stacking gel (upper gel). Ten and 3% polyacrylamide gels were used as the separating and stacking gels, respectively, of which the ratio of acrylamide to N,N'-methylene-bis-acrylamide was 25. Polymerization of both gels was catalyzed by 0.02% ammonium persulfate.

One mL of whey solution (2-4 mg protein/mL) was treated with 5% SDS and 0.2 mM 2-mercaptoethanol in boiling water for 1.5 min, followed by the addition of 200 mg sucrose and 50 µL of 0.05% bromphenol blue tracking dye solution. Twenty five µL of the treated whey solution was applied to the sample slot after the sample slots and upper electrode chamber were filled with Tris-glycine electrode buffer (3g Tris + 14.4g glycine + 1g SDS in 1 L, pH 8.3).

Electrophoresis was performed at room temperature with a constant voltage of 90 volts until the tracking dye marker migrated to 1 cm from the gel bottom, in approximately 4.5 hr. The gel was then removed, placed on a net plastic floater (a gel supporter), immersed in 0.25% Coomassie Brilliant Blue R-250 dye solution (Weber and Osborn, 1969), and stained for 1.5 hr. The gel was rinsed with water, transferred to a diffusion destainer (model 172A, Bio Rad

Laboratories, Richmond, CA), and destained vertically for 18 to 20 hr with a circulation of destaining solution (a mixture of 10% acetic acid and 7.5% methanol) through a cartridge of activated carbon. Glycoproteins were stained by using periodic acid-Schiff (PAS) technique described by Zacharius et al. (1969).

2. Gradient SDS-PAGE

Solutions containing 3% and 20% acrylamide were prepared from a stock solution of 60% acrylamide with 4% crosslinking. The solutions were made in 0.25 M Tris-HCl (pH 8.3) containing 0.2% SDS and 0.125% tetramethyl ethylenediamine (TEMED). Ammonium persulfate for polymerization was added to the solutions immediately before mixing. Gradients were generated using a two chamber device containing 20 ml of 20% acrylamide in the mixing chamber and 20 ml of 3% acrylamide in the reservoir chambers. The mixture was then pumped into a vertical slab mould of the Atto SJ 1060 SDH Electrophoresis Unit (Atto Co., Tokyo, Japan), at a flow rate of 2 mL/min. Sample preparation, electrophoresis conditions, staining and destaining were performed as described in Section (C-1).

D. IMMUNOCHEMICAL ANALYSIS

1. Immunoelectrophoresis and immunodiffusion

Immunoelectrophoresis and immunodiffusion analysis were carried out according to the method of Williams and Chase (1971) with modifications. Nine mL of 1% agarose in 0.05 M Na-barbital acetate buffer, pH 8.3 was gelatinized over Gelbond film (0.02 x 7.5 x 10 cm, FMC Corporation Marine Colloid Division Bioproducts, Rockland, MA). Three µL of whey sample was applied to a punched sample well with a diameter of 2 mm and immunoelectrophoresis was performed at room temperature for 45 min with a constant voltage of 60 volts. Sixty µL of

antibody (Miles Laboratories Inc.) was added to the trough and diffusion was performed overnight in a cold room. After deproteinization by shaking in 0.3 M and 0.15 M NaCl solutions and then in water, each for 1 day, the gel was air-dried, and stained with Amido Black 10B dye solution.

Quantitative immunochemical analysis of IgG was carried out by radial immunodiffusion (R.I.D.) with a R.I.D. kit (Miles Laboratories Inc.). Whey or protein samples were dialyzed against 20 mM sodium phosphate buffer pH 7.0 for 2 days and freeze-dried. Whey samples were then dissolved in 0.05 M barbital acetate buffer pH 8.3 to give a concentration within the range of the kit used for determination of immunoglobulins. After deproteinization, the gel was air-dried and then stained with Amido Black 10B dye solution.

2. Enzyme linked immunosorbent assay for anti-lipopolysaccharide activity determination

The method of Stephens (1984) was used with slight modifications. Immulon 2 flat-bottomed microtitre plates were coated with 100 µL of 0.01% lipopolysaccharides (LPS) in coating buffer (0.05 M sodium carbonate, pH 9.6) for 2 hr at room temperature and washed three times in 0.01 M sodium phosphate bufferred saline (PBS) containing 0.05% Tween, pH 7.2. Serial dilutions of immunoglobulins, made in PBS/Tween, were dispensed in 100 µL volumes and the plates incubated for 2 hr at room temperature. After further washing with PBS/Tween (3 times), 100 µL of alkaline phosphatase conjugated rabbit antibovine IgG (1:750 dilution in PBS/Tween) were added and incubated for 2 hr. Plates were washed and 100 µL of substrate (p-nitrophenyl phosphate, 1 mg/mL in 1 M diethanolamine buffer at pH 9.8 containing 0.5 mM magnesium chloride and 0.2% sodium azide) was added. After 30 min the reaction was stopped by addition of 20 µL 5N NaOH and the absorbance change was read on an ELISA plate reader

(Titertek Multiscan, Flow Laboratories, Scotland) with a 405 nm filter. Corrections were made for non-specific adsorption of Igs.

3. Sandwich enzyme linked immunosorbent assay for IgG assays

The method of Troncone et al. (1986) was used with modifications. Immulon 2 flat-bottom microtitre plates were coated with 100 µL rabbit anti-bovine IgG (1:100 dilution in PBS/Tween) and incubated for 2 hr at room temperature and washed three times in PBS containing 0.05% Tween. Serial dilutions of immunoglobulin samples, made in PBS/Tween, were dispensed in 100 µL volumes and the plates incubated for 2 hr at room temperature. After further washing, 100 µL of alkaline phosphatase conjugated rabbit anti-bovine IgG (1:750 dilution in PBS/0.05% Tween) were added and incubated for 2 hr. Plates were washed and 100 µL of substrate (p-nitrophenyl phosphate disodium 1 mg/mL in 1 M diethanolamine buffer at pH 9.8 containing 0.5 mM magnesium chloride and 0.2% sodium azide) was added. After 30 min the reaction was stopped by the addition of 20 µL 5 N NaOH and the absorbance was read on an ELISA plate reader with a 405 nm filter. Corrections were made for non-specific adsorption of Igs.

E. SODIUM HEXAMETAPHOSPHATE TREATMENT OF CHEESE WHEY

An aliquot of 10% sodium hexametaphosphate (SHMP) solution was added to 25 mL of pH adjusted cheese whey while maintaining the pH by dropwise addition of 3 N NaOH or 3 N HCl. The mixture was held for 1 hr, then centrifuged at 10,000 x g for 15 min. The precipitate was dispersed in 5 mL of 0.5 M Tris-HCl buffer, pH 6.8, and made up to 25 mL after further pH adjustment to 6.8. The supernatant was neutralized to pH 6.8 with 3 N NaOH. The samples were dialyzed against distilled water for 48 hr and then freeze dried (Figure 1).

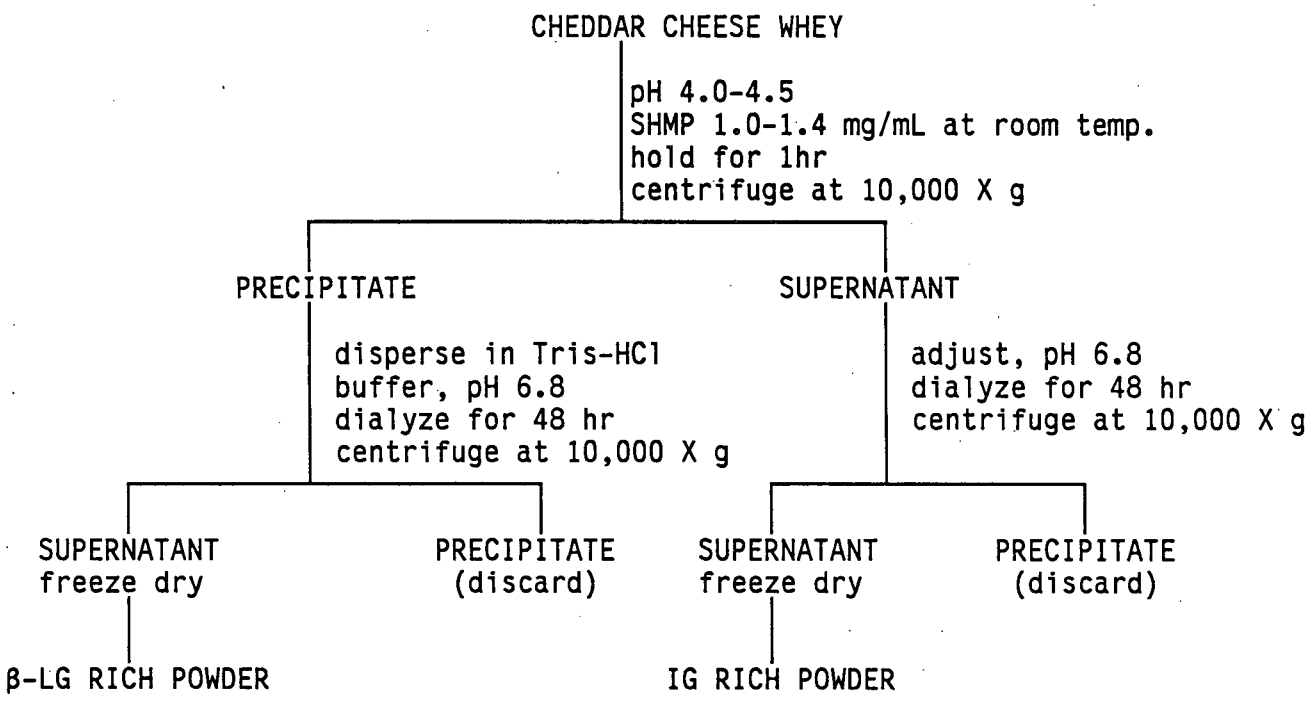


Figure 1. Flow diagram of the procedure for elimination of β-lactoglobulin from Cheddar cheese whey with SHMP.

F. OPTIMIZATION PROCEDURE

The mapping super simplex optimization (MSO) of Nakai et al. (1984) was used to find the most suitable conditions for the polyphosphate treatment of cheese whey which would give the maximum separation efficiency of Igs and a minimum amount of β-lactoglobulin in the supernatant. An IBM PC computer was used for computation for the MSO and centroid mapping optimization (CMO) by the method of Aishima and Nakai (1986). The experimental conditions (factors) used in MSO and CMO were within the following ranges: pH 4.0-4.5, SHMP concentration 1.0-1.4 mg/mL. All experiments were carried out at room temperature (22°C).

1. Mapping super simplex

Mapping super simplex introduced by Nakai et al. (1984) and written for the IBM-PC was used in order to speed up the iterative optimization procedure and graphically illustrate the experimental response surface. After doing nine experiments, the level values for each factor used in the optimization were divided into four groups based on their locations on the scale within large, medium and small limits. The large and small limits were determined from individual plots of response value (Separation efficiency) vs. each factor level (initial and final concentration of SHMP and pH). The medium limit was an average of both large and small limits. These limits were used for grouping the data. Data points for one factor which belonged to the same groups of other factors were joined together thus giving an estimate of the response surface. The maps for all factors provided new level values for each factor.

2. Centroid mapping optimization and simultaneous factor shift

Centroid mapping optimization (Aishima and Nakai, 1986) was used in order to improve the optimization efficiency and to allow for a series of experiments

to be run simultaneously. After doing another six experiments, the map for each factor was generated in similar manner as in (F-1). The maps provided target values where the high separation efficiencies were located.

A Simultaneous Factor Shift Program (Nakai et al., 1984) written for an IBM-PC was used. Target values (estimated best separation efficiency) were determined from the graphs. The program is designed to shift all factor levels obtained from the mapped graphs simultaneously one fifth the distance between the present best value and the target value. The new experimental conditions (vertices) resulting from the Simultaneous Factor Shift Program were investigated and their response values were calculated.

G. EVALUATION OF SEPARATION EFFICIENCY (RESPONSE VALUE)

Peak areas of whey proteins on the electrophoretograms were analysed using a Kontes fiber optic scanner (Model K-494800, Kontes Scientific Instruments, Vineland, NJ) together with a Varicord variable response recorder (Model 42 B, Photovolt Corp, NY). Separation efficiency (SE) was expressed as the "Igs to β-Lg ratio" calculated from peak area of Igs (PA_{Igs}) and β-Lg ($PA_{\beta-Lg}$) on the densitometric patterns as:

$$SE = PA_{Igs} / (PA_{Igs} + PA_{\beta-Lg})$$

PA_{Igs} was estimated by multiplying the heavy chain peak area by a coefficient of 1.4 since the determination of light chain peak area was difficult due to overlapping with other minor proteins. The coefficient 1.4 was derived from analysis of IgG standards.

For quantitative analysis, the variation of staining and destaining conditions during electrophoresis was standardized using an internal standard of ovalbumin. Ten microliters of 0.1% ovalbumin solution treated with SDS and 2-mercaptoethanol (similar to the treatment of sample) was added to each whey

sample solution and analyzed simultaneously. The ovalbumin peak area measured for every run was compared with the peak areas measured for a series of ovalbumin standard. The ratio of ovalbumin values, thus obtained, was used as a correction factor.

H. SURFACE PLOT

Contour and 3-dimensional surface plots were obtained using the UBC Surface Visualization Routines program (Mair, 1982) on an Amdahl 470 V/8 computer. The 3-dimensional plot was rotated and tilted for the best view of the surface: (a) The "about" angle was the angle of turn, in degrees, of rotation about the z-axis, measured clockwise from the positive x-axis; (b) the "above" angle was the angle of tilt, in degrees, of rotation about the y-axis, measured above the xy plane. In this work, x=pH, y=SHMP and z=SE (Separation efficiency).

I. PHOSPHORUS DETERMINATION

The phosphorus distribution of the fractions obtained by SHMP treatment was determined according to the method of Morrison (1964).

J. FRACTIONATION PROCEDURES OF BIOACTIVE COMPONENTS

1. Gel filtration chromatography

Immunoglobulins were isolated from colostral whey, acid whey and cheese whey using Sephacryl S-300 (Pharmacia Fine Chemicals, Uppsala, Sweden) (94 x 2.5 cm) and Fractogel TSK HW-55 (EM Science, Gibbstown, NJ) (40 x 2.6 cm). The column of Sephacryl S-300 was equilibrated with 0.1 M Tris-HCl buffer, pH 8.0 containing 0.5 M NaCl (Pharmacia Fine Chemicals, 1978), while the column of Fractogel TSK was equilibrated with 0.07 M imidazole - 0.05 M KCl buffer, pH 6.5.

2. Silica adsorption Chromatography

Chromatographic conditions were based on the process recommended by Spring and Peyrouset (1982) for silica, controlled pore glass and alumina. Small columns (1.3 x 7.5 cm) were equilibrated with 0.005 M sodium phosphate solution, pH 8.2, then whey containing 0.005 M phosphate was passed through. After washing off the unbound materials, the bound lactoferrin was eluted with 0.1 M acetic acid containing 0.5 M NaCl, and the immunoglobulin fraction was eluted with 0.1 M Tris-HCl buffer containing 0.5 M NaCl, pH 9.0.

3. Heparin-Sepharose Chromatography

Lactoferrin was isolated from cheese whey by using heparin-Sepharose column after equilibration with 0.005 M Veronal-HCl containing 0.05 M NaCl, pH 7.4 (Blackberg and Hernell, 1980).

4. Metal Chelate-interaction Chromatography

Sepharose 6B was activated according to the method of Sundberg and Porath (1974). One hundred grams of suction-dried Sepharose 6B was washed on a glass filter-funnel with water and then mixed with 100 mL of 1,4-butanediol diglycidyl ether (BGE) and 100 mL of 0.6 M sodium hydroxide solution containing 2 mg of sodium borohydride per millilitre. The suspension was mixed by rotation for 8 hr at 25°C and the reaction stopped by washing the gel on a glass filter-funnel with large volumes of water.

Epoxyactivated gel obtained above was coupled to iminodiacetic acid according to the method of Porath and Olin (1983). To 100 grams of epoxyactivated gel 250 mL of 2 M Na_2CO_3, 12.5 g of disodium iminodiacetate, and 0.15 gram of sodium borohydrate were added. The suspension was kept at 60°C overnight with slow stirring. The gel was washed thoroughly on a Büchner funnel

with water, with diluted acetic acid (5%) and again with water until the washings were neutral (Figure 2).

Iminodiacetic acid 1,4-butanediol diglycidyl Sepharose 6B (IDA-BGE Sepharose Sepharose 6B) was packed into glass columns with distilled water. The upper one-half to two-thirds of the chelating Sepharose was saturated with copper ions as indicated by their blue color, followed by washing off with distilled water and equilibration with the starting buffer (0.05 M Tris-acetate containing 0.5 M NaCl, pH 8.2). Liquid whey containing 0.05 M Tris-acetate and 0.5 M NaCl, pH 8.2 was passed through the copper loaded column. After washing off the unbound whey protein fractions with the starting buffer, linear gradient of elution was used to elute the bound proteins; the pH gradient was formed using equal volumes of starting buffer at pH 8.2 and a limit buffer at pH 2.8. Alternatively, bound proteins were eluted with the same buffer at pH 4.0 and 0.01 M imidazole as a two step elution.

The eluent was collected in fractions and protein peaks were detected by UV absorbance at 280 nm using Cary 210 Spectrophotometer (Varian Instrument Division, CA).

Following gradient elution, the chelating gel was regenerated with 0.05 M Na_2EDTA solution to strip off the copper ions, followed by 6 M urea to remove any remaining bound proteins. After washing with distilled water, the chelating Sepharose was ready for the next cycle of copper loading and whey treatment (Figure 3).

K. DETERMINATION OF CAPACITY OF MCIC

1. Immunoglobulins

Crude Ig was isolated form bovine colostral whey by ammonium sulfate precipitation, according to the method of Fey et al. (1976). A solution of the

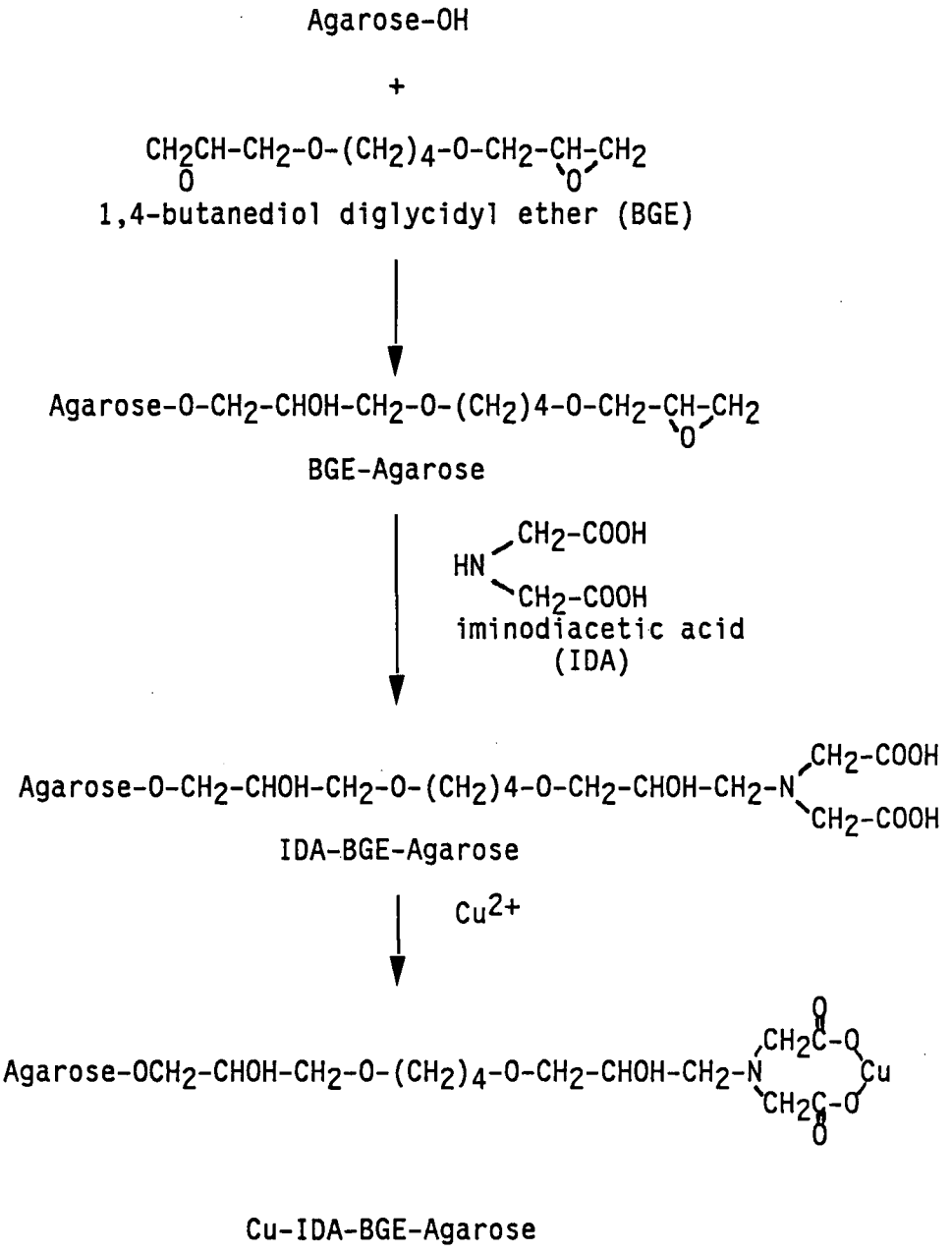
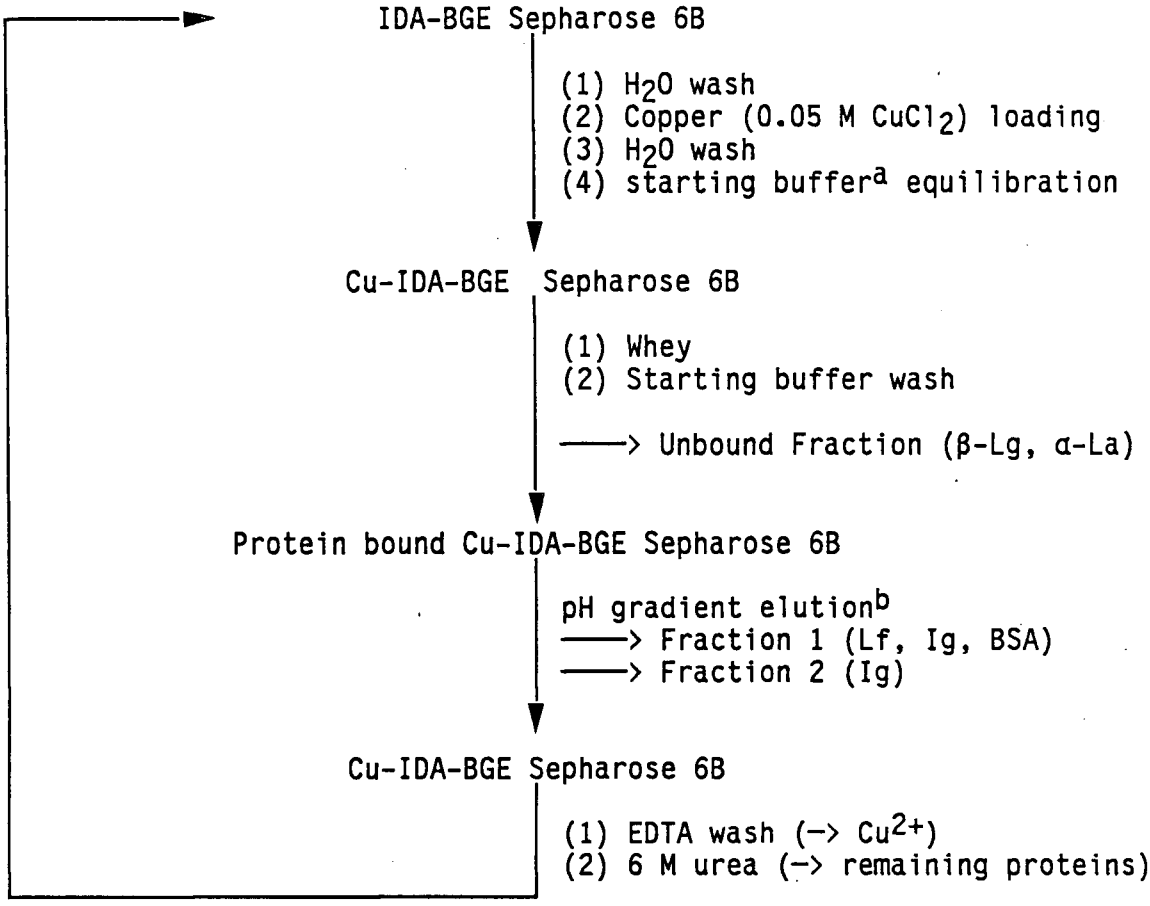


Figure 2. Preparation of metal chelate agarose



[a] starting buffer = 0.05 Tris acetate, pH 8.2, 0.5 M NaCl
[b] linear gradient: starting buffer as in Footnote a, limit buffer = 0.05 M Tris acetate, pH 2.8, 0.5 M NaCl

Figure 3. Flow chart of MCIC process of cheese whey treatment for isolation of Ig

crude Ig (roughly 0.3% w/v) with A_{280} of approximately 2.1 was passed through a small column (1.4 x 7 cm) containing 2.2 mL copper-loaded chelating Sepharose 6B equilibrated in starting buffer (0.05 M Tris-acetate pH 8.2, 0.5 M NaCl). While the crude Ig solution was being applied to the top of the column, the eluted fractions were continually monitored with respect to A_{280}. Saturation of the column (i.e., no further binding of protein) was indicated when A_{280} of the eluted fractions became equal to A_{280} of the original crude Ig solution. The binding capacity of the copper-loaded gel was calculated, at this saturation point, as the amount of applied crude Ig minus the amount of unbound Ig (This amount of Ig was calculated from the A_{280} and volume of each fraction). After washing off unbound protein with the starting buffer, the bound proteins were eluted with 0.05 M Tris-acetate buffer at pH 4.0 containing 0.5 M NaCl. The recovery of Ig was calculated as the percentage of eluted protein compared to bound protein.

2. Ovotransferrin

A solution (0.2% w/v) of commercial OVT with A_{280} of approximately 1.94 was passed through a small column (1.4 x 7 cm) containing 3 mL copper-loaded chelating Sepharose 6B equilibrated with the starting buffer. While the OVT solution was being applied to the top of the column, the eluted fractions were continually monitored by measuring A_{280}. Saturation of the column (i.e., no further binding of protein) was indicated when A_{280} of the eluted fractions became equal to 1.94. The binding capacity of the copper-loaded gel was calculated as the difference between the amounts of OVT applied and unbound. After washing off the unbound protein with the starting buffer, the bound proteins were eluted first with 0.05 M acetate-Tris buffer at pH 4.0 containing 0.5 M NaCl, and then with 0.01 M imidazole. The recovery of OVT was calculated as the percentage of eluted protein compared to bound protein.

3. Transferrin

A solution of 0.2% (w/v) transferrin in 0.05 M Tris-acetic acid/0.5 M NaCl buffer, pH 8.2, was passed through the column charged with copper ion. The absorbance at 280 nm (A_{280}) of the effluent from the column was monitored. The binding capacity was calculated from the differences of the absorbance of the eluted protein as compared to the absorbance at the saturation point of the column. The percentage of protein eluted was calculated by comparing the amount of protein bound to the column with the protein eluted from the column.

L. PRETREATMENT OF EGG WHITE

Eggs were obtained from the University of British Columbia Experimental Farm. To obtain a homogeneous and less viscous sample with suitable flow properties, the separated egg whites were blended (2000-2500 rpm, 7-10 sec) in a Lourdes MM-1A MultiMixer (Lourdes Instrument Corporation, Old Bethpage, NY) as reported by Li-Chan et al. (1986).

M. PREPARATION OF APO, DIFERRIC AND DICUPRIC OVOTRANSFERRIN

Iron free (apo) OVT was prepared by dialyzing standard OVT first against 0.1 M citric acid, pH 2-3 for 36 hr at 4°C, then against deionized water prior to lyophilization. Diferric OVT was prepared by dialyzing the apo-OVT against 1.7 mM ferrous ammonium sulfate for 36 hr, then excess iron was removed by gel filtration on a Shephadex G-25 column equilibrated with 0.05 M Tris-acetate/0.5 M Nacl, pH 8.2 (Cole et al., 1976). In a similar manner, dicupric OVT was prepared by dialyzing against 0.01M cupric chloride.

N. HISTIDINE MODIFICATION OF PROTEINS

Histidine residues of Ig, TF, OVT and casein fractions were modified according to the method of Rogers et al. (1977) with modifications. Diethylpyrocarbonate (DEP) to make the final concentration of 20 mM was added directly to a 5-10 mg/mL protein solution in 0.05 M phosphate buffer pH 6.6 containing 8 M urea while stirring. After 20 min stirring the extent of ethoxyformyl histidine formation was determined by an increase in A_{240} of the reaction solution (E_{240} = 5.9×10^3 L M^{-1} cm^{-1}) (Roosemont, 1978). The purity of DEP used was determined according to Holbrook and Ingram, (1973).

O. ISOELECTRIC FOCUSING

Analytical horizontal polyacrylamide gel isoelectric focusing (IEF-PAGE) was carried out in a Bio-Rad Model 1415 electrophoresis cell, according to the manufacturer's instructions. Gel slabs were 45 mm x 125 mm and 0.8 mm thick. Bands were located by means of a Coomassie Blue protein stain.

P. PREPARATION OF ANTISERA

Antiserum to egg white and other proteins were produced by immunizing adult female New Zealand white rabbits (UBC, Animal Care Unit) each time with 1-10 mg of antigen emulsified in Freund's complete adjuvant (FCA). Immunizations were given in multiple subcutaneous sites, and repeated intravenously (I.V.) in a two to six week period by replacing FCA with phosphate buffered saline, pH 7.2, as a carrier until a satisfactory response was obtained. Serum was tested by double diffusion in gel against egg white proteins as reported by Garvey et al. (1977).

Q. MEASUREMENT OF BACTERIOSTATIC ACTIVITY

The method of Dolby and Stephens (1983) was used for the determination of bacteriostatic activity of the isolated proteins. Immunoglobulin and transferrin prepared by the MCIC method were added to 5 mL of Trypticase Soy Broth (TSB) at concentrations of 10 mg/mL each or in a mixture of 5 mg/mL each. A half mL of 0.05 M $NaHCO_3$ was added to each broth and sterilized by filtration (Millex-HA, 0.45 µm, Millipore Corp. Bedford, MA). The broths including the control (TSB only) were inoculated with 10^4 colony forming units (cfu)/mL of the test culture; samples of the broth cultures were taken after 1, 3, and 5 hr. Serial dilutions of the bacteria in the broth cultures was accomplished by plating on Trypticase Soy Agar (TSA) with the spiral plater (Anonymous, 1985). The inoculated plates were incubated at 37°C for 18 hr.

R. EXTRACTION OF LIPOPOLYSACCHARIDES

Lipopolysaccharides of E. coli, S. typhimurium and B. parapertussis were extracted by using phenol/water according to the method of Jann (1985). After cultivation in TSB, the bacteria were killed by the addition of 1% phenol, centrifuged at 5000 x g for 30 min and washed with 0.15 M saline and centrifuged again. They were then freeze-dried. One gram of dry bacteria was suspended in 20 mL of water at 68°C. Twenty mL of 90% phenol, prewarmed to 68°C were added to the bacterial suspension and the mixture was kept at 68°C with vigorous stirring for 15 min. After cooling to about 10°C in an ice-bath, the suspension was centrifuged at 5000 x g for 30 min. This resulted in the formation of two phases. A precipitate was formed between the layers and a bacterial pellet in the lower phase. The upper aqueous phase was collected by suction, then the lower phenol phase together with the pellet was treated with 20 mL water at 68°C as described above. The combined aqueous phases were

dialyzed against distilled water for 48 hr in the cold room to remove phenol and low-molecular weight material. The solution was then freeze-dried to give a white powder.

S. LACTOPEROXIDASE ASSAY

The lactoperoxidase content of fractions obtained by MCIC method were analyzed by using the procedure described by Sigma Chemical Co. (Bull. No. 8-84 for peroxidase prod. No. p.8250). A substrate mixture of 0.1 M potassium phosphate buffer, pH 6.0 (0.32 mL), 0.147 M hydrogen peroxide (0.16 mL), 5% (w/v) pyrogallol (0.32 mL) and distilled water (2.1 mL) was mixed by inversion. The initial A_{420} was monitored until constant with a cuvette containing H_2O as the reference. To this mixture, at zero time, 0.1 mL of a lactoperoxidse containing fraction (10 mg lactoperoxidase per mL of 0.1 M potassium phosphate buffer, pH 6.0) was added. The solution was mixed by inversion and the increase in the in A_{420} was recorded every 10 seconds for about two minutes, using a Cary 210 Spectrophotometer (Varian Canada Inc.). The initial linear rate of increase in absorbance was determined using linear regression, and was used to determine the units of lactoperoxidase activity per mg solid:

$$\text{Units/mg solid} = \frac{A_{420}/20 \text{ second}}{(12)^* \times (\text{mg enzyme as solid/mL reaction mix})}$$

12* = Extinction coefficient as determined by Sigma

Units obtained were compared to that of bovine lactoperoxidase (80 units/mg protein using pyrogallol as substrate). Lactoperoxidase was calculated as the percentage of lactoperoxidase content in the pooled fractions.

T. SEPARATION OF HEAVY AND LIGHT CHAINS OF IMMUNOGLOBULINS

Reduction and alkylation of S-S groups in the Ig rich fraction were performed according to the method of Garvey et al. (1977). A 10 mL solution of 2% Ig in 0.55 M Tris-HCl buffer pH 8.2 was bubbled with N_2 for 15 min. Five mL of 0.15 M dithiothreitol in 0.55 M Tris-HCl buffer were added, and the mixture was allowed to react for 1 hr under a positive N_2 atmosphere. Ten mL of 0.25 M 2-iodoacetamide (IAA) in 0.55 M Tris-HCl buffer pH 8.2 were then added to the reduced Ig sample and the mixture was kept in the cold room for 1 hr. The reduced and alkylated Ig was then equilibrated with 1 M propionic acid or 0.1 M Tris-HCl buffer containing 4 M guanidine-HCl and 1 mM IAA, pH 8.2 and fractionated on a Sephadex G-75 and an Ultrogel ACA 54 column (40 x 2.6 cm), respectively.

RESULTS AND DISCUSSIONS

PART I

REDUCTION OF β-LACTOGLOBULIN CONTENT OF CHEESE WHEY BY USING SODIUM HEXAMETAPHOSPHATE

Since elimination of β-lactoglobulin by ferric chloride methods (Kaneko et al. 1985; Kuwata et al. 1985) can saturate iron binding proteins present in whey and may result in loss of the antimicrobial activity of lactoferrin, non-ferric methods were investigated (Appendix 1). Polyphosphates have been extensively used as additives in food processing. Gordon (1945) in his patent used polyphosphate to extract whey proteins from cheese whey. In this part, polyphosphates were investigated as a possible means for the selective precipitation of β-lactoglobulins from cheese whey leaving immunoglobulins in the supernatant.

A. OPTIMUM CONDITIONS FOR SEPARATION OF IMMUNOGLOBULINS AND β-LACTOGLOBULIN

Mapping simplex optimization with two factors generated three experiments called the "initial simplex". After obtaining the response values which were, in our case, the separation efficiency (SE) of immunoglobulins, the values were reported back to the computer to obtain new vertices (experimental conditions) in a form of the repetitive sequences of centroid, reflection, and curve-fitting. After performing nine vertices for the MSO, mapping was done by plotting the response values against the factor levels. A crude approximation of the response surface appeared to direct the search for higher SE towards more acidic conditions (pH 4.0-4.2); therefore, the range for lower and upper limit of pH was narrowed down to 4.0-4.2. In a similar manner, higher SE could be expected at higher polyphosphate concentrations (1.2-1.4 mg/mL); therefore, the range of concentration of SHMP was restricted to 1.3-1.4 mg/mL.

With the new lower and upper limits for the pH and SHMP, the CMO program was applied. After entering the new ranges, a new initial simplex (three vertices) was created. The experiments were carried out and the response values were reported. Upon improvement in the response values, simultaneous shift was implemented after six experiments in the centroid search. Figures 4A and 4B show

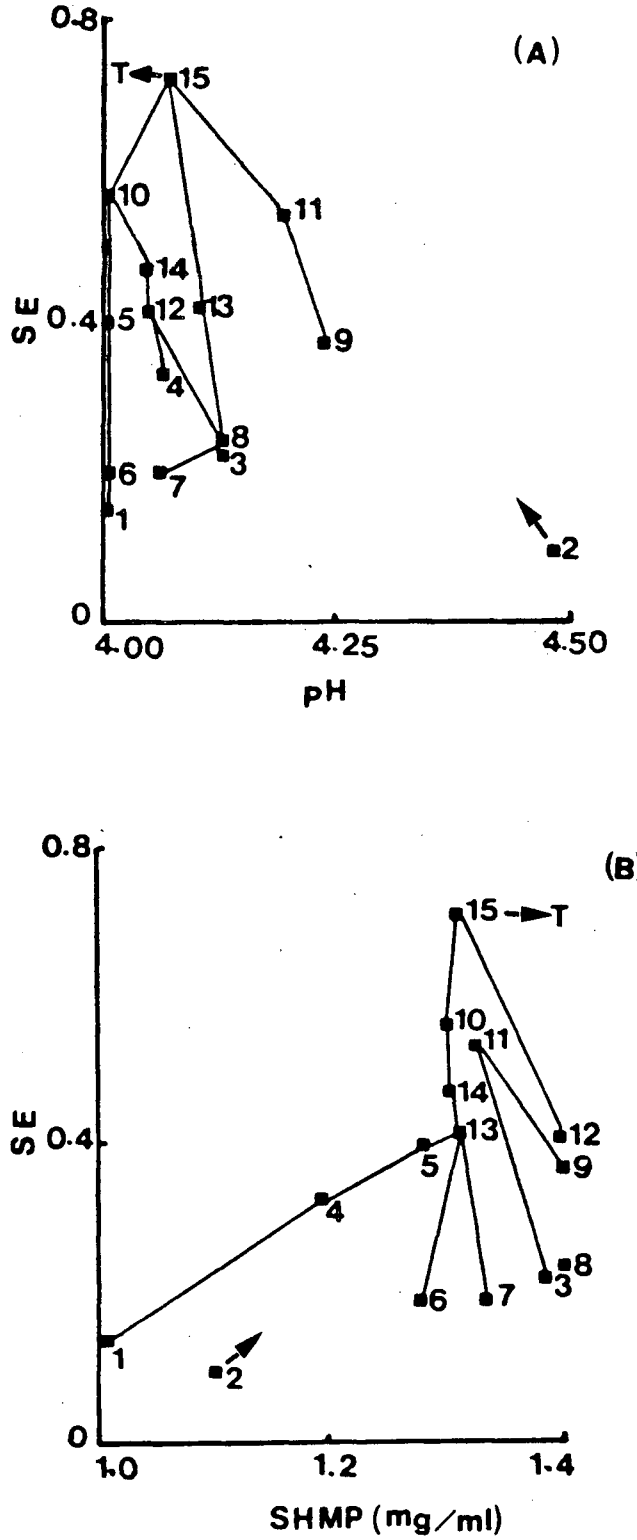


Figure 4. Approximate response surface patterns for (A) pH and (B) SHMP concentration obtained by mapping accumulated data from simplex optimization (Vertices 1-9) and centroid optimization (Vertices 10-15). T, target values of pH and SHMP.

the approximate response surface for both factors from which, based on the present best values of 4.07 and 1.33, the target values (T) of 4.03 and 1.36 were set for pH and SHMP, respectively. However, because of the failure to achieve further improvement in response values, further experimentation was discontinued.

Examining the best response value (Vertex 15 Figures 4A and 4B), it was found that pH 4.07 and 1.33 mg SHMP/mL yielded about 80% elimination of β-lactoglobulin from cheddar cheese whey (into the precipitate), with almost complete recovery of immunoglobulins in the supernatant. The majority of α-lactalbumin was found in the supernatant as indicated by SDS-PAGE (Figure 5). However, most of the bovine serum albumin was precipitated along with the β-lactoglobulin with only a small amount remaining in the supernatant. Recovery of Igs in the supernatant was evident immunochemically as shown in Figure 6. The supernatants showed a long, outer precipitating line corresponding to the standard IgG arc; while the precipitates showed no such precipitating line. The inner precipitating lines (by the sample application well) might represent lactoferrin, transferrin and IgM. Serum albumin and β-lactoglobulin arcs are indicated by the inner and the outer precipitating lines, respectively, formed toward the anode, far from the application well. The response of anti-bovine whey protein antiserum toward α-lactalbumin was rather weak (not shown), probably due to its low molecular weight and consequently lower antigenicity. Approximately 90 % of Igs in the supernatant were determined to be IgG by means of R.I.D. (not shown).

B. THREE DIMENSIONAL ILLUSTRATION OF EFFECTS OF pH AND HEXAMETAPHOSPHATE ON SEPARATION EFFICIENCY

Contour and 3-dimensional surface plots were generated by computer to aid in visualization of the relationship between pH, SHMP and SE of immunoglobulins in Cheddar cheese whey. Figures 7A and 7B showed that, in general, combinations of

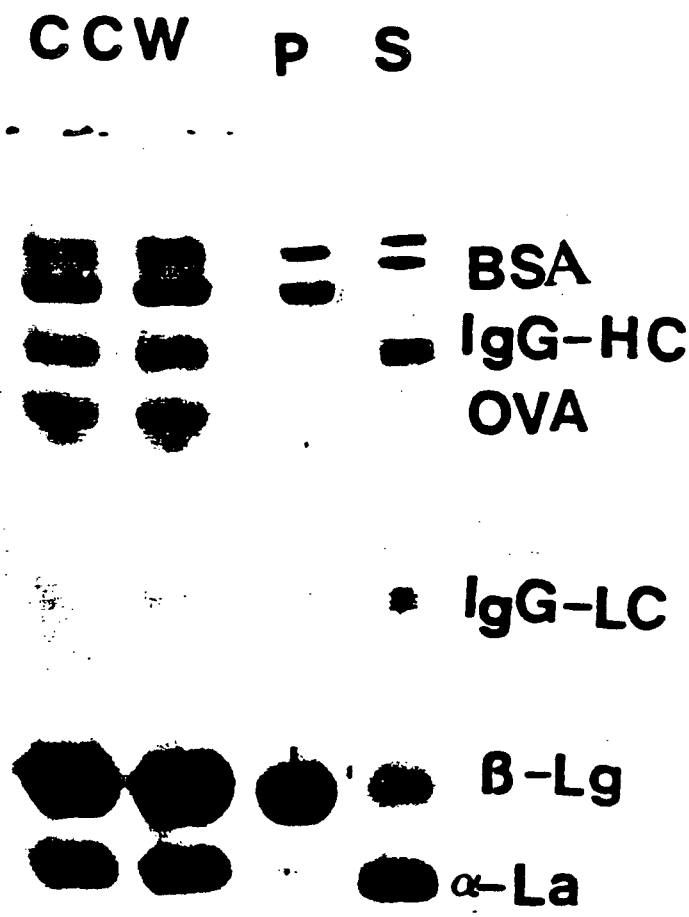


Figure 5. SDS-PAGE of supernatant (S) and precipitate (P) obtained after treatment with 1.33 mg/mL SHMP at pH 4.07. CCW, Cheddar cheese whey; α-La, α-lactalbumin; β-Lg, β-lactoglobulin; IgG-HC, immunoglobulin G heavy chain; IgG-LC, immunoglobulin G light chain; BSA, bovine serum albumin; OVA, ovalbumin.

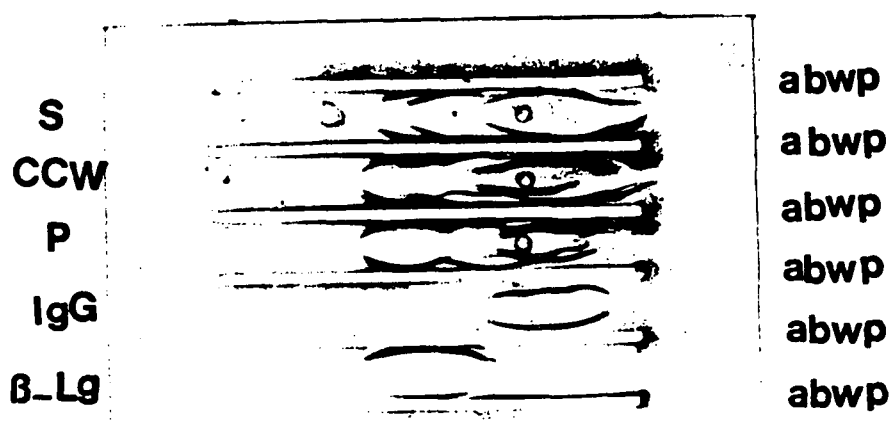


Figure 6. Immunoelectrophoretic pattern of cheddar cheese whey. S, supernatant; CCW, Cheddar cheese whey; P, precipitate; IgG, immunoglobulin G; β-Lg, β-lactoglobulin; abwp, antibovine whey proteins.

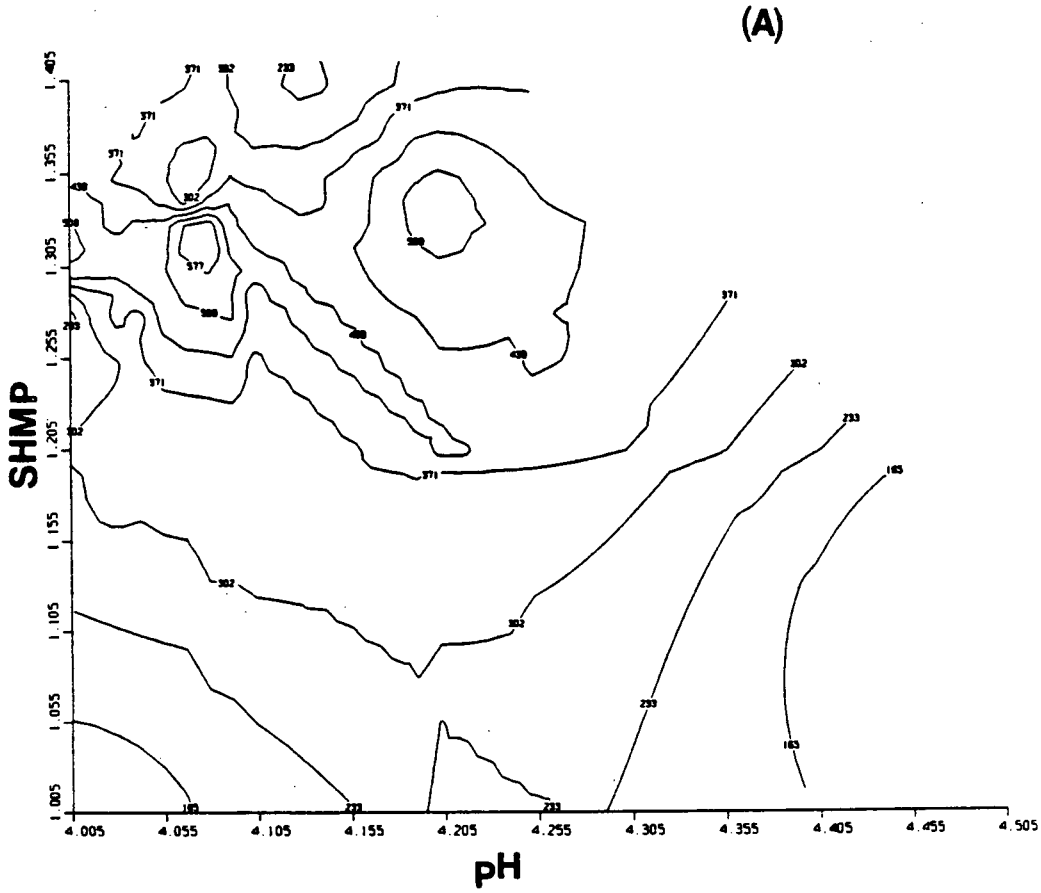
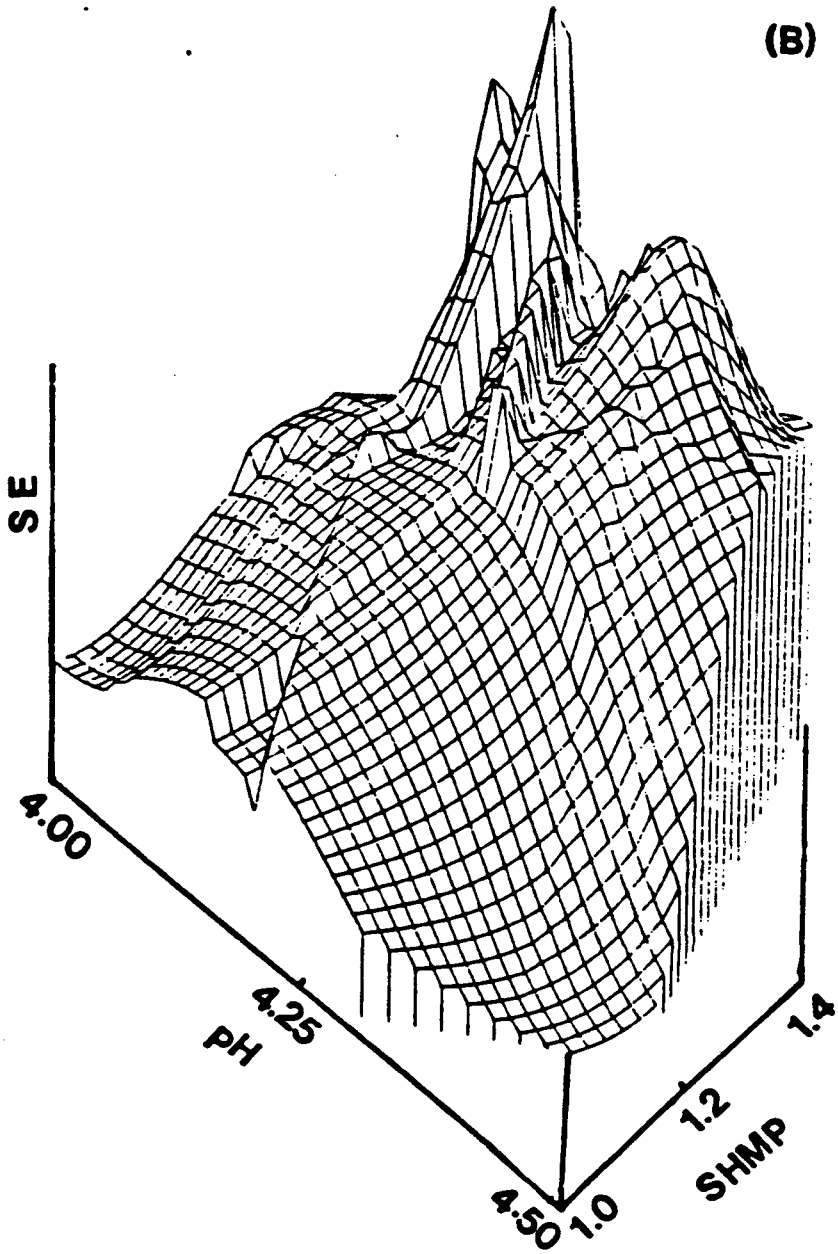


Figure 7. Contour (A) and 3-dimensional (B) surface plots of relationship between pH, SHMP and Separation effeciency (SE) of cheese whey treatment. ("about" angle=60 and "above" angle=35 for 3-dimensional plot).


(B)
SE
4.00
4.25
4.50
pH
1.0
1.2
1.4
SHMP

low pH and high SHMP concentration resulted in good separation efficiency. Separation efficiency was improved by decreasing pH values below 4.25 and by increasing SHMP concentration above 1.2 mg/mL. By lowering the pH below the isoelectric point of whey proteins, the positive side chain amino groups could interact with the negative groups surrounding phosphate molecules by which polyphosphates act as cross-linking agents. However, different proteins may interact differently via polyphosphates to form aggregates. Surface exposed amino groups and unfolding or expansion of protein molecules when the polyphosphate is bound may play an important role in that interaction, leading to preferential precipitation of β-lactoglobulins (Melachouris, 1972).

The contour plot (Figure 7A) shows three humps with SE of 0.508, 0.577 and 0.302, which can be seen also in Figure 7B. Multiple peaks might have been caused by the absence of data points between SE 0.508 and the other two peaks with SE's of 0.302 and 0.577; in other words, more experiments under the conditions with closer intervals between pH 4.2 and 4.08 might be required in order to obtained a smoother surface.

C. ELIMINATION OF PHOSPHORUS

The phosphorus distribution in the supernatant and the precipitate are shown in Table 5. Removal of 72.2% and 45.3% of the total phosphorus from the supernatant and the precipitate, respectively, was achieved by dialysis against distilled water for 48 hr. Facile removal of polyphosphate from whey preparations by dialysis might indicate weak binding of phosphorus with whey proteins. Since no precipitation of whey proteins by SHMP was observed at pH values higher than 5, it was assumed that ionic interaction might be involved in the interaction between SHMP and proteins. At pH values lower than the isoelectric point, positively charged groups (basic amino acid residues) in protein molecules might interact

Table 5. Phosphorus distribution in supernatant and precipitate obtained by SHMP treatment. P, phosphorus.

Fraction	mg P/100 mL	mg P/100 mL after dialysis	% removed by dialysis
Supernatant	65.0	18.1	72.2
Precipitate	16.0	8.75	45.3
Cheese whey	50.0	8.6	82.8

with each other via SHMP and cause the aggregation of the proteins. By increasing the pH above the isoelectric point and increasing net negative charges, this interaction might be disrupted and the free SHMP could be removed by a simple dialysis process, thereby separating a whey protein fraction containing a phosphorus content within the level recommended for infant formulas (0.033%) (Friend et al., 1983).

D. PROPOSAL OF NEW INFANT FORMULA

Table 6 compares the composition of the new infant formula to that of human and cow's milk and the current commercial SMA (whey-based) formula. In the commercial SMA formula, the ratio of casein/whey proteins of cow's milk (79/21) has been changed to 40/60 in order to mimic the ratio found in human milk. However, simple adjustment of casein/whey protein ratio does not minimize the compositional differences between cow and human milk proteins, i.e. higher contents of α-lactalbumin, lactoferrin, immunoglobulins and lysozyme in human milk as compared to cow's milk.

By eliminating β-Lg completely with full retention of other whey proteins, the whey protein composition of the new β-Lg-free formula would be as shown in Table 6. In this proposed formula, immunoglobulins and lactoferrin are much closer in their quantities to that found in human milk. In addition, lysozyme separated from egg white can be incorporated in this new infant formula as proposed by Friend et al. (1983), in order to improve the therapeutic value of infant formula. Thus, the β-Lg-reduced supernatant which is rich in immunoglobulins when incorporated into infant formulae, may be an additional benefit to infant feeding.

Table 6. Protein composition of human and cow's milks and whey-based and proposed β-lactoglobulin (β-Lg)-free infant formula.

Protein	Human[a] Total %	Cow Total %	Whey-based formula Total %	β-Lg free formula Total %
Total	100	100	100	100
Caseins	35.0[a]	79.0[a]	40.0[c]	40.0
Total whey	65.0[a]	21.0[a]	60.0[c]	60.0
α-lactalbumin	17.0[a]	2.8[b]	8.0	17.2[d]
β-lactoglobulin	--	11.2[b]	32.0	--
immunoglobulins	11.0[a]	2.3	6.6[d]	14.1[d]
serum albumin	6.0[a]	1.8	5.1[d]	10.9[d]
lactoferrins	17.0[a]	1.7[b]	4.9[d]	10.5[d]
lysozyme	6.0[a]	--	--	--
others	8.0[a]	1.2[b]	3.4[d]	7.3[d]

[a] Gurr, 1981
[b] Calculated from electrophoretic scanning of Cheddar cheese whey
[c] Friend et al., 1983
[d] Whey protein composition is calculated based on our data (b)

PART II

SEPARATION OF BOVINE IMMUNOGLOBULINS AND LACTOFERRIN FROM WHEY PROTEINS BY GEL FILTRATION TECHNIQUES

Although benefits expected by feeding infants with non-immunized immunoglobulins compared to feeding hyperimmunized Ig (Hilpert et al., 1974/1975; Ballabriga, 1982; Ebina et al., 1984, 1985) are unknown, some uses may be justified in collaboration with other antimicrobial components in cow's milk as discussed by Packard (1982). Lactoferrin together with secretory immunoglobulin A from human milk showed a considerable bacteriostatic effect against human enteropathogenic strains of E. coli (Dolby and Stephens 1983; Stephens et al., 1980). In this part of the thesis, gel filtration techniques were assessed for immunoglobulins and lactoferrin fractionation.

A. GEL FILTRATION ON SEPHACRYL S-300

Gel filtration is the method of choice for the preparative isolation of proteins (Van Oss, 1982-1983). Bovine colostrum contains proteins with a wide range of molecular sizes from α-lactalbumin (MW ca. 14,500) to IgM (MW ca. $1X10^6$). Gel filtration of high molecular weight proteins requires a highly porous and rigid gel to provide a good stability and short separation times, therefore, Sephacryl S-300 was chosen for this experiment. The yield of immunoglobulin isolated by gel filtration can be as high as 90% (Van Oss, 1982-1983).

Figures 8 and 9 show the separation patterns of the untreated bovine colostral whey and the ammonium sulfate treated whey, respectively. Results indicated that two major peaks were obtained in the elution profile of treated whey (Figure 9) which were also in the elution profile of untreated whey (Figure 8). The first fraction (F1) appeared in the void volume of the column which indicated that the molecular weight was in the vicinity of a million, and the second peak indicated a lower molecular weight protein. Fraction 3 (F3) of both untreated bovine colostral whey and the ammonium sulfate treated whey contained

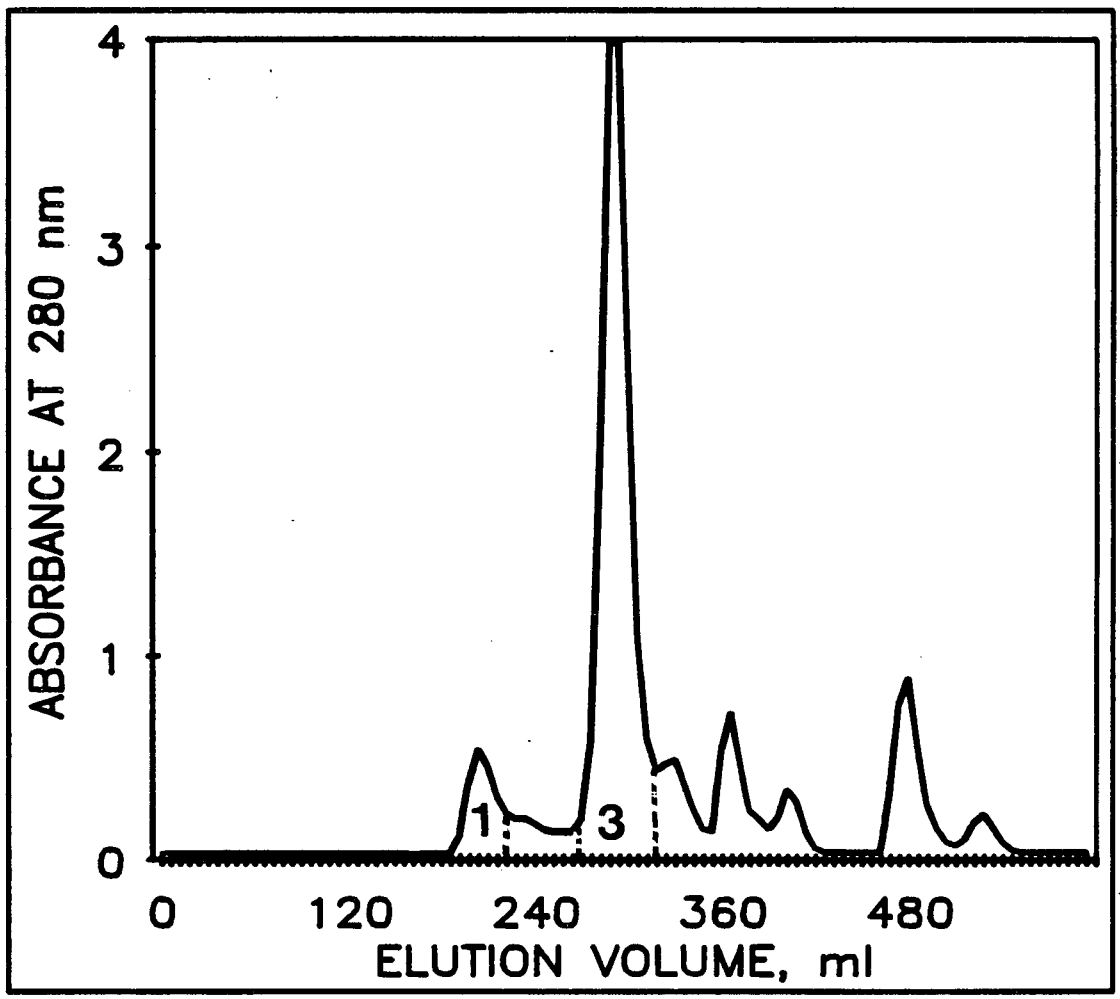


Figure 8. Gel filtration of bovine colostral whey on Sephacryl S-300 Superfine column (94 x 2.5 cm) eluted with 0.1 M Tris-HCl buffer pH 8.0 containing 0.5 M NaCl. Flow rate, 12 mL/hr. 1 and 3 are fractions 1 and 3, respectively.

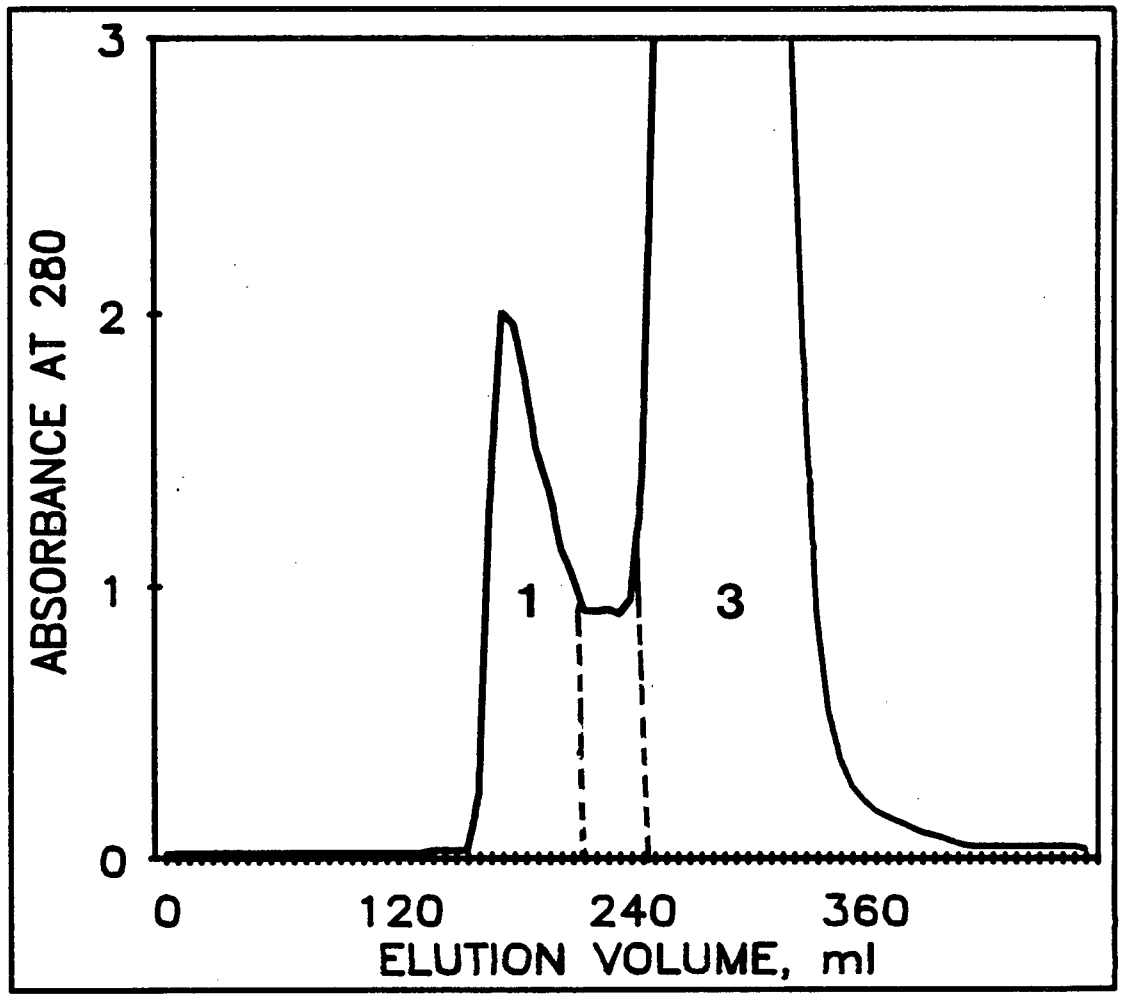


Figure 9. Gel filtration of crude Ig obtained from ammonium sulfate treatment on Sephacryl S-300 Superfine column (94 x 2.5 cm), eluted with 0.1 M Tris-HCl buffer pH 8.0 containing 0.5 M NaCl, flow rate 12 mL/hr. 1 and 3 are fractions 1 and 3, respectively.

99% IgG when analyzed by radial immunodiffusion (Table 7) and double sandwich ELISA.

Figure 10 shows the electrophoretic patterns of colostral whey and fractions obtained by gel filtration fractionation (Figure 8: F1, F3). It was observed that F3, which represented IgG, was a highly purified fraction containing IgG. However, the less pure F1 fraction contained some contaminants which moved the same distance as lactoferrin. Lactoferrin may have bound firmly to the high molecular weight compound (IgM) through hydrogen-bonding since both proteins are glycoproteins.

Immunoelectrophoresis is one of the most important techniques for the identification and characterization of immunoglobulins and their classes, because of its sensitivity and specificity (Ohtani and Kawai, 1981). Interpretation of immunoelectrophoretic patterns depends upon the specificity and the potency of the anti-sera employed. Immunoelecrophoretically, F1 and F3 showed a single precipitin arc against anti-whole bovine antiserum, attesting to the purity of these fractions (Figure 11). Since F1 diffused a short distance, it represents a high molecular weight (IgM) while F3 which diffused a longer distance may represent the lower molecular weight IgG. The diffusion rate of antibody or antigen is inversely proportional to the molecular weight (Atassi et al., 1984).

B. GEL FILTRATION ON TSK HW-55

Figure 12 is an elution pattern of colostral whey on a TSK HW-55 column which is basically similar to the elution profile of colostral whey on Sephacryl S-300. SDS-PAGE indicated that the major portion of F1 and F2 were immunoglobulins. However, F1 and F2 were not as pure as F1 and F3 obtained from Sephacryl S-300 (Figure 13). Immunoelectrophoresis clearly showed that the

Table 7. Immunoglobulin G contents* of fractions obtained from gel filtration on Sephacryl S-300 and crude Ig prepared by ammonium sulfate treatment.

Sample	Protein content** (mg/mL)	IgG content (mg/mL)	Purity %
F3 (Figure 8)	10	9.90	99
F3 (Figure 9)	10	9.90	99
Crude Ig	10	5.10	51
AW-fraction 2 (Figure 15)	7.2	6.00	83.3
CCW-fraction 2 (Figure 16)	7.6	7.00	92.1

* Determined by radial immunodiffusion analysis.
** Determined by BioRad reagent.

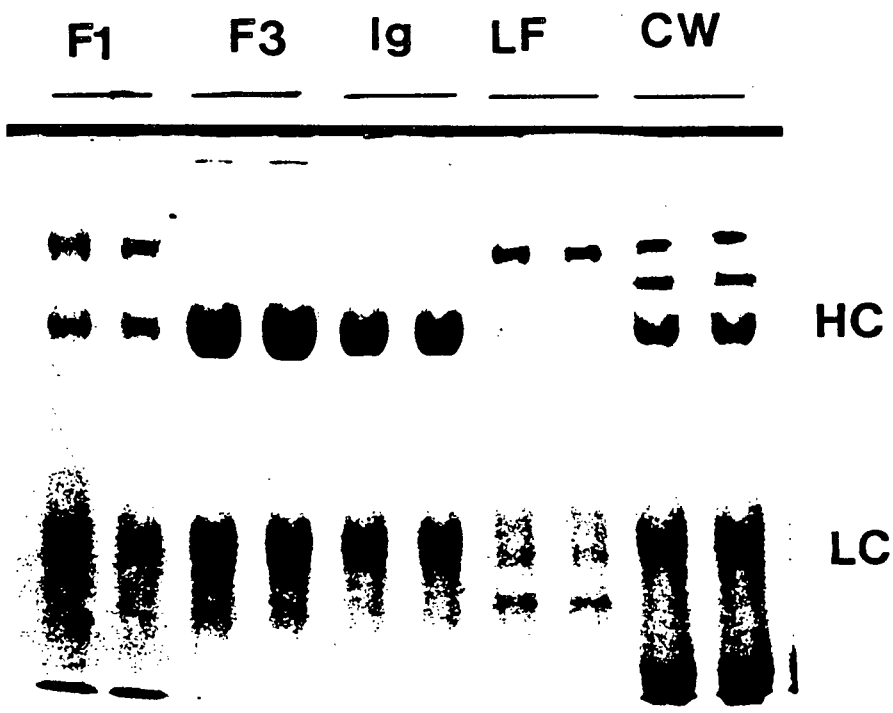


Figure 10. SDS-PAGE of fractions obtained from gel filtration on Sephacryl S-300. F1, fraction 1; F3, fraction 3 (Figure 8); Ig, crude immunoglobulin; LF, lactoferrin; CW, untreated colostral whey; HC, LC, immunoglobulin heavy and light chains, respectively.

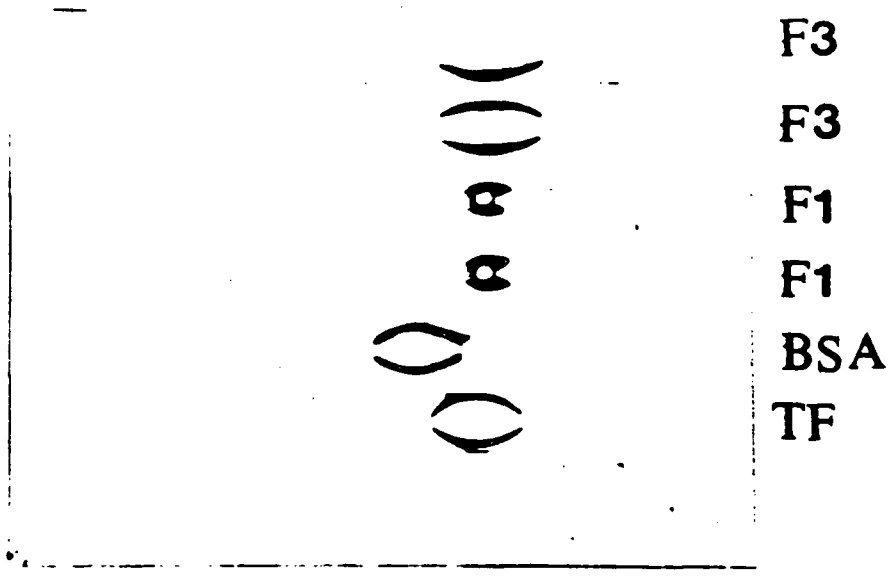


Figure 11. Immunoelectrophoretic analysis against anti-whole bovine antiserum of fractions obtained from Figure 8. F3, fraction 3; F1, fraction 1; ; BSA, bovine serum albumin; TF, transferrin.

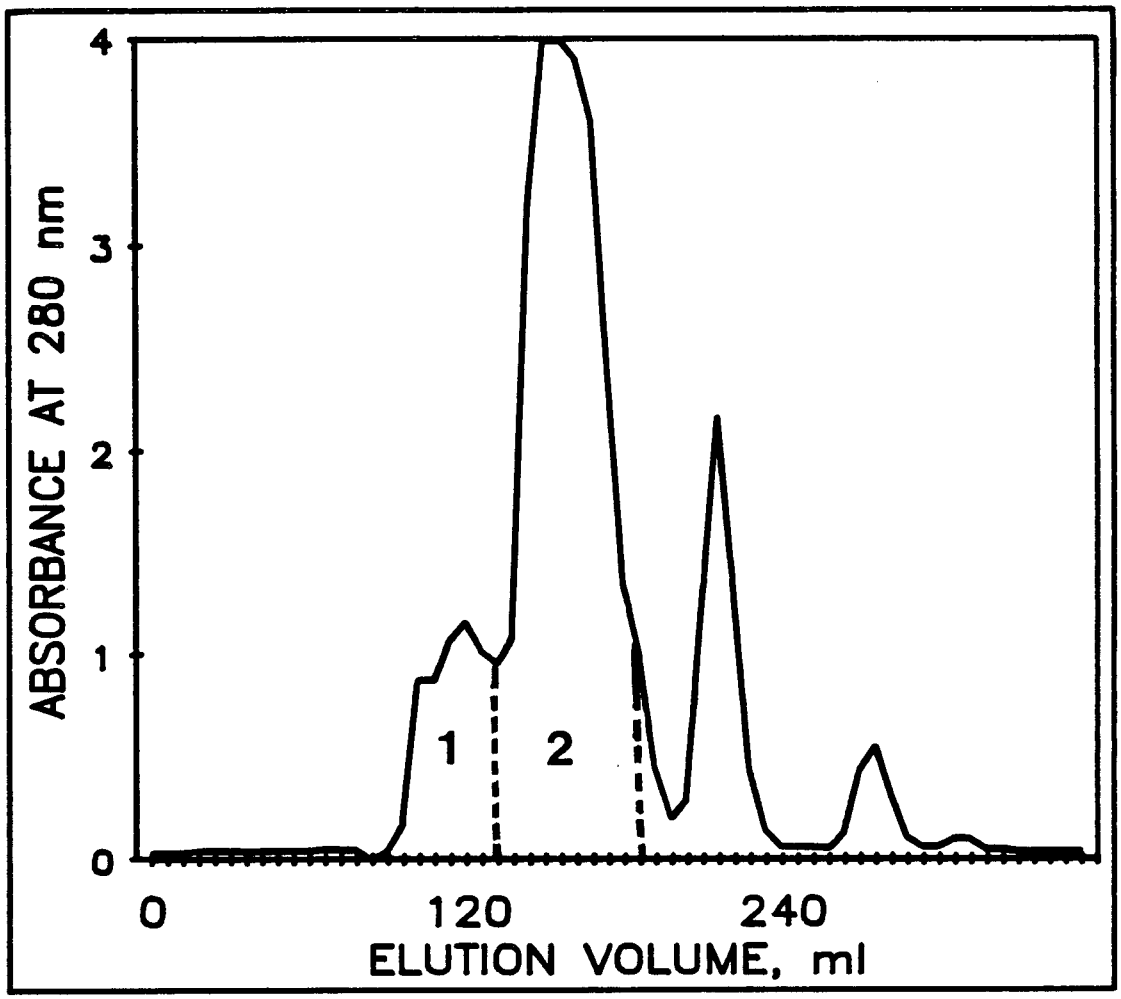


Figure 12. Gel filtration pattern of colostral whey on TSK column (40 x 2.6 cm) eluted with 0.07 M imidazole-0.05 M KCl buffer, pH 6.5; flow rate, 50 mL/hr. 1 and 2 are fractions 1 and 2, respectively.

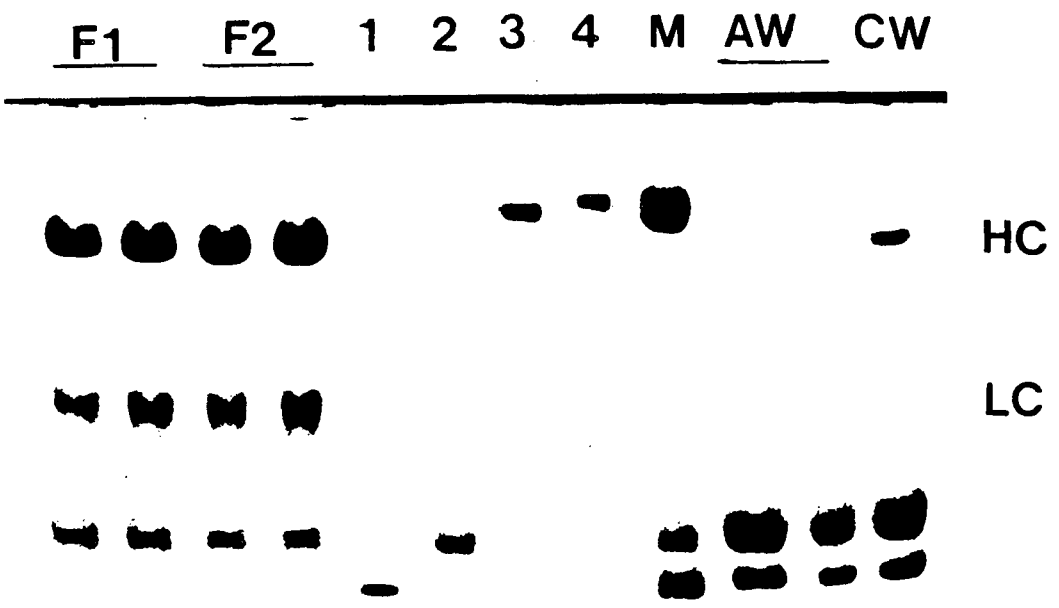


Figure 13. SDS-PAGE of fractions (F1 and F2) obtained from Figure 12 as compared to standards. Lane 1, α-lactalbumin; Lane 2, β-lactoglobulin; Lane 3, bovine serum albumin; Lane 4, transferrin; M, standard mixture; AW, acid whey, CW, colostral whey; HC and LC, immunoglobulin heavy and light chain, respectively.

fractions obtained by TSK column contained some impurities, mainly bovine serum albumin, not found in the fractions obtained by Sephacryl S-300 column (Figure 14).

By comparing fractions obtained from gel filtration on Sephacryl S-300 (F1, F3) with those obtained on TSK HW 55 (F1, F2), it is obvious that the former contains immunoglobulins of higher purity than those obtained from TSK HW 55 (Figure 14). Therefore, the Sephacryl S-300 type column was chosen to isolate Ig from acid and cheese whey.

C. ISOLATION OF IMMUNOGLOBULINS FROM WHEY PROTEINS

Figure 15 shows the elution profile of acid whey on Sephacryl S-300 while Figure 16 is the elution profile of Cheddar cheese whey on the same column. The first fraction (F1) which eluted at the void volume, was a turbid solution containing high molecular weight components. The F1 fraction was sharper for acid whey than that for the cheese whey. The F1 fraction may represent the lipoprotein fraction in both wheys. The second fraction (F2), a shoulder of fraction 3, contained immunoglobulins from acid whey and cheese whey as indicated by SDS-PAGE (Figure 17 and 18). The immunoglobulin fraction contained some impurities which were probably lactoferrin and bovine serum albumin (Table 7). Immunoelectrophoresis showed that fraction F2 of both acid whey and cheese whey contained mainly immunoglobulins with smaller amounts of bovine serum albumin (Figure 19).

D. ISOLATION OF LACTOFERRIN FROM WHEY PROTEINS

Figure 20 shows the elution profile of Cheddar cheese whey when eluted from a haparin-Sepharose column with a linear NaCl gradient. Lactoferrin was selectively adsorbed to the column from cheese whey and was eluted at about 0.5 M

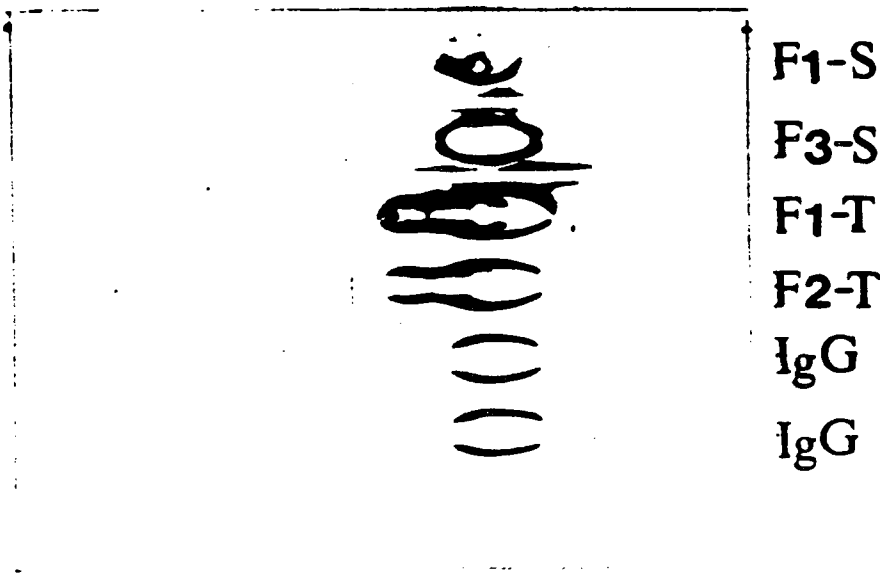


Figure 14. Immunoelectrophoresis of fractions obtained by Sephacryl S-300 and Fractogel TSK column against anti-whole bovine serum antiserum. (F1-S and F3-S, fraction 1 and 3 of Figure 8, respectively) (F1-T and F2-T fraction 1 and 2 of Figure 12, respectively) IgG, immunoglobulin G.

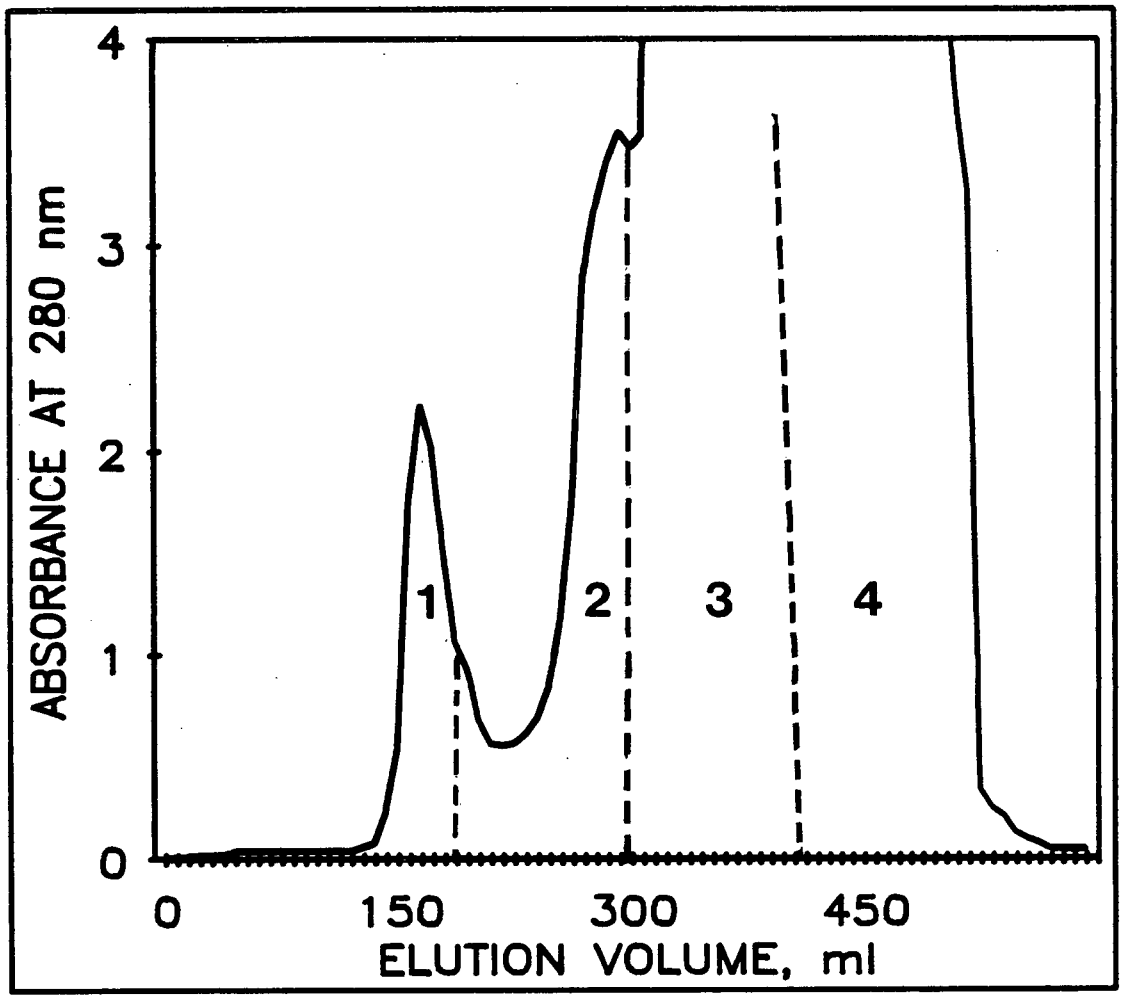


Figure 15. Gel filtration of acid whey on Sephacryl S-300 Superfine column (94 x 2.5 cm), eluted with 0.1 M Tris-HCl buffer, pH 8.0 containing 0.5 M NaCl. Flow rate 18 mL/hr. 1, 2, 3 and 4 are the fractions obtained.

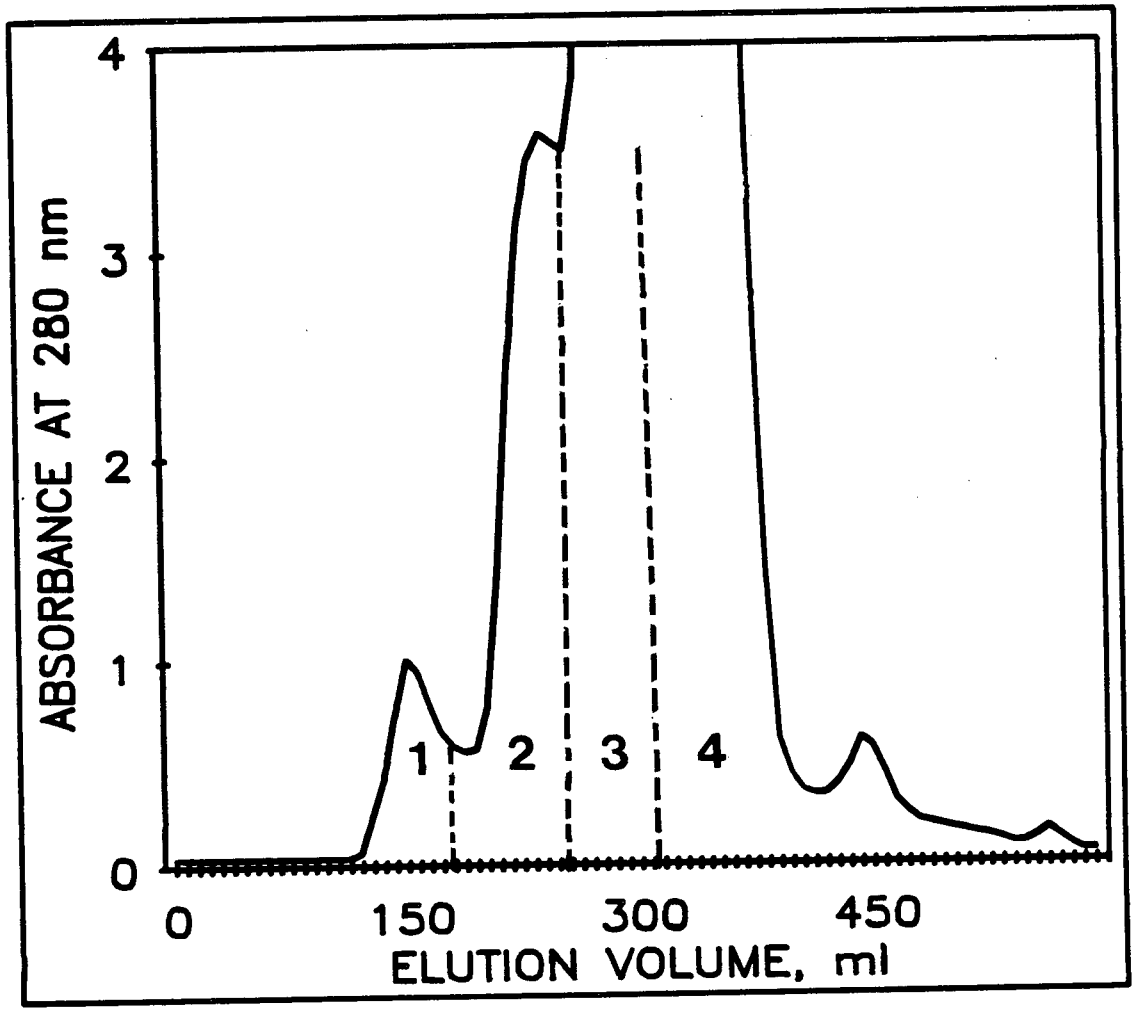


Figure 16. Gel filtration of Cheddar cheese whey on Sepharyl S-300 Superfine column (94 x 2.5 cm), eluted with 0.1 M Tris-HCl buffer, pH 8.0 containing 0.5 M NaCl. Flow rate 18 mL/hr. 1, 2, 3 and 4 are the fractions obtained.

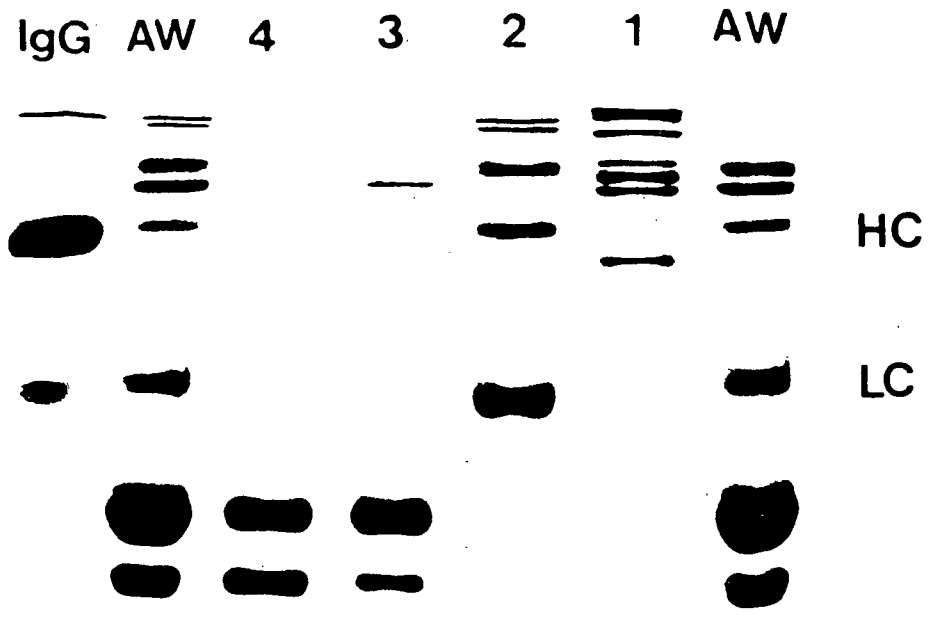


Figure 17. SDS-PAGE of fractions (1, 2, 3, 4) obtained from Figure 15. AW, acid whey; IgG, immunoglobulin G; HC and LC, heavy and light chains of immunoglobulins, respectively.

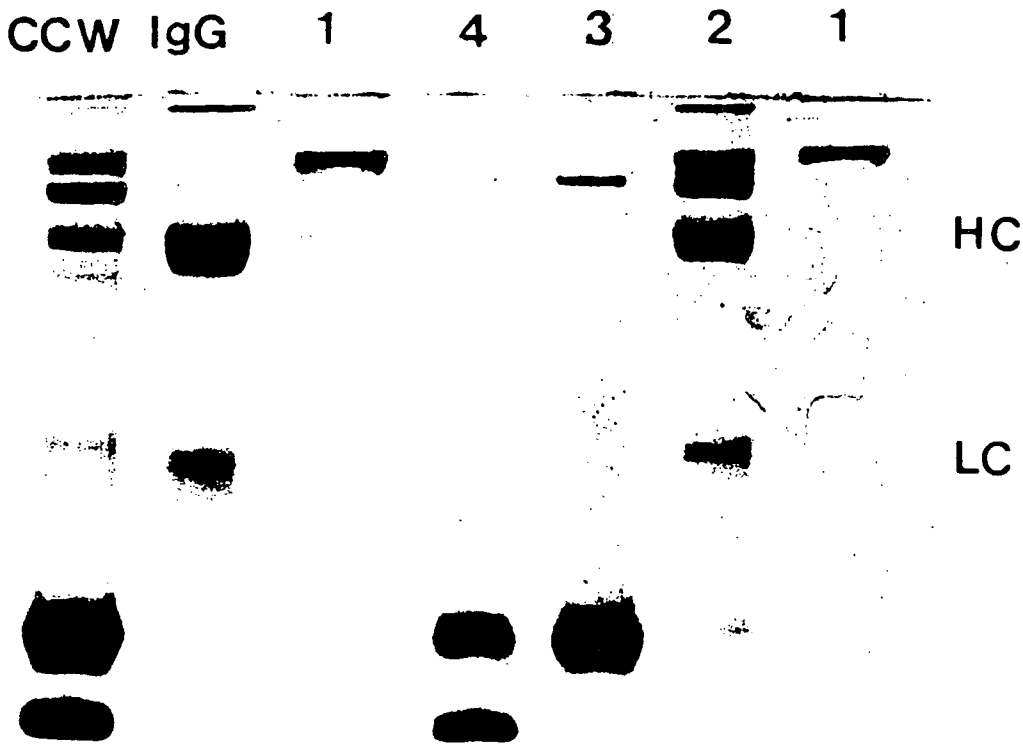


Figure 18. SDS-PAGE of fraction (1, 2, 3, 4) obtained from Figure 16. IgG, immunoglobulin G; CCW, Cheddar cheese whey; HC and LC, heavy and light chains of immunoglobulins, respectively.

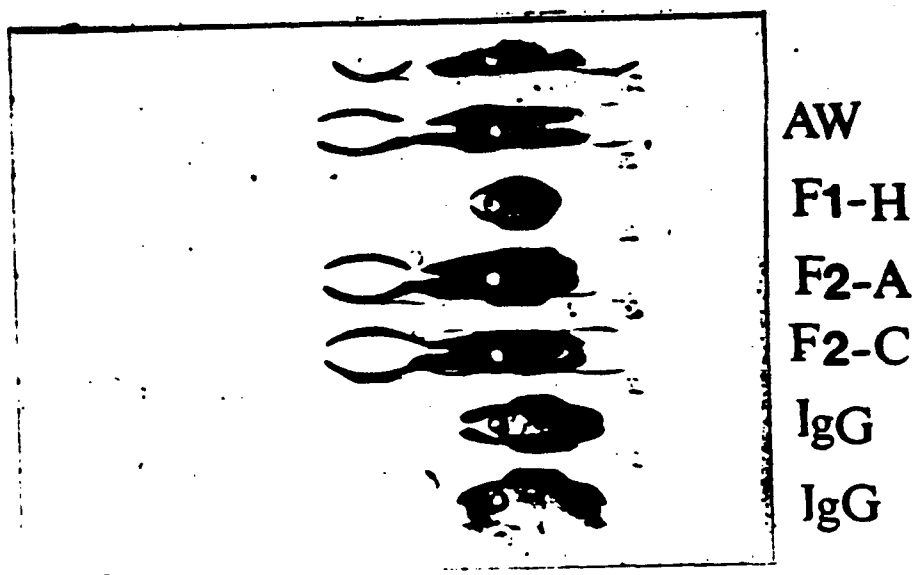


Figure 19. Immunoelectrophoresis of fractions obtained from gel filtration of whey protein against anti-whey protein antiserum. AW, acid whey; F1-H, fraction 1 from Figure 20; F2-A, fraction 2 from Figure 15; F2-C, fraction 2 from Figure 16; IgG, immunoglobulin G.

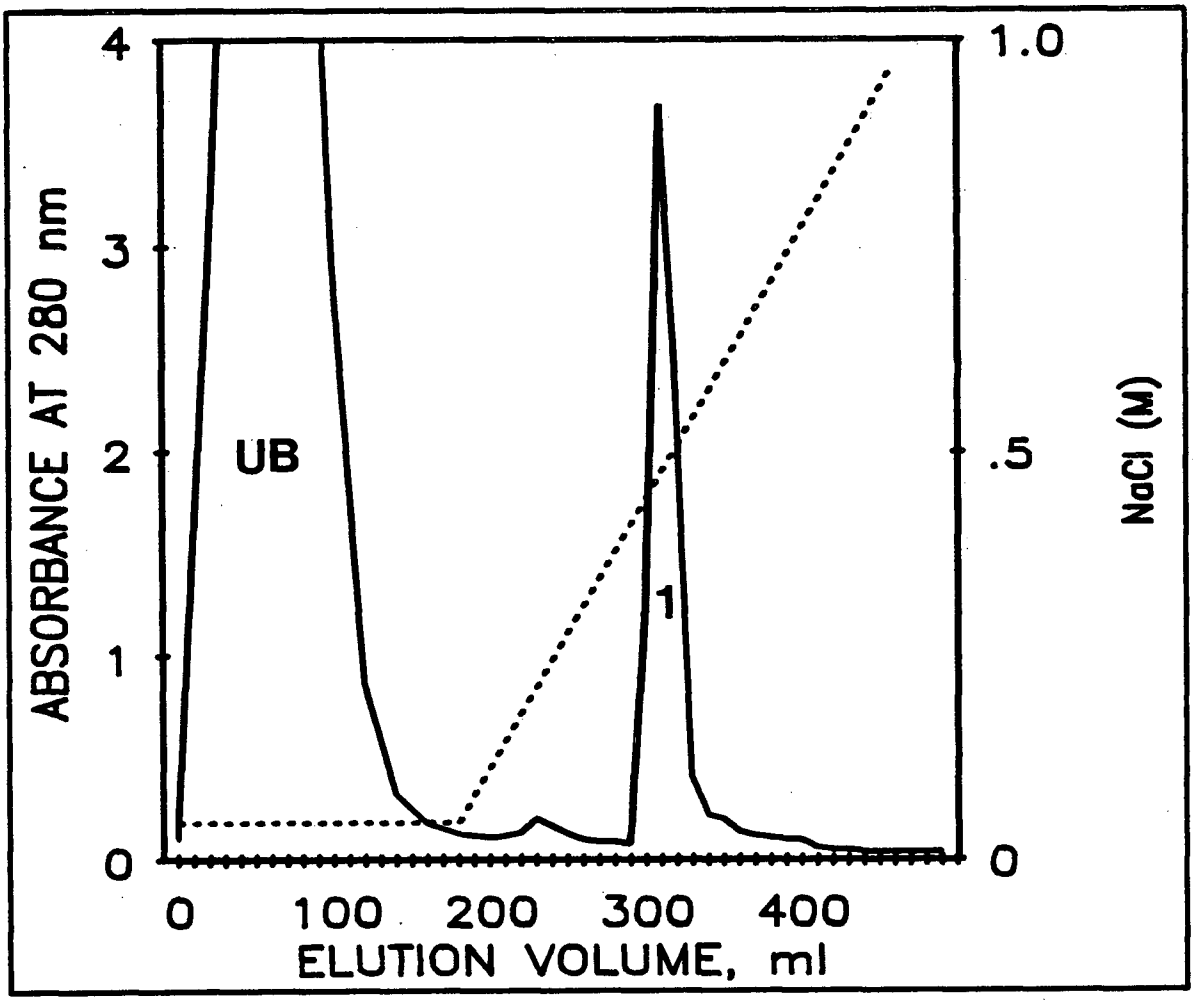


Figure 20. Heparin-Sepharose chromatography of Cheddar cheese whey. Cheese whey (400 mL) dialyzed against 0.05 M NaCl in 5 mM veronal-HCl, pH 7.4 was applied to the column (10 mL settled gel). The column was washed with the same buffer and then eluted with a linear gradient of NaCl (....) as indicated. The flow rate was 50 mL/hr. UB, unbound proteins; 1, fraction 1.

NaCl isocratically. The isolated lactoferrin gave only one precipitant line with immunoelectrophoresis (Figure 19), which indicated the high purity of this fraction. SDS-PAGE (Figure 21) also yielded a single band, confirming the purity of this fraction.

E. ANTI-LIPOPOLYSACCHARIDES ACTIVITY OF ISOLATED IMMUNOGLOBULINS

Figure 22 shows the anti-lipopolysaccharide activity of immunoglobulins isolated from colostrum using a gel filtration technique. Lipopolysaccharides were extracted from the pathogenic bacteria, E. coli, S. typhimurium and Bordetella parapertussis and their binding with isolated Ig was measured using an enzyme linked immunosorbent assay (ELISA). The isolated IgG was shown to have binding activity against LPS isolated from E. coli and to a lesser extent against S. typhimurium. Higher recognition of colostral Ig to LPS from E. coli as compared LPS from S. typhimurium may indicate that the dairy cow was infected more often by E. coli than by S. typhimurium. Surprisingly isolated Ig showed activity and recognition of LPS isolated from B. parapertussis which causes whooping cough in infants, and which may indicate the presence of similarities in the antigenic structure of LPS between this bacterium and those extracted from E. coli.

The strain of E. coli used in this study is known to cause diarrhea in infants by producing one or both of two classes of enterotoxins. E. coli enterotoxin mainly affects fluid transport processes of the small intestine, therefore, any changes in either or both absorption and secretion may result in diarrhea (Holmgren, 1985). Holmgren (1985) discussed possible approaches for the prevention of and treatment of E. coli pathogenic action. One approach was to use receptor blockade by using a non-toxic binding agent. This approach was to prevent binding of toxin to the epithelium. Therefore, high binding activity

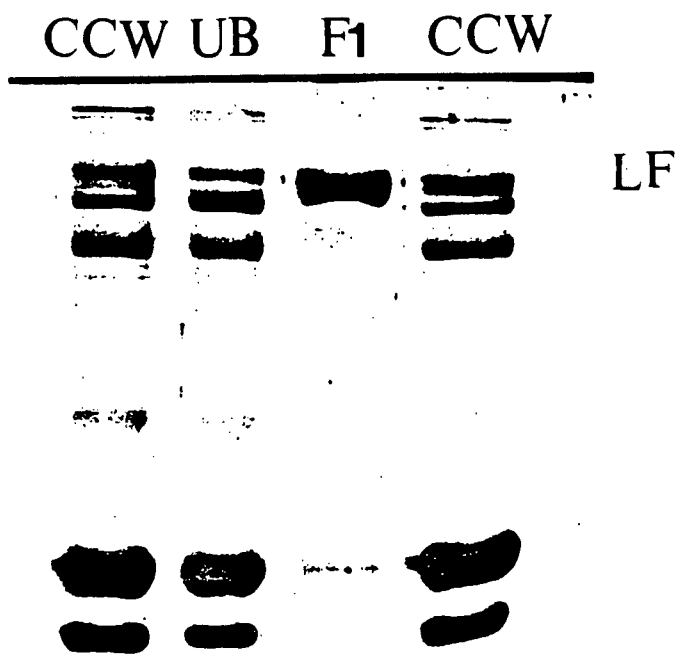


Figure 21. SDS-PAGE of fractions obtained from Figure 20, CCW, Cheddar cheese whey; UB; unbound whey proteins to Heparin-Sepharose column; F1, lactoferrin rich fraction; LF, lactoferrin.

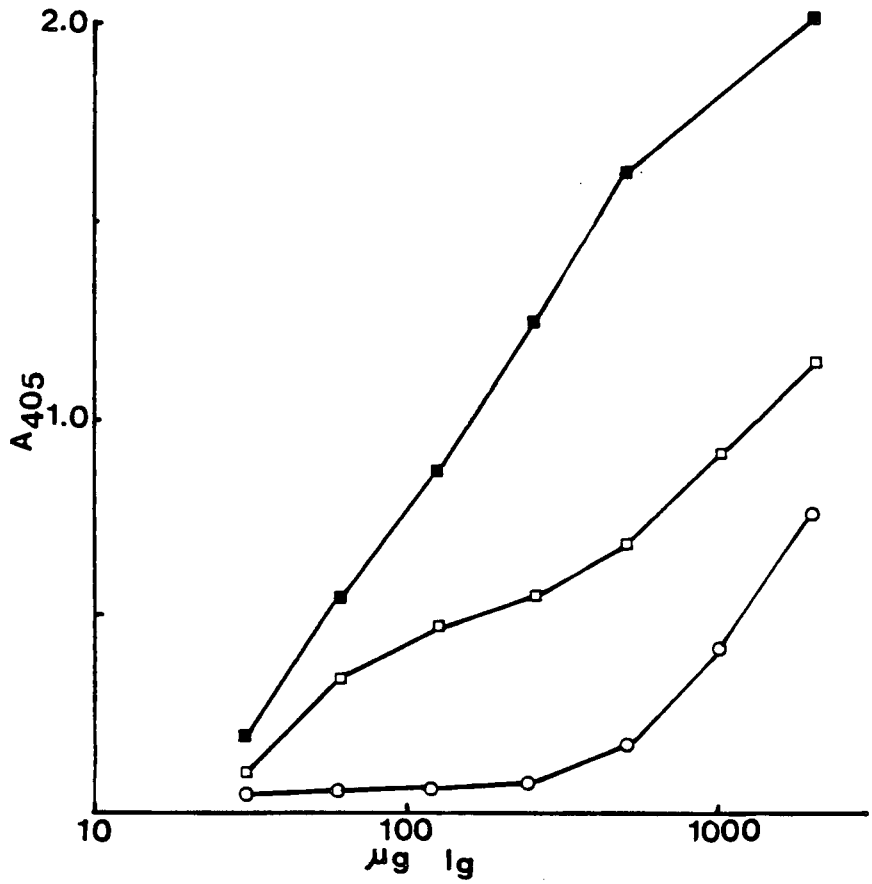


Figure 22. Anti-lipopolysaccharide activity of colostral IgG isolated by gel filtration on Sephacryl S-300. ■—■, E. coli LPS; □—□, S. typhimurium LPS; ○—○, B. parapertussis LPS.

of isolated Ig toward LPS extracted from E. coli may interfere with the binding of bacterial toxin to the epithelium thus preventing diarrheal diseases when Ig is used for infant feeding.

These studies demonstrate that Cheddar cheese whey could be an important source for the isolation of immunoglobulins and lactoferrin. Importance of lactoferrin as a bacteriostatic agent has been well established (Reiter, 1983). Immunoglobulins isolated from cheese whey as an immunoglobulin rich fraction (Fraction 2) can be incorporated into infant formulae. The lipopolysaccharide binding activity of immunoglobulins may give a clear evidence to add this fraction to commercial infant formulae in order to protect infants from gastrointestinal infection.

PART III

SEPARATION OF IMMUNOGLOBULINS AND LACTOFERRIN FROM CHEESE WHEY BY ADSORPTION AND CHELATING CHROMATOGRAPHY TECHNIQUES

When considering the treatment of a large amount of cheese whey, the difficulties in mechanization of gel filtration techniques for extracting immunoglobulins and lactoferrin renders a large scale operation less feasible and more difficult. Therefore, adsorption and metal chelating chromatographic methods were investigated to extract these proteins by an easy and efficient method.

A. ADSORPTION CHROMATOGRAPHY METHODS

In searching for a method to isolate immunoglobulins and other bioactive compounds from whey protein, the economic drive, ease to mechanize and capacity of the method were used as criteria.

Figure 23 shows the elution profiles of adsorbed proteins from the chromatographic treatment of 1 litre of Cheddar cheese whey (adjusted to pH 8.2) on silica, controlled pore glass and alumina, eluted with acetic acid solution followed by Tris-HCl buffer.

The amount of protein in these fractions from the silica column was very small, as indicated by the low A_{280} values and small peak areas. SDS-PAGE (Figure 24) shows that the acetic acid eluted fraction contained primarily lactoferrin (Lane 2). The amount of protein in the Tris-HCl eluted fraction was too small to be identified (lanes 5 and 12). The silica-treated cheese whey (i.e., unadsorbed protein fraction, lane 8) showed little difference from the electrophoretic pattern of untreated cheese whey (lane 1 & 11). Similar results were obtained when the chromatography was carried out at the starting pH of 7.5 instead of 8.2.

As shown by SDS-PAGE profiles (Figure 24), the large A_{280} peak eluted by acetic acid from controlled pore glass contained mainly lactoferrin and bovine serum albumin (BSA) (lane 3), while the peak eluted by Tris-HCl contained a mixture of proteins including lactoferrin, BSA and Ig (lane 6).

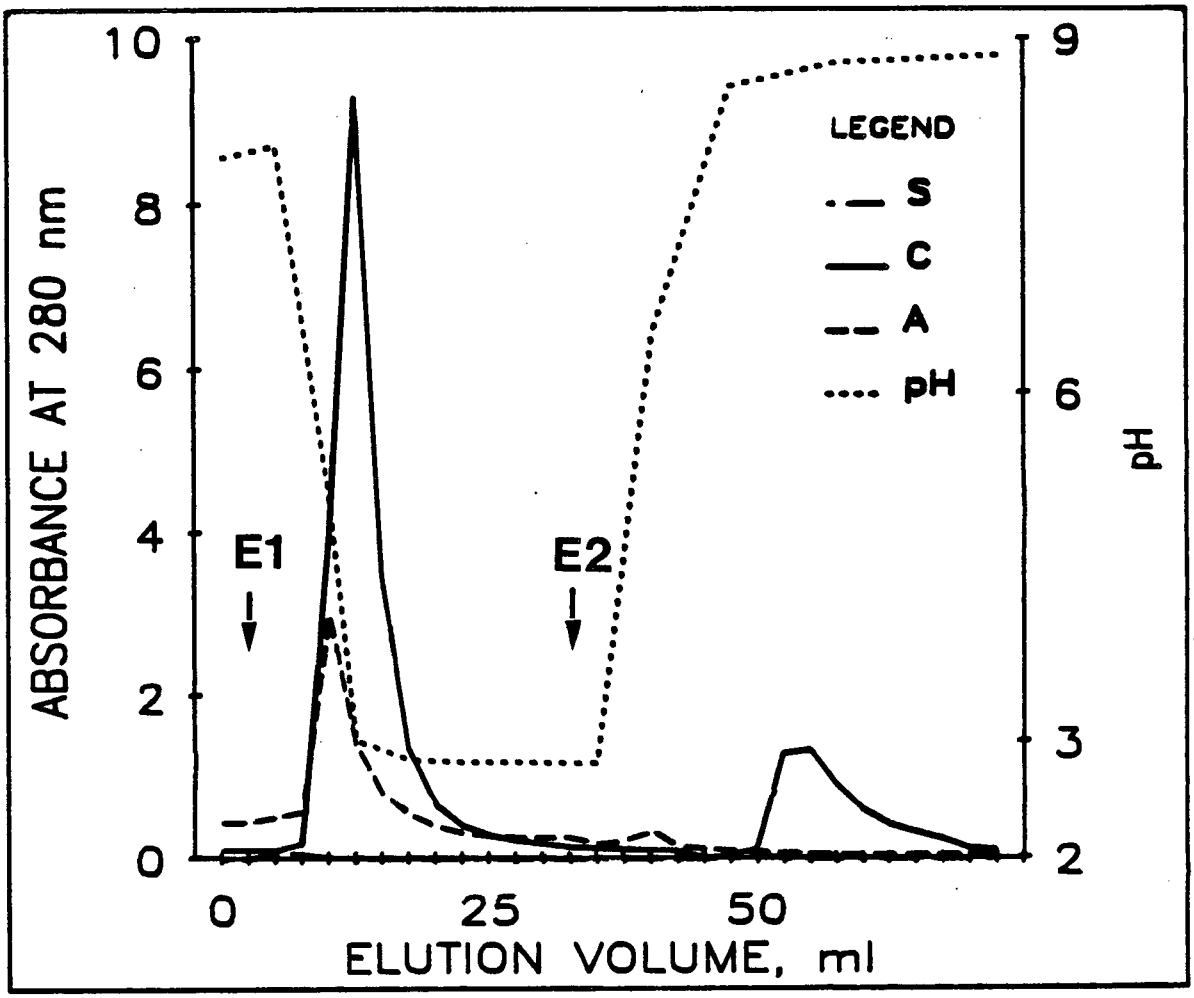


Figure 23. Elution profiles of adsorbed proteins from silica (S) (close to the baseline), controlled pore glass (C) and alumina (A) chromatographic treatment of Cheddar cheese whey. One litre of Cheddar cheese whey in 0.005 M Na_2HPO_4, pH 8.2 was passed through 1.3 x 7.0 cm column of (S), (C) or (A) equilibrated with 0.005 M phosphate buffer at pH 8.2. After washing with 30 mL of equilibrating buffer, the adsorbed proteins were eluted with E1 (50 mL 0.1 M acetic acid pH 2.77 containing 0.5 M NaCl), then E2 (60 mL 0.1 M Tris-HCl pH 9.0 containing 0.5 M NaCl). The flow rate was 1 mL/min.

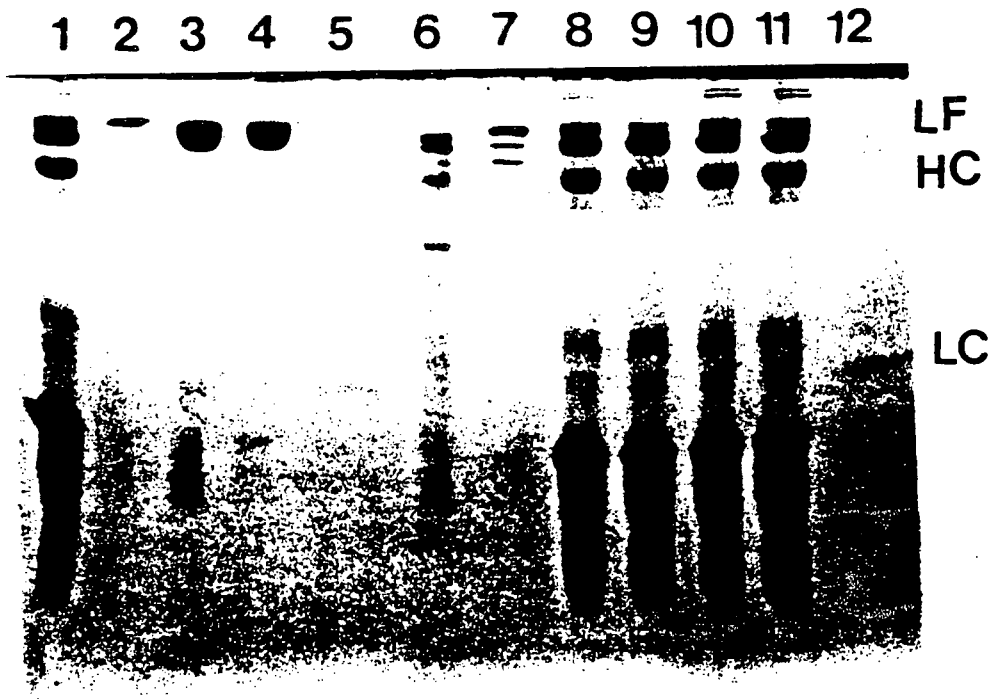


Figure 24. SDS-PAGE profiles of cheese whey and fractions obtained from Figure 23. Lane 1, untreated Cheddar cheese whey; Lane 2, acetic acid fraction from silica sand; Lane 3, acetic acid fraction from controlled pore glass; Lane 4, acetic acid fraction from alumina; Lane 5, Tris-HCl fraction from silica sand; Lane 6, Tris-HCl fraction from controlled pore glass; Lane 7, Tris-HCl fraction from alumina; Lane 8, unbound fraction from silica; Lane 9, unbound fraction from controlled pore glass; Lane 10, unbound fraction from alumina; Lane 11, untreated cheddar cheese whey; Lane 12, acetic acid fraction from alumina, LF, lactoferrin; HC and LC, immunoglobulin heavy and light chains, respectively.

The acetic acid elution of alumina column yielded a small peak followed closely by a second larger peak. The larger peak contained mainly lactoferrin and BSA according to SDS-PAGE (Figure 24 lane 4). Tris-HCl elution yielded only a very broad peak with low A_{280}; this fraction contained mainly lactoferrin (Figure 24, lane 7). The Tris-HCl eluant emerging from the column was observed to be turbid, starting at the 80 mL elution volume, which may be due to lipid fractions eluted from the alumina.

Of the adsorption chromatographic support materials investigated, controlled pore glass appeared to be the most promising for isolation of Ig. However, reducing the amount of cheese whey sample to 250 mL and analyzing the unbound fraction indicated that appreciable quantities of Ig were not adsorbed to the CPG column. This suggested that the capacity of CPG for Ig adsorption was not very high. Increasing the CPG bed volume from 10 to 20 mL improved recovery somewhat, but the capacity was still insufficient for efficient removal of Ig from whey.

B. METAL CHELATE INTERACTION CHROMATOGRAPHY

1. Acid whey

Acid whey (obtained from acidification of raw skimmilk) was applied to a column containing copper ions immobilized on Sepharose 6B. After washing unbound proteins with the starting alkaline buffer, the adsorbed proteins were eluted using a linear gradient. Figure 25 shows the elution profile of adsorbed proteins from MCIC treatment of 1 litre of acid whey. Two major peaks were detected by monitoring A_{280} of the effluent. The fractions comprising the first peak were yellowish, while the second peak was colorless. SDS-PAGE (Figure 26 lane 1) indicated that the first peak was the lactoferrin rich fraction; however, it also contained other proteins which were adsorbed to the

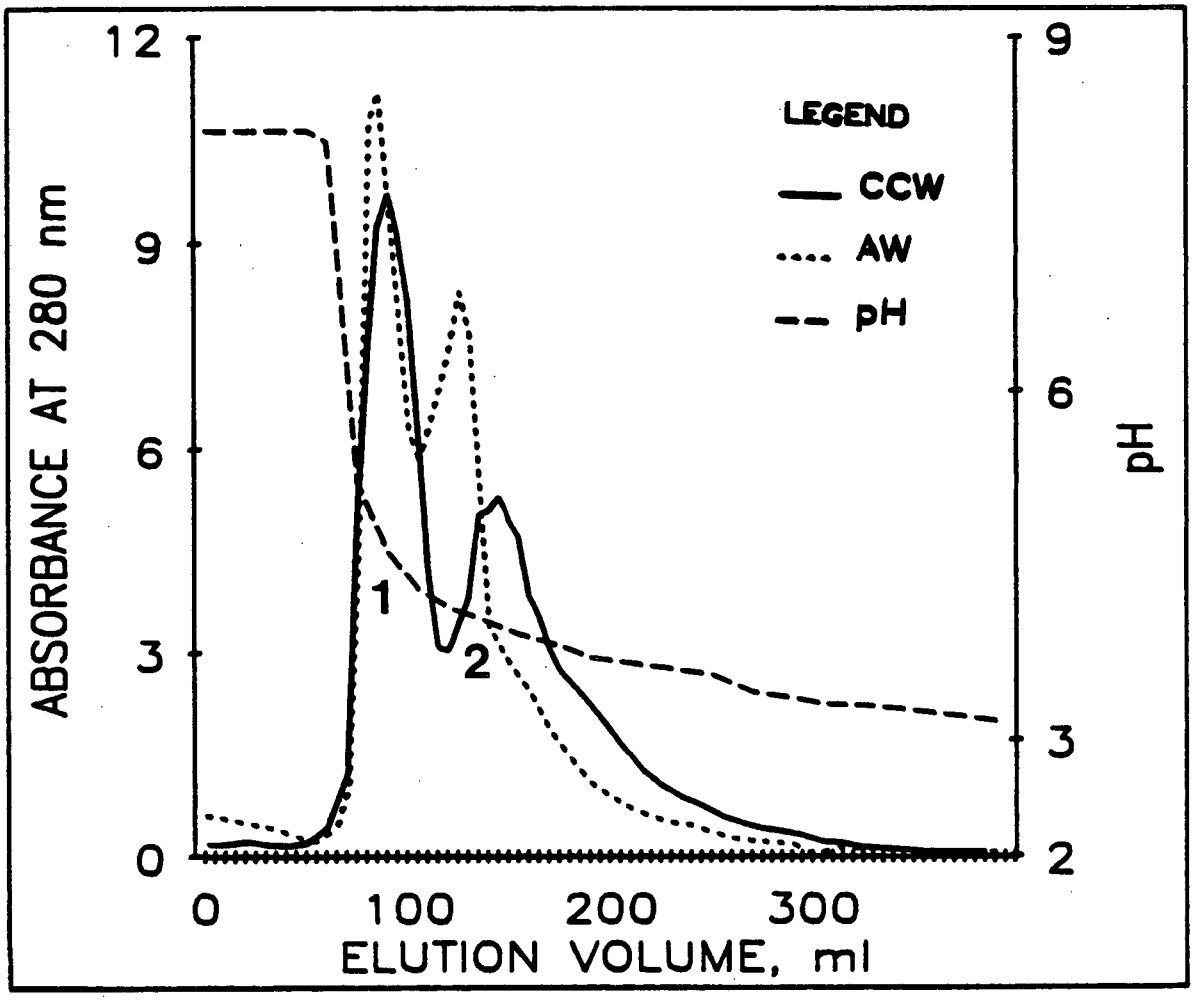


Figure 25. Elution profiles of adsorbed proteins from MCIC on Sepharose 6B treatment of 1 L Cheddar cheese whey (CCW) and 1 L acid whey (AW) (obtained from raw milk), using linear gradient elution of 0.05 M Tris-acetate containing 0.5 M NaCl, pH 8.0 to 2.8. Flow rate was 0.8 mL/min. CCW, Cheddar cheese whey; AW, acid whey.

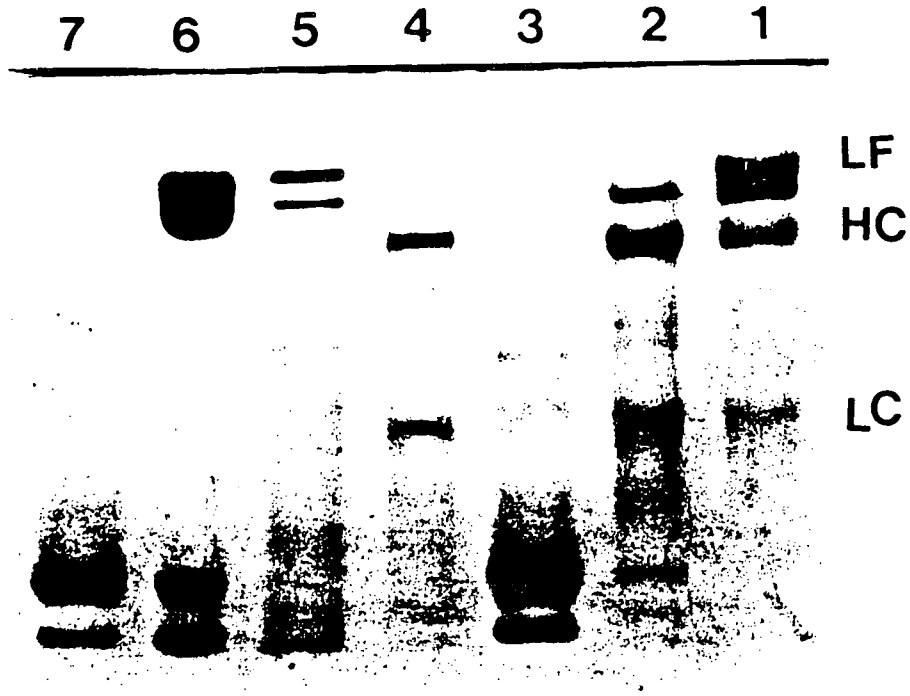


Figure 26. SDS-PAGE profiles of acid whey and fractions obtained by MCIC and gel filtration. Lanes 1 and 2, are fractions obtained by MCIC; Lane 3, unbound material to MCIC column; Lanes 4 and 5, peak 1 and 2 obtained by gel filtration on Sephacryl column; Lane 6, mixture of standard proteins (transferrin, bovine serum albumin, β-lactoglobulins and α-lactalbumin); Lane 7, acid whey. LF, lactoferrin; HC and LC, heavy and light chains of immunoglobulins, respectively.

column and eluted in the same fraction; these proteins were Ig and BSA. The second smaller peak was mainly composed of Ig (Figure 26 lane 2). Figure 26 (lane 3) also indicated that the unbound fraction contained no Ig but mainly α-La and β-Lg.

Immunochemical analyses (Table 8) showed that the majority of active IgG (i.e., over 77%), was present in the second pooled peak, over 20% of active IgG was present in the first pooled peak and was undetectable in the unbound fractions.

Table 9 shows the distribution of whey proteins in comparison to the pooled fractions obtained by the MCIC process as calculated from the densitometric scanning of SDS-PAGE. Lactoferrin and immunoglobulins were predominant proteins in peak one (F1) (48% and 20%, respectively) while bovine serum albumin represented 27% of this fraction. This fraction was almost free from α-La and β-Lg. Immunoglobulins were the major proteins in peak 2, representing 88.4% of the total proteins in this fraction. Immunoglobulins and lactoferrin in both fractions represented more than 75% of the total proteins.

Immunoelectrophoresis conducted against anti-bovine whole serum antibodies (Figure 27) also indicated the presence of IgG (the predominant type of immunoglobulins in bovine milk) as well as the presence of BSA in both fractions.

2. Cheddar cheese whey

Figure 25 shows an elution profile of adsorbed proteins from MCIC treatment of 1 L Cheddar cheese whey at pH 8.2. A similar elution pattern was obtained from acid whey. Two major peaks were detected by monitoring A_{280} of the effluent. The fractions comprising the first peak were slightly yellowish, while the fractions from the second peak were clear.

Table 8. IgG activity of peak and unbound fractions from MCIC treatment of whey.

	Concentration mg/mL		
	IgG	protein	IgG purity
Acid whey			
unbound	ND	80.3	ND
1st peak	5.0	20.3	24.6
2nd peak	18.0	23.4	77.2
Cheddar cheese whey			
unbound	ND	79.3	ND
1st peak	1.25	51.4	2.4
2nd peak	40.0	75.4	53.0
ED whey powder			
unbound	ND	65.2	ND
1st peak	5.00	31.3	16.0
2nd peak	20.00	70.7	28.3

ND not detectable

[a] IgG activity of dialyzed fractions was determined by RID

[b] Protein concentration was determined by Kjeldahl N x 6.38

Table 9. Whey proteins distribution* in acid whey, fractions obtained from MCIC of bovine acid whey, and the unbound materials to MCIC column.

Protein components %	Acid whey %	F1 %	F2 %	Unbound Materials %
α-lactalbumin	13.9	---	---	27.9
β-lactoglobulin	56.9	4.8	2.1	67.8
Immunoglobulins	11.4	20.1	88.4	---
Bovine serum albumin	9.2	27.1	9.5	3.3
Lactoferrin	8.6	48.0	---	---

* Calculated from peak area of the electrophoretic patterns.

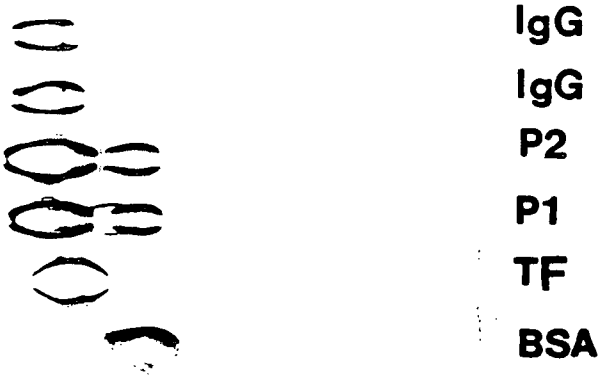


Figure 27. Immunoelectrophoretic analysis of fractions obtained by MCIC of acid whey. IgG, immunoglobulin G; P2 and P1 are Peak 2 and 1, respectively of Figure 25; TF, transferrin; BSA, bovine serum albumin.

SDS-PAGE (Figure 28) indicated that the first large peak was composed of lactoferrin and bovine serum albumin (lanes 5 and 7) while the second smaller peak was mainly composed of immunoglobulins (lanes 6 and 8). Some β-Lg and α-La were also adsorbed on Cu^{2+}-immobilized Sepharose, as indicated in the unbound fraction at the early stage of whey application; no whey proteins were present in the unbound fraction after 120 mL of whey had been applied (lane 2). However, after 460 mL of applied whey, β-Lg and α-La were not adsorbed (lane 3 and 4) indicating that the column had lower affinity for these components than for LF, BSA and IgG. The absence of β-Lg and α-La in the eluted peaks (lanes 5 to 8) can be explained by the fact that they were weakly bound and were either displaced by LF, BSA and IgG during later stages of whey application or were eluted during the washing stage prior to the elution of adsorbed proteins.

Immunochemical analysis (Table 8) showed that the majority of active IgG was present in the second peak with very minor activity in the first peak and undetectable amount in the unbound fractions. The IgG activity of the second peak (Ig rich fraction) of Cheddar cheese whey was lower than that of acid whey obtained from raw skimmilk. This may be due to pasteurization (73°C, 15 sec) of milk used for cheese manufacturing.

3. Electrodialyzed and sweet whey powders

a. IgG activity and protein content

IgG activity and protein content of reconstituted electrodialyzed (ED) whey and sweet whey (after reconstitution to 6.5% total solids solution) are compared to those of liquid cheese whey (Table 10). Similar values for % protein were obtained for liquid whey and the reconstituted wheys. However, the IgG activity was much lower for the reconstituted wheys (especially the sweet whey) than liquid cheese whey.

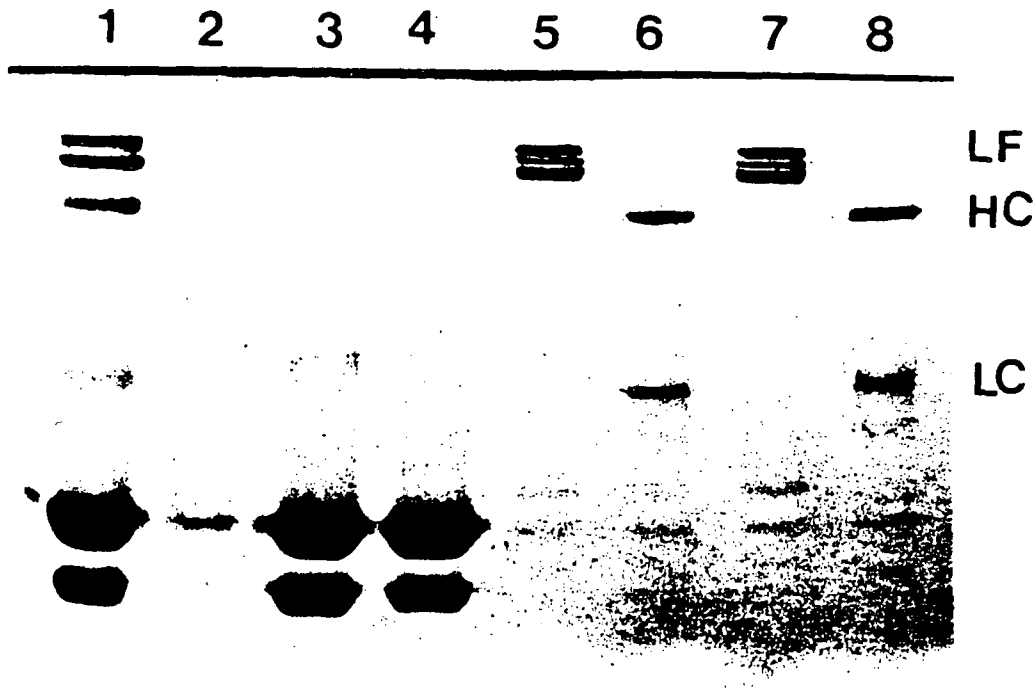


Figure 28. SDS-PAGE of Cheddar cheese whey and fractions obtained by MCIC on Sepharose 6B treatment. Lane 1, control cheese whey; Lanes 2, 3 and 4, are unbound fraction; Lanes 5 and 7, first eluted peak; Lanes 6 and 8, second eluted peak; LF, lactoferrin; HC and LC heavy and light chains of immunoglobulin, respectively.

Table 10. IgG and protein contents of reconstituted ED and sweet whey powders compared to liquid cheese whey.

	g IgG/100g protein[a]	% protein[b]
Liquid cheese whey	4.0	0.88
Sweet whey	1.2	0.84
ED whey	2.2	0.87

[a] IgG content was determined by R.I.D.

[b] Protein content was determined as Kjeldahl N x 6.38; units for % protein are g protein/100 mL for liquid cheese whey, and for sweet and ED whey powders after reconstitution to 6.5% (total solids) solution.

b. Effect of pH adjustment

Figure 29 shows the SDS-PAGE profile of the supernatant and precipitate fractions of fresh liquid cheese whey after pH adjustment in the pH 4.5 - 8.5 range. No precipitates were observed and only small amounts of pellets were obtained after centrifugation of the pH adjusted liquid whey. The precipitates contained trace amounts of β-Lg and an unidentified component "C" (presumed to be a casein fraction, from its position on the electrophoretic profile).

Figure 30 shows the SDS-PAGE profile of the supernatant and precipitate fractions of ED whey at pH 4.5 and 8.2. As indicated, the precipitate at pH 4.5 (lane 3) from ED whey contained a large amount of immunoglobulins, some BSA, β-Lg, α-La and all of the LF. At pH 8.2, the precipitate from ED whey (lane 1) contained some LF, BSA, β-Lg and only a small amount of Igs.

It was found from immunochemical analyses (RID, Table 11) that the Ig in the precipitates from sweet whey at both pH values were inactive while those in the supernatant fractions showed very low immunochemical activity. Little difference in IgG activity was observed between the two pH treatments of sweet whey. In fact, supernatant and precipitate fractions from sweet whey adjusted to varying pH values from 4.5 to 8.5 showed little effect of pH, indicating that pH may not be the only factor causing precipitation. Denaturation of the proteins during processing of the whey powder may be the other factor causing precipitation.

On the other hand, for ED whey the amount of total precipitate as well as IgG activity in the precipitate was dependent on the pH. SDS-PAGE profiles show much greater tendency for precipitation of Ig from ED whey at pH 4.5 to 5.5 than at higher pH.

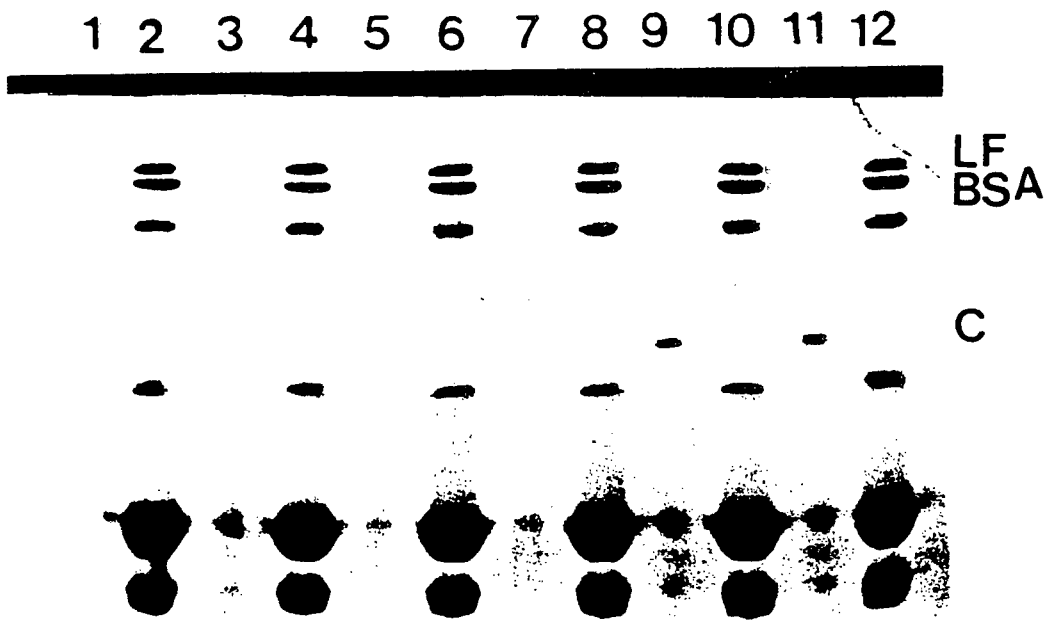


Figure 29. SDS-PAGE profile of liquid cheese whey after pH adjustment and centrifugation. Lanes 1, 3, 5, 7, 9 and 11 are the precipitate and Lanes 2, 4, 6, 8, 10 and 12 are the supernatant of samples treated at pH 8.5, 8.0, 7.0, 6.0, 5.0 and 4.5, respectively, LF, lactoferrin; BSA, bovine serum albumin; C, casein.

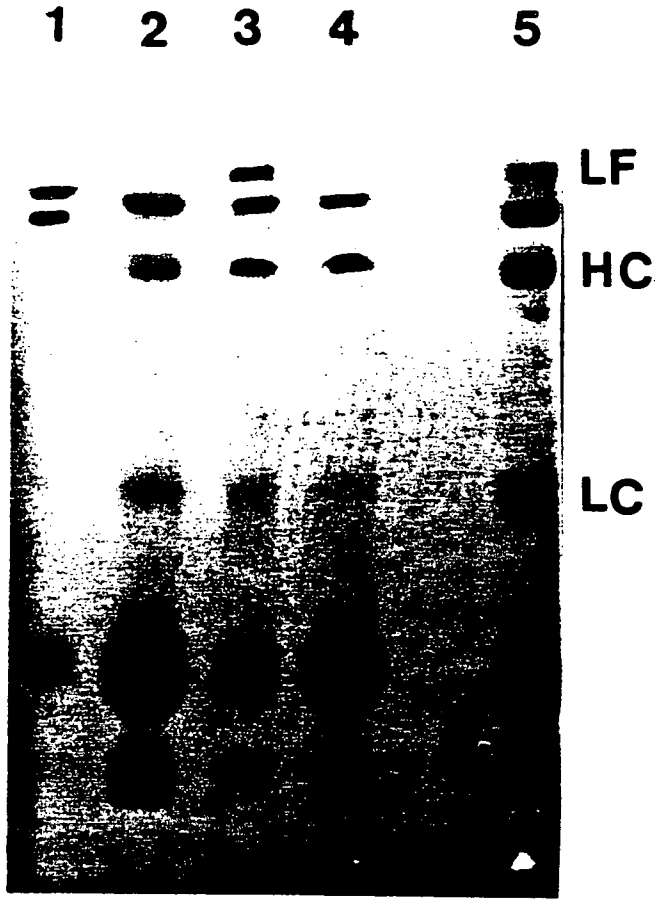


Figure 30. SDS-PAGE profiles of fractions from ED whey after pH adjustment and centriguation. Lanes 1 and 3, are precipitate at pH 8.2 and 4.5 respectively; Lanes 2 and 4, are supernatant at pH 8.2 and 4.5 respectively; Lane 5, electrodialyzed whey; LF, lactoferrin, HC and LC, heavy and light chains of immunoglobulins, respectively.

Table 11. IgG activity of pH 4.5 and pH 8.2 supernatant (S) and precipitate (P) fractions from sweet whey and ED whey[a].

	Concentration, mg/mL		
	IgG[b]	protein[c]	gIg/100g protein
Sweet whey			
pH 8.2 P	ND[d]	18.3	ND[d]
S	1.25	77.0	1.6
pH 4.5 P	ND[d]	16.9	ND[d]
S	1.25	70.2	1.8
ED whey			
pH 8.2 P	ND[d]	12.4	ND[d]
S	5.00	80.6	6.2
pH 4.5 P	1.10	29.9	3.7
S	1.41	57.6	2.5

[a] No data were obtained for liquid cheese whey, which showed almost no precipitation over the entire pH 4 - 8 range.

[b] IgG activity of dialyzed fractions (10X concentrated) was determined by RID.

[c] Protein concentration was determined by Kjeldahl N x 6.38.

[d] ND = not detectable.

The supernatant fraction from ED whey at pH 8.2 showed higher immunochemical activity than the precipitated fraction, which showed no detectable IgG activity due to the low amount of total protein as well as IgG. However at pH 4.5, activity was higher in the precipitated fraction and less activity was found in the supernatant fraction (Table 11). These results suggest that at pH 4.5 IgG is preferentially precipitated from the ED whey. Unlike the precipitated Ig from sweet whey which was immunochemically inactive, precipitated Ig from ED whey remained active.

c. Isolation of immunoglobulins by controlled pore glass

Figure 31 shows the elution profiles of adsorbed proteins from controlled pore glass (CPG) (10 mL bed volume) chromatography of 250 mL of sweet whey (SW) and ED1 whey, respectively. In each case, a single peak was eluted by buffer E1 (0.1 N acetic acid pH 2.8 - 2.9 containing 0.5M NaCl) and a single peak was eluted by buffer E2 (0.1 N Tris-HCl pH 9.0 containing 0.5 M NaCl). The size of the first peak was decreased for ED1 and SW compared to ED2, while the size of the second peak remained fairly constant, despite the four-fold reduction in volume of applied whey (250 mL in ED1 and SW vs. 1 litre in ED2, Figure 31).

Electrophoretic analysis of the unbound fraction of whey from CPG indicated that appreciable quantities of Ig were not adsorbed to the CPG but were eluted in the unbound fraction (Figure not shown). This suggests that the capacity of CPG for Ig adsorption is not very high. Although the quantity of whey applied in these studies had already been reduced to 250 mL (ED1), compared to 1 litre (ED2), Ig recovery was still far from quantitative. Increasing the CPG bed volume from 10 to 20 mL improved recovery somewhat, but the capacity was still insufficient for quantitative removal of Ig from whey.

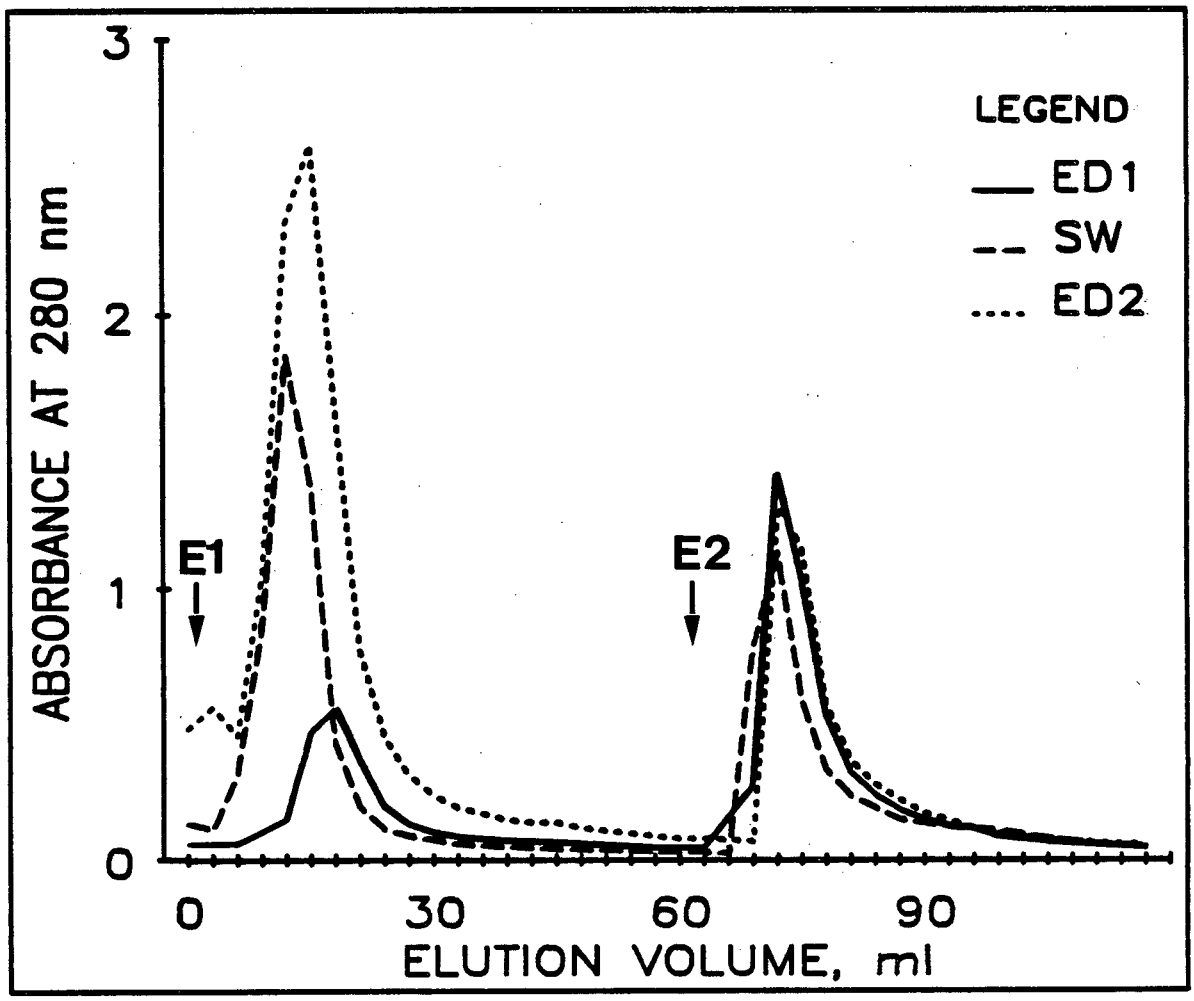


Figure 31. Elution profile of adsorbed proteins from CPG (10 mL) treatment of 250 mL of electrodialyzed whey (ED1), 250 mL of sweet whey (SW), and 1 L of ED whey (ED2). Arrows indicate start of elution with E1 (0.1 N acetic acid pH 2.8 containing 0.5 M NaCl) and E2 (0.1 M Tris-HCl, pH 9.0 containing 0.5 M NaCl) buffers.

d. Isolation of immunoglobulins by metal chelate-interaction chromatography

Figure 32 is an elution profile of adsorbed proteins from MCIC treatment of 960 mL ED whey reconstituted in water (pH adjusted to 8.2). An elution profile similar to that from fresh liquid cheese whey was obtained. However, the two peaks were broader and smaller than those from fresh liquid cheese whey. SDS-PAGE (Figure 33) showed that the first large peak was composed of BSA, some Ig and β-Lg while the second smaller peak was composed mainly of Igs and a trace amount of BSA (lanes 6 and 7, respectively). The profiles of the unbound whey fractions (Figure 33, lanes 2, 3 and 4) indicated that some Igs were not adsorbed. Even at the early stage with 120 mL of applied whey (lane 2), some BSA, LF, β-Lg and trace amounts of Igs were found in the unbound fractions suggesting that MCIC may have less affinity for these proteins from the ED whey than from fresh liquid cheese whey.

Radial immunodiffusion showed the purity of Igs recovered from ED whey by MCIC were not as good as from fresh cheese whey (Table 8). The first peak contained about 16% IgG while the second peak was composed of about 28% IgG on a protein basis. Unbound fractions showed no detectable IgG activity, despite the presence of Ig band in the SDS-PAGE profile (Figure 33).

The elution profile of adsorbed proteins from MCIC treatment of 720 mL sweet whey reconstituted in starting buffer at pH 8.2 is shown in Figure 32. Possibly two peaks were eluted, with the second peak appearing as a shoulder from the first larger peak. Both the large and the shoulder peak were composed of Igs, BSA, LF, some β-Lg and α-La, based on SDS-PAGE (Figure 34, lanes 10 and 11 respectively). The difference between the two peaks was that the shoulder peak (lane 11) contained less β-Lg and more α-La than the larger peak (lane 10). The wash fractions (lanes 6, 7, 8 and 9) were composed mainly of β-Lg, α-La, some BSA and trace amounts of LF and Igs. Unbound fractions (lanes 3, 4, 5)

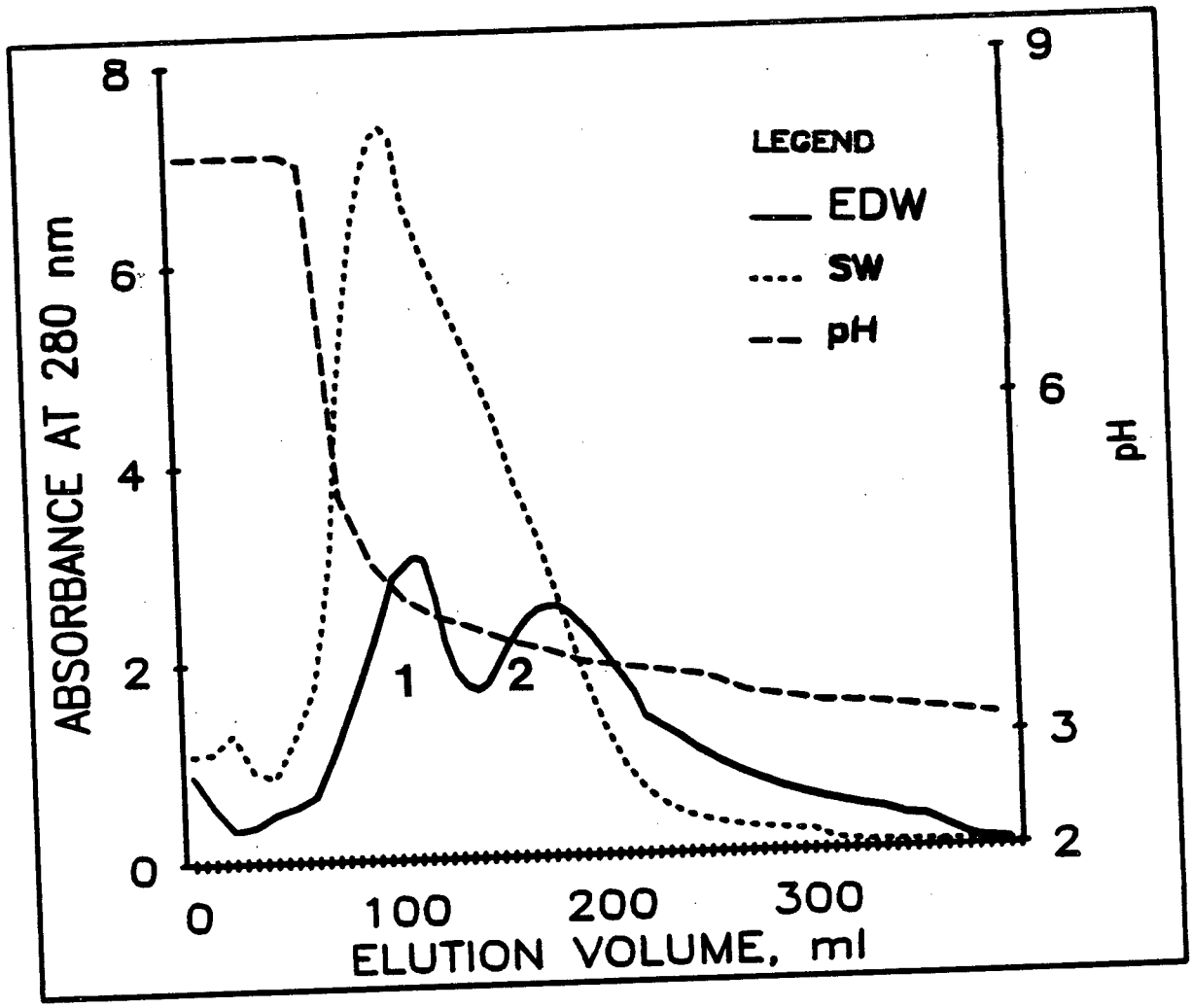


Figure 32. Elution profile of adsorbed proteins from MCIC on Sepharose 6B treatment of 960 mL electrodialyzed whey (EDW) and 720 mL sweet whey (SW) powders reconstituted in water, using linear gradient elution of 0.05 M Tris-acetate containing 0.5 M NaCl, pH 8.2 to 2.8. Flow rate was 0.8 mL/min. 1 and 2 are fractions obtained.

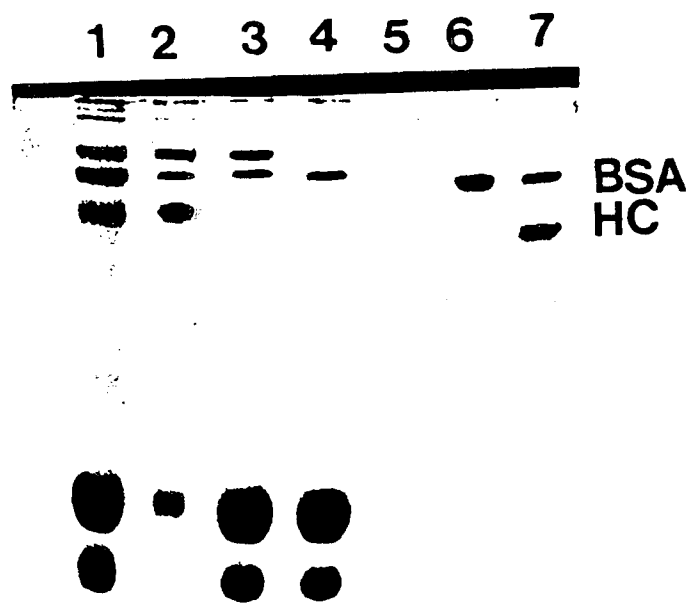


Figure 33. SDS-PAGE profiles of ED whey and fractions obtained by MCIC on Sepharose 6B treatment. Lane 1, control untreated whey; Lanes 2, 3 and 4, are unbound fractions; Lane 5, wash fraction; Lane 6, first eluted peak; Lane 7, second eluted peak; BSA, bovine serum albumin; HC, heavy chain of immunoglobulins.

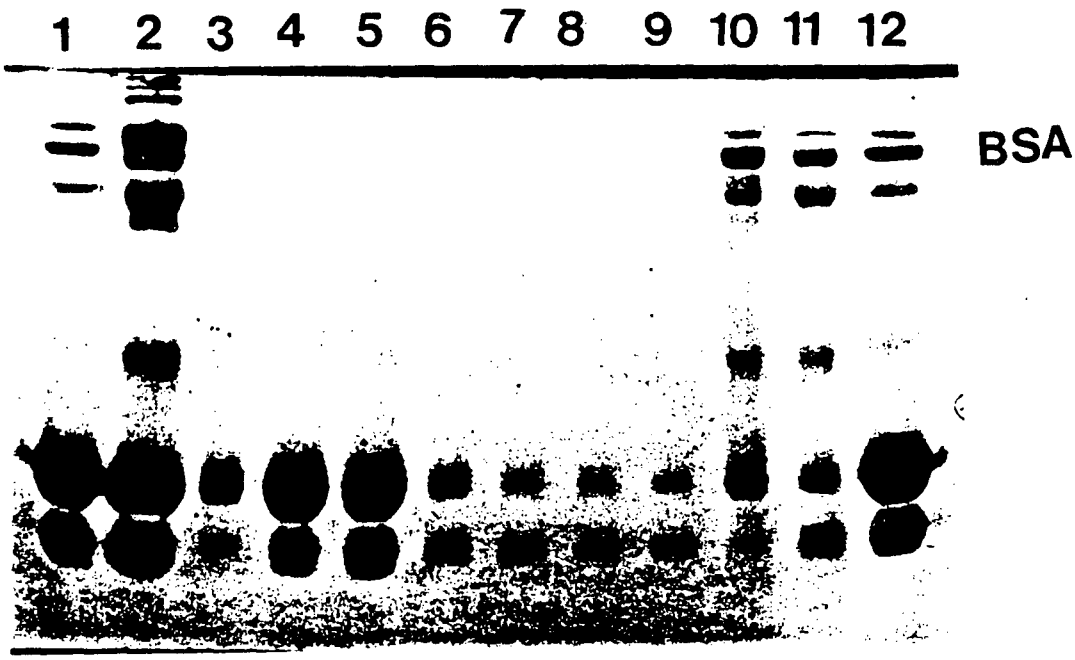


Figure 34. SDS-PAGE profiles of sweet whey and fractions obtained by MCIC on Sepharose 6B treatment. Lanes 1, 12, control untreated whey; Lane 2, precipitate; Lanes 3, 4 and 5; unbound fractions; Lanes 6, 7, 8 and 9, wash fractions; Lane 10, first eluted peak; Lane 11, second (shoulder) eluted peak; BSA, bovine serum albumin.

were composed mainly of β-Lg and α-La with some BSA, LF and Ig at all stages of whey application, with the exception that at the early stage there was relatively less β-Lg and α-La (lane 3).

Precipitation occurred in the sweet whey which was made up in the starting buffer (pH 8.2 containing 0.5 M NaCl) prior to application to MCIC. Figure 34 (lane 2) shows the profile of the precipitate containing all major whey proteins including Ig. Another problem that arose during the chromatography of both ED and sweet reconstituted wheys was the build up of pressure. The Sepharose gel became tightly packed during whey application, shrinking the bed height from 9.0 cm to 8.0 cm. The bed height increased and swelled back only slightly during the subsequent washing and eluting stage. Unbound proteins from liquid whey application were normally washed from the column with starting buffer of half the volume of applied whey to obtain effluent A_{280} of 0.3 or less; whereas with sweet whey it required almost twice the volume of applied whey in order to reduce A_{280} to 0.9. This indicates that sweet whey proteins have less affinity to bind to the Cu-loaded column than liquid cheese whey and can easily be removed during the washing step.

IgG activity of ED and sweet whey powders were much lower than liquid Cheddar cheese whey. Adjustment of the pH of reconstituted ED and sweet whey powders resulted in large amounts of precipitate, particularly under acidic conditions. The precipitates contained large quantities of LF and Ig, as well as other proteins. Even at a higher pH (8.2), where no precipitation was observed unless the whey was centrifuged, the reconstituted ED and sweet whey samples had a tendency to clog the MCIC columns.

These results suggest that these powders (i.e., ED and sweet whey) are not good starting materials for isolation of active Ig. It is possible that some process during the manufacture of these powders (e.g. heating or drying) caused

denaturation and aggregation of the whey proteins. It is therefore recommended that either liquid whey or powders produced by a milder process should be used.

C. BINDING CAPACITY AND RECOVERY OF IMMUNOGLOBULINS FROM METAL CHELATE-INTERACTION CHROMATOGRAPHY COLUMN

Crude Ig (0.3%) separated from colostral whey by ammonium sulfate precipitation was passed through a 2.2 mL Cu-loaded Sepharose 6B column as shown in Figure 35. It was found that the capacity of the column was 101 mg Ig/mL column. The proportion of immunoglobulin eluted from the column under acidic conditions was 94% of the amount bound to the column. Figure 35 also shows that the capacity of 62 mg Ig/mL of Cu-Loaded IDA-BGE Sephacryl S-300 column was lower than that of the Sepharose 6B counterpart. However, the proportion of immunoglobulins eluted from this column was 100% of the amount adsorbed to the column. Thus, the binding capacity for bovine Ig appears to fall in the same range as that reported by Lonnerdal et al. (1977) for human lactoferrin, which was 70 mg LF/mL gel containing 50 µmole copper ions.

Using a 2.5 X 9 cm column containing a 50 mL bed volume of chelating Sepharose gel, approximately half (25 mL) of which was copper-loaded gel, near-quantitative recovery of IgG was obtained in the peak containing Ig, from application of 750 mL of liquid whey (6.5% total solids, containing 250 mg IgG). Radial immunodiffusion indicated that peak 1 and peak 2 contained 6 and 200 mg IgG respectively, while the IgG content of the unbound fraction was not detectable (below the lower detection limit of R.I.D.). The recovery of IgG was 82.2% for liquid whey. The purity of IgG was also increased from about 4% in cheese whey to 53% in the IgG rich fraction (F2). These results indicate that MCIC capacity for Ig isolation from cheese whey is at least 200 mg IgG per 25 mL of copper-loaded gel or about 8 mg IgG/mL copper-loaded gel; thus, approximately

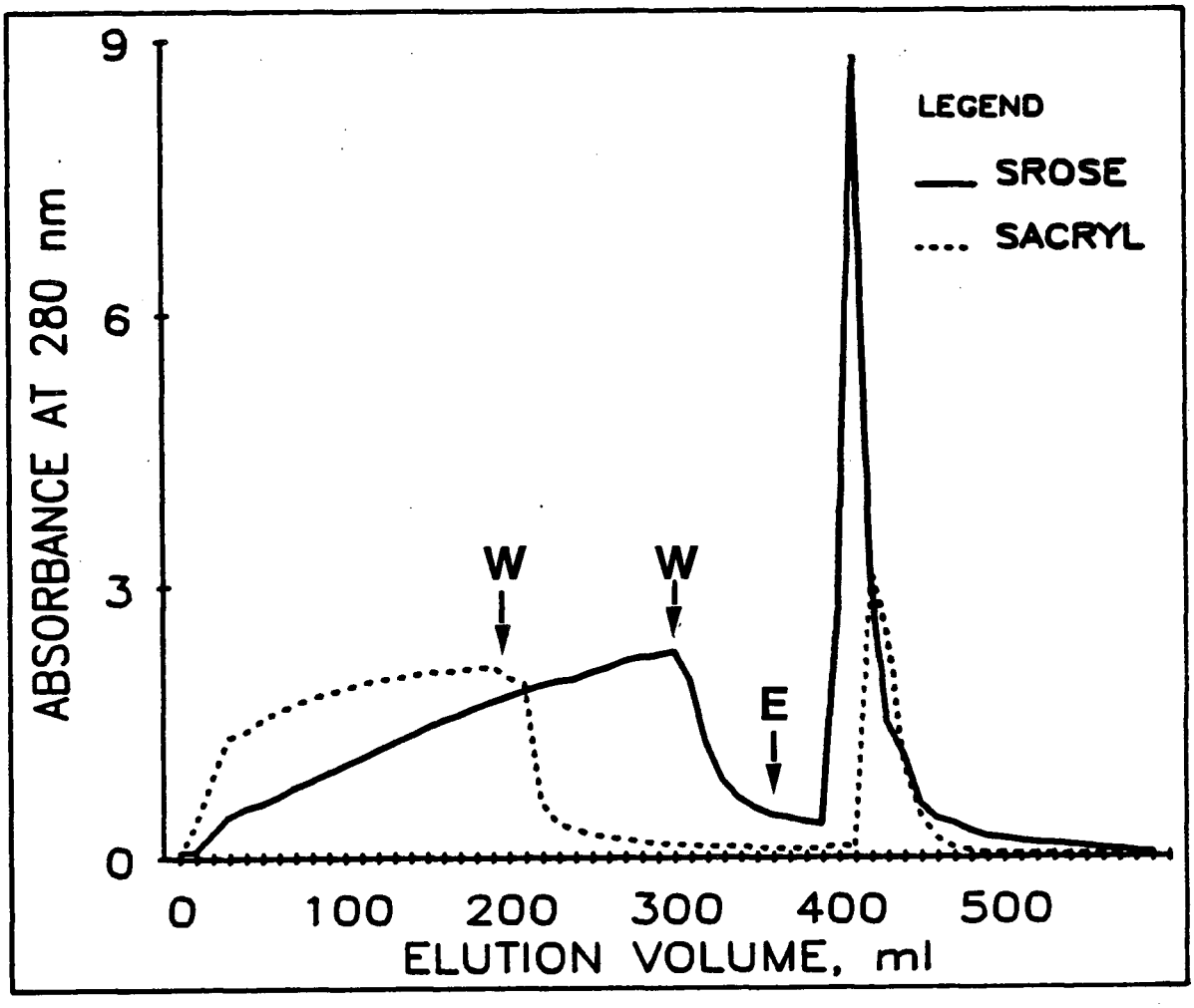


Figure 35. Saturation point for adsorption of crude Ig (prepared from colostrum by ammonium sulfate method) on Cu-loaded IDA-BGE Sepharose 6B (SROSE) and Sephacryl S-300 (SACRYL). 0.3% crude Ig was passed through a 10 mL column (7.0 x 1.4 cm) equilibrated with 0.05 M Tris-acetate/0.5 M NaCl, pH 8.2. W, washing with the starting buffers; E, elution with 0.05 M Tris-acetate /0.5 M NaCl, pH 4.0. The flow rate was 20 mL/hr.

1 litre of whey could be treated with about 25 mL of copper-loaded gel.

The discrepancy between the apparent capacity of MCIC for crude Ig (101 mg/mL) and for Ig in whey proteins may be due to the presence of other proteins in cheese whey. In addition to isolation of Ig, both LF and BSA from cheese whey were also bound to the copper-loaded gel. Binding of these two proteins may have lowered the apparent binding capacity for Ig from whey. However, LF and BSA are probably less strongly bound to the immobilized copper than Ig, since they were eluted before Ig during pH gradient elution.

D. ANTI-LIPOPOLYSACCHARIDE ACTIVITY OF IMMUNOGLOBULINS RICH FRACTION

Figure 36 shows the anti-lipopolysaccharide activity of an IgG rich fraction isolated from cheddar cheese whey by MCIC method. Immunoglobulins isolated from cheese whey recognized and bound to LPS extracted from E. coli, S. typhimurium and B. parapertussis. Among the three O antigens, immunoglobulins showed higher recognition and binding ability to the LPS isolated from E. coli indicating that this antigen is fairly common to the dairy cow's from which the wheys were produced. Lower recognition was obtained for LPS isolated from S. typhimurium. The isolated Ig showed binding ability to LPS isolated from B. parapertussis.

LPS, which is serologically called as O antigen and pharmacologically known as endotoxins, are the integral components of the outer membrane of Gram-negative bacteria. They contain protein, lipids and lipopolysaccharides. Potentially all of these components are more or less exposed on the cell surface and thus can interact with living cells or substances in the environment. Some proteins form pores through which substances can be exchanged across the cell wall. Other proteins participate in iron scavenging or cell adhesion. With respect to pathogenicity of Gram-negative bacteria, the cell wall

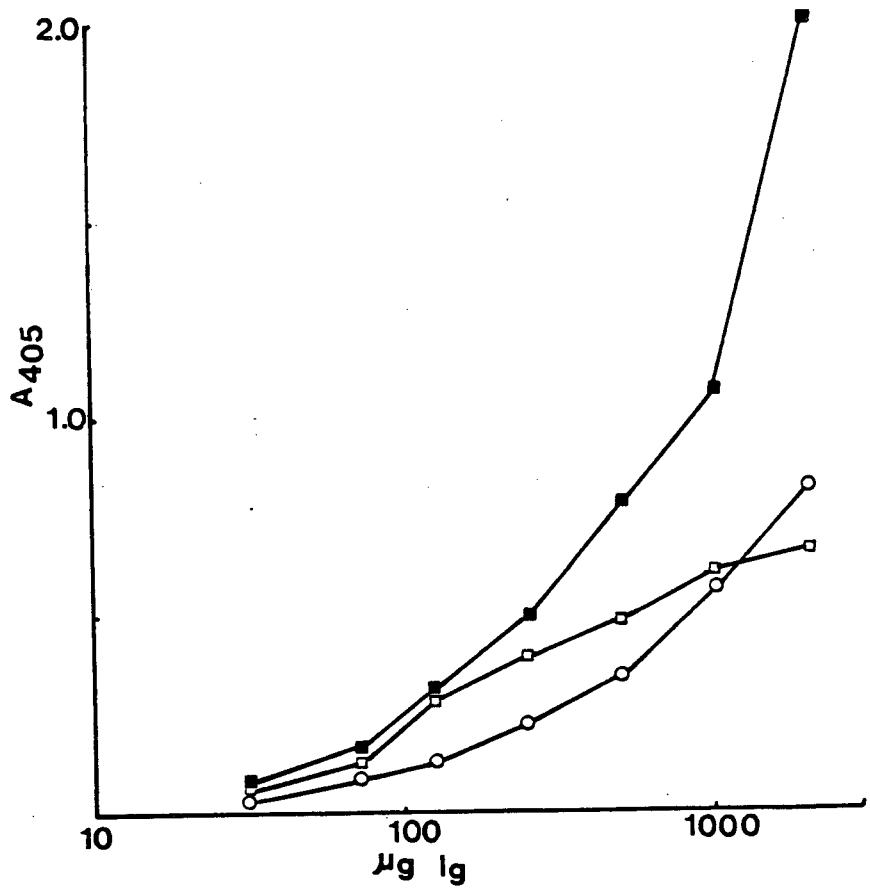


Figure 36. Anti-lipopolysaccharide activity of Ig isolated from cheese whey by MCIC method. ■—■, E. coli LPS; □—□ S. typhimurium LPS; ○—○, B. parapertussis LPS.

lipopolysaccharides are not only dominant antigens but also mediators of a great many biological activities (Jann and Jann, 1985, Rietschel et al., 1982). Even though it is hard to speculate on what follows the binding of Ig with LPS, the binding may interfere with the process by which bacteria adhere to and colonize the intestinal lining (Packard, 1982). The interaction of Ig with LPS may also disturb the biological processes which are involved in the transport of materials across the bacterial cell wall.

E. LACTOPEROXIDASE CONTENT OF LACTOFERRIN RICH FRACTION

The lactoferrin rich fraction obtained by MCIC treatment was yellowish in color and became greenish on freezing. The color was thought to be due to the presence of lactoperoxidase which constitutes about 1% of the whey proteins. The color of lactoperoxidase is due to the iron content (it contains 0.071% iron which represents one atom of iron per molecule) (Paul and Ohlsson, 1985). The lactoperoxidase containing fraction lost its color when dialyzed against 0.1 M citric acid for 36 hr. Moreover, the lactoperoxidase assay indicated the presence of less than 2% lactoperoxidase in LF fraction. The presence of lactoperoxidase in the LF rich fraction would give extra antimicrobial activity to the isolated fraction (Pruitt and Tenovuo, 1985).

F. IDENTIFICATION OF GLYCOPROTEINS IN LACTOFERRIN-RICH FRACTION

Figure 37 shows the SDS-PAGE of cheese whey, the F1 fraction obtained from MCIC column, standard lactoferrin (LF) and lactoperoxidase (LP). Samples were stained with Coomassie Brilliant Blue (Figure 37A) as well as periodic acid-Schiff (PAS) stain (Figure 37B). Using Coomassie stain, LF rich fraction was shown to contain Ig, LP and lactoferrin. According to the SDS-PAGE of

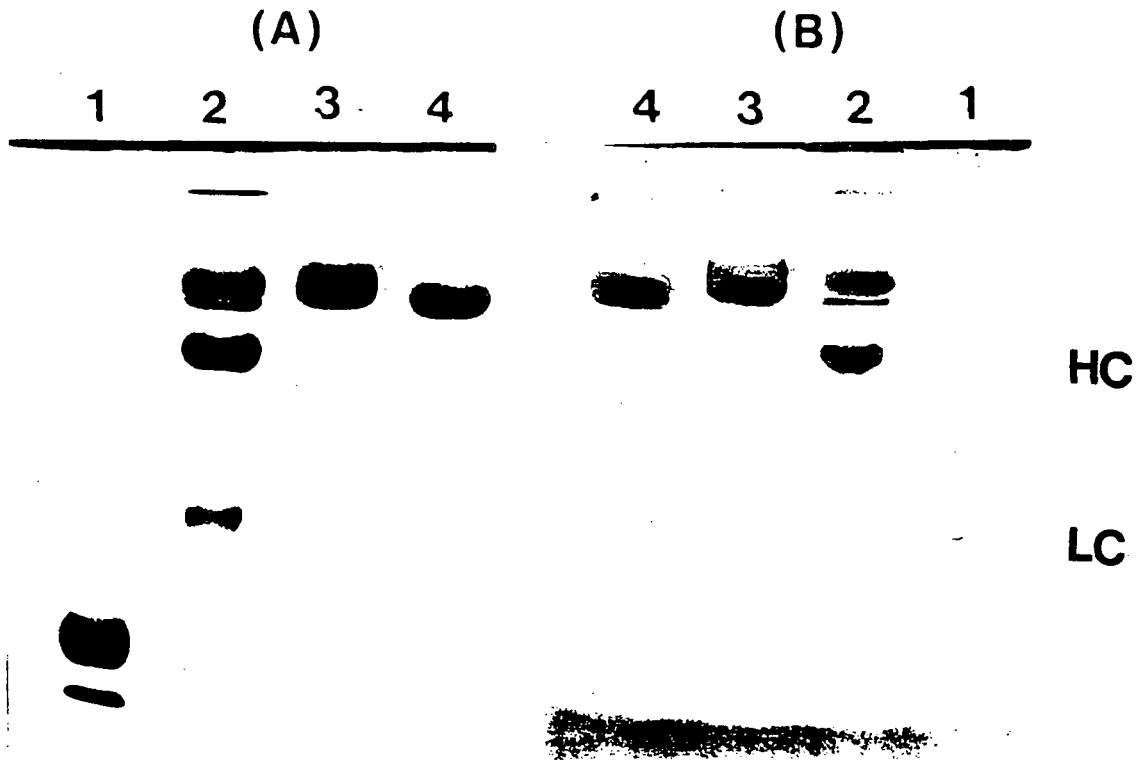


Figure 37. SDS-PAGE of whey proteins (1), lactoferrin rich fraction (2), lactoferrin (3) and lactoperoxidase (4). (A) stained with Commassie Brillant Blue and (B) stained with periodic acid Schiff (PAS). HC and LC heavy and light chains, respectively.

standard LF and LP, lactoferrin has slightly higher molecular weight than LP. PAS technique, which was applied to detect glycoproteins following electrophoresis on SDS-PAGE, indicated that LF, LP and the heavy chain of Ig contained covalently bound carbohydrate. This staining method also indicated that the majority of carbohydrate in Ig was located in the heavy chains of the molecule.

G. ISOELECTRIC POINTS OF LACTOFERRIN AND IMMUNOGLOBULINS RICH FRACTIONS

The isoelectric point of fractions obtained by MCIC treatment of cheese whey were found to be in the range of 5.2-6.6 which covered the isoelectric points of standard lactoferrin, 6.0, and immunoglobulins 5.5-6.8 (Josephson et al., 1972). However, the isoelectric point of lactoperoxidase was 8.63 which was lower than the reported data of 9.16 - 9.8 (Righetti and Caravaggio, 1976).

H. HISTIDINE MODIFICATION AND METAL CHELATE-INTERACTION CHROMATOGRAPHY

Among all of the amino acids comprising proteins, Rassi & Horvath (1986) found that histidine and cysteine gave the highest retention factors when passed through a Cu-IDA column. However, studying the retention behavior of free amino acids might not represent the real behavior of that amino acid when it is found in protein. The role of histidine in immunoglobulins and the lactoferrin rich fraction isolated by MCIC treatment was investigated using MCIC. When control Ig was applied to MCIC column, almost no protein was eluted in the washing step (Figure 38), indicating that all the sample applied was adsorbed to the column. This fraction was subsequently eluted with 0.01 M imidazole solution. However, modification of histidine groups of Ig by diethyl pyrocarbonate (DEP-Ig) greatly inhibited the interaction with the copper ion. Most of histidine-modified Ig was found in the washing solution, while only a small amount of protein was

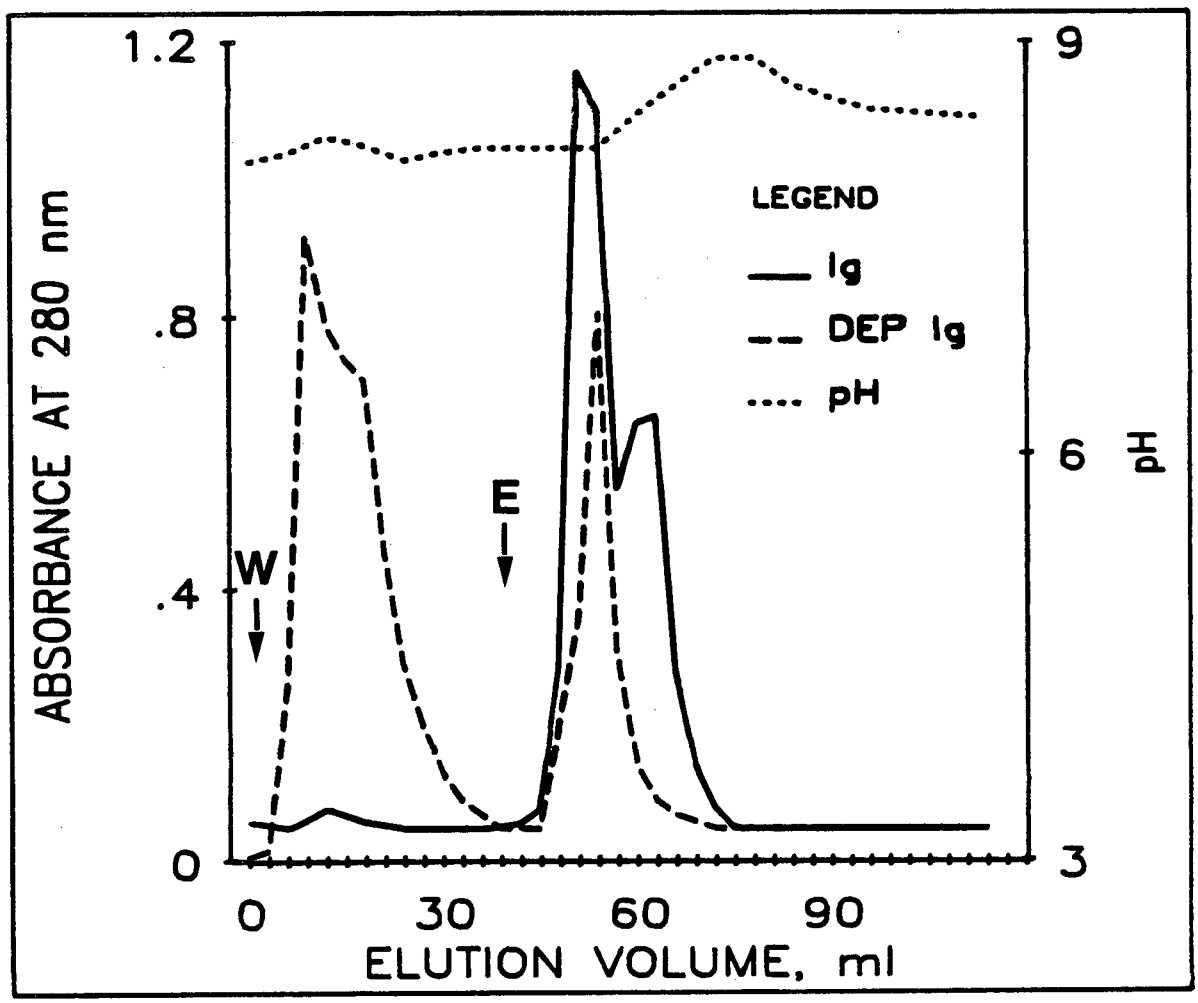


Figure 38. Elution profiles of control (Ig) and diethyl pyrocarbonate treated immunoglobulins (DEP Ig). Samples (30 mg/5 mL 0.05M Tris-acetate containing 0.5 M NaCl, pH 8.2) were applied to the column (1.4 x 7.0 cm) and washed (W) with the starting buffer then eluted (E) with 0.01 M imidazole. Flow rate was 30 mL/hr.

adsorbed. These data strongly suggest the involvement of histidine groups of Ig in the interaction with copper ion immobilized on agarose. The small amount of DEP-Ig adsorbed on the column may suggest the involvement of other amino acids in the interaction, especially cysteine which is considered to be the second major force contributing to the interaction (Rassi & Horvath, 1986).

Figure 39 shows the elution profiles of the lactoferrin rich fraction obtained from cheese whey before and after histidine modification. Before histidine modification, all proteins in the sample applied were adsorbed to the column of MCIC, however, after blocking histidine groups most of the proteins applied were desorbed by washing the column with the starting buffer. Appreciable amounts of protein were eluted with 0.01 M imidazole. This may indicate that some other forces are involved in the interaction or the modification process of histidine was not complete (since this fraction was mixture of Ig, LF and BSA, it was difficult to calculate mole modified histidine per mole protein).

I. SEPARATION OF HEAVY AND LIGHT CHAINS OF IMMUNOGLOBULINS

A typical elution pattern of reduced and alkylated Ig from Sephadex G-75 column is shown in Figure 40. The first peak which eluted at the void volume represents the heavy chain while the second peak consists of light chains. The third peak is eluted at a volume corresponding to the total bed volume, and therefore represents small molecules, specifically the reducing and alkylating agents. The homogeneity of the heavy and light chain preparation was demonstrated by SDS-PAGE (Figure 41). Better resolution of heavy and light chains was obtained when Ultrogel ACA 54 was used instead of Sephadex G-75 as indicated in Figure 42. Based on the total absorbance unit calculation, the percentage of heavy chains (first peak) and the light chains (second peak) obtained from these figures were 70-75% and 25-30% respectively. These values

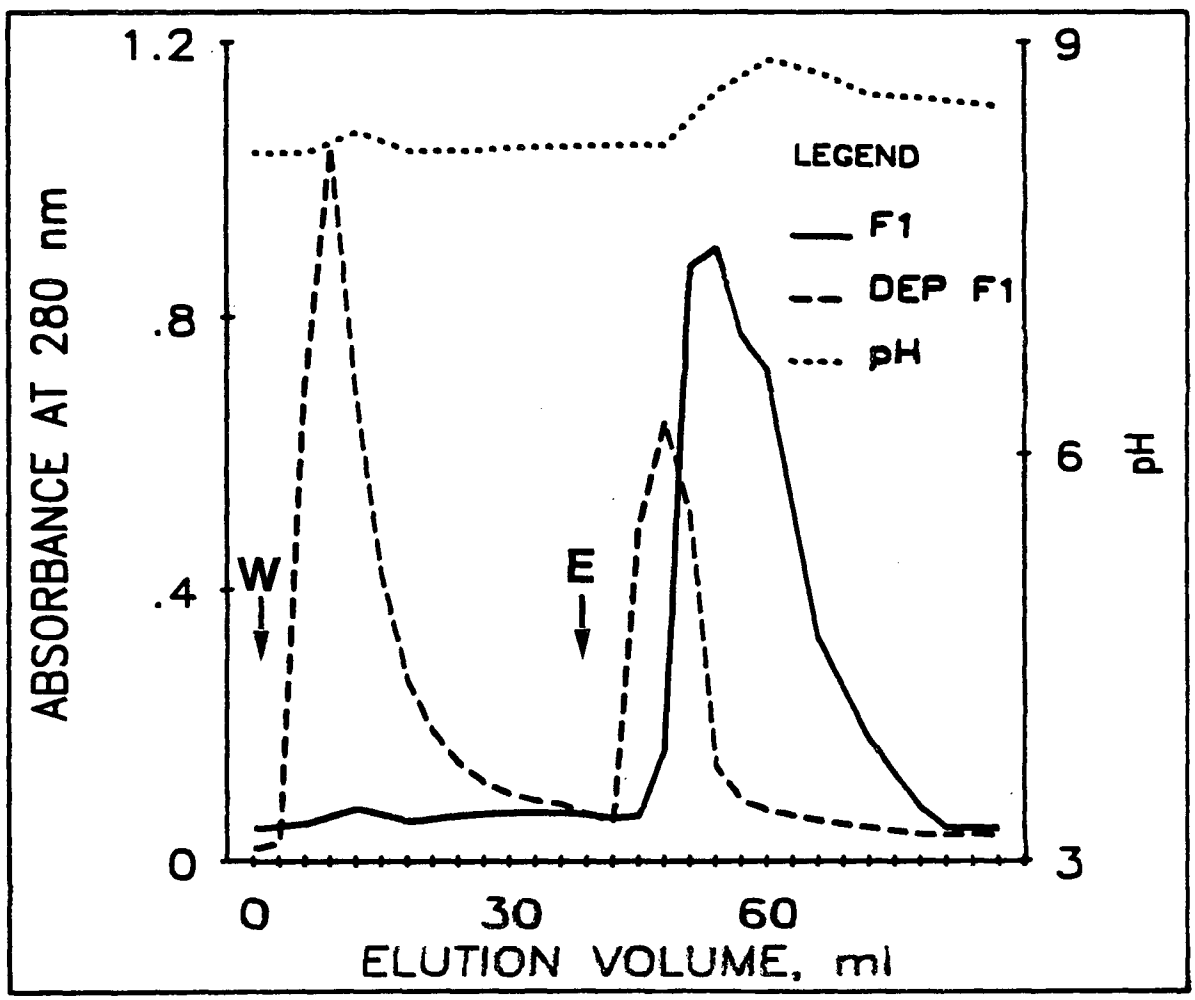


Figure 39. Elution profiles of F1-MCIC fraction before (F1) and after diethyl pyrocarbonate treatment (DEP F1). Samples (30 mg/5 mL 0.05 M Tris-acetate containing 0.5 M NaCl, pH 8.2) were applied to the column (1.4 x 7.0 cm) and washed (W) with the starting buffer then eluted (E) with 0.01 M imidazole. Flow rate was 30 mL/h.

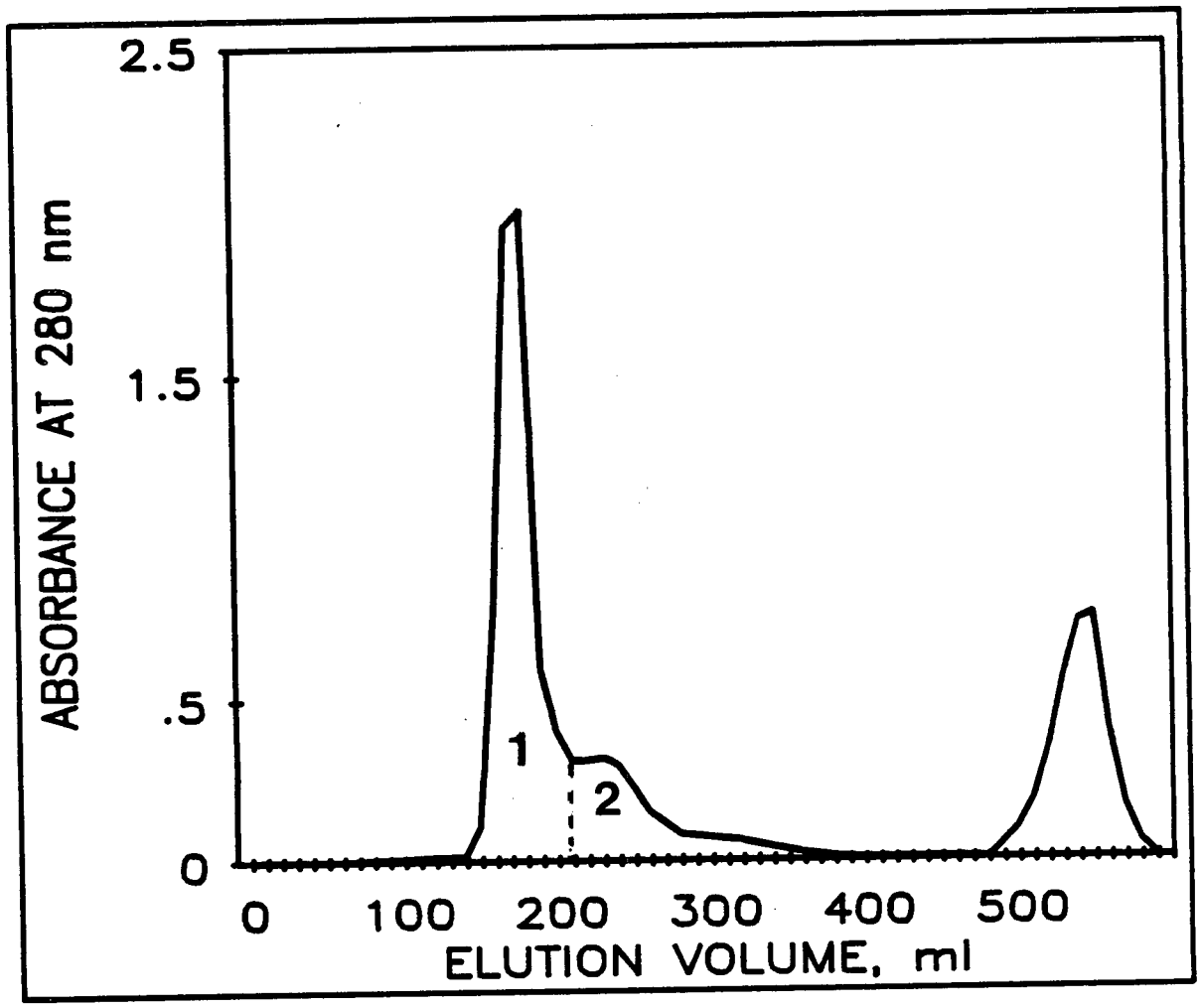


Figure 40. Elution profiles of reduced and alkylated heavy and light chains of immunoglobulin on Sephadex G-75 eluted with 1 M propionic acid. 1 and 2 are fractions obtained.

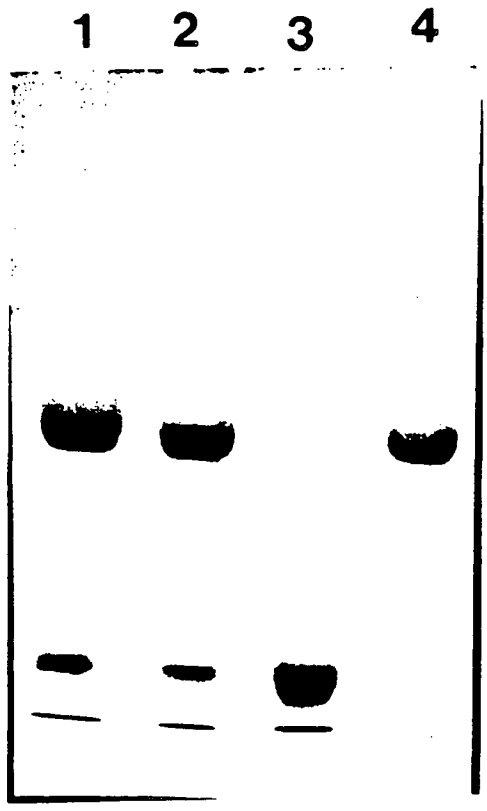


Figure 41. SDS-PAGE profiles of heavy and light chains of immunoglobulins isolated by gel filtration. Lanes 1 and 2, crude immunoglobulins; Lanes 3 and 4, immunoglobulin light and heavy chains, respectively obtained from Figure 40.

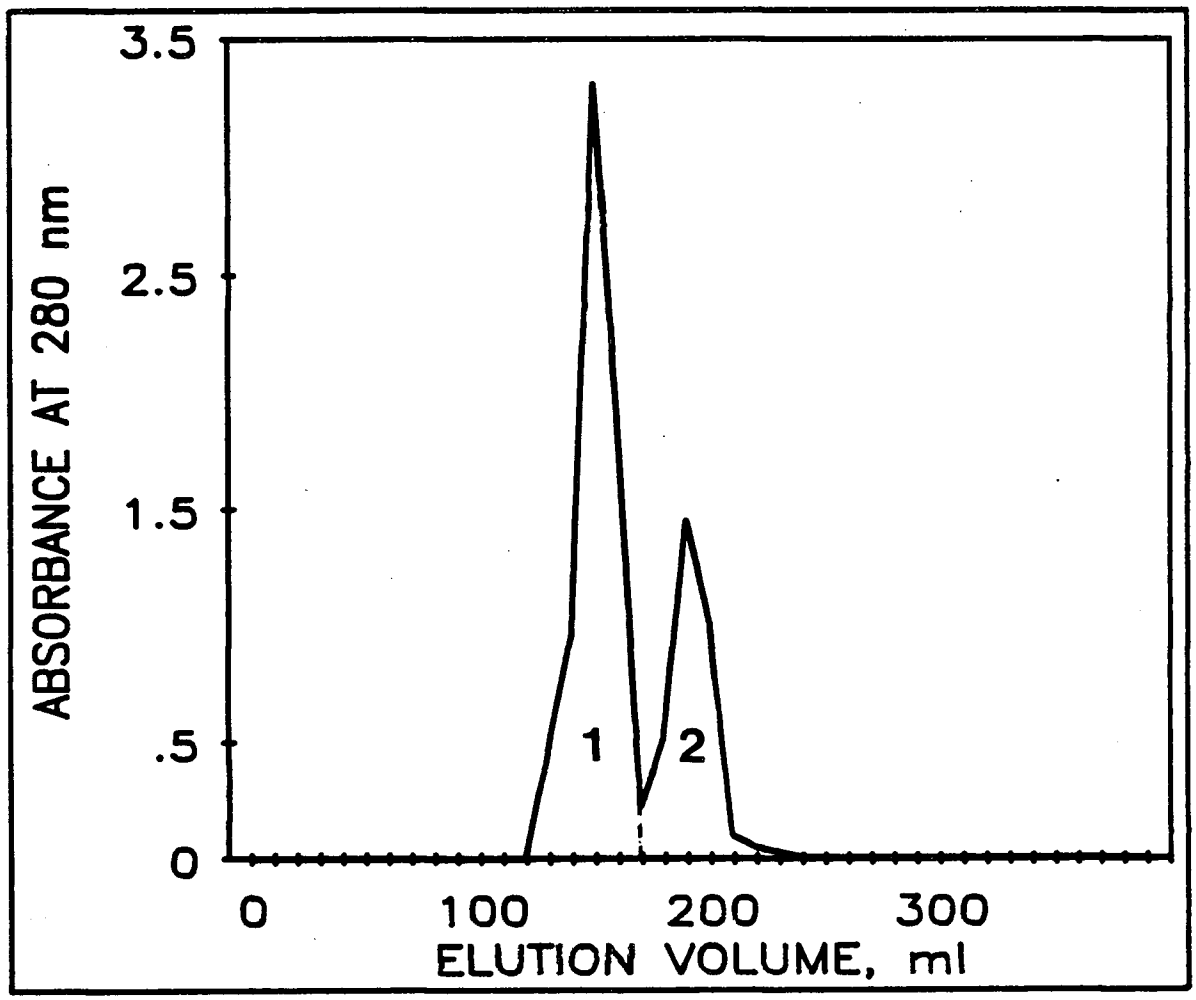


Figure 42. Elution profile of reduced and alkylated heavy and light chains of Ig on Ultrogel ACA 54 eluted with 0.1 M Tris-HCl buffer containing 4 M Guanidine-HCl and 1 mM iodoacetamide, pH 8.2. 1 and 2 are fractions obtained.

are within the range of reported data for Ig of different species (Fleischman et al., 1962; Small and Lamm, 1966).

J. SEPARATION OF LACTOFERRIN AND LACTOPEROXIDASE IN LACTOFERRIN RICH FRACTION

In addition to the gel filtration process used to isolate the LF from LF rich fraction, several attempts were made in order to obtain better resolution of LF from other protein contaminants.

1. Gel filtration method

Figure 43 shows the elution pattern of lactoferrin rich fraction (peak 1) from MCIC of bovine acid whey on Sephacryl S-300 column. Two major peaks were obtained, the first peak was colorless while the second peak was yellowish. SDS-PAGE analysis (Figure 26) indicated that the first peak consisted mainly of immunoglobulins while the second peak contained predominantly lactoferrin.

2. Stepwise pH elution

Figure 44 represents a stepwise elution process for separating LF rich fraction on MCIC column loaded with copper. Elution with the starting buffer, 0.5 M NaCl in 0.05 M Tris-acetate at pH 7 and 6 did not remove any of the bound proteins from the column; however, elution with the same buffer at pH 5 removed some of the bovine serum albumin. Further elution with the same buffer at pH 4.0 gave two peaks, i.e., LF and Ig, which were not well-separated. Using pH 5 as a cleaning step for eluting bovine serum albumin, then elution with a pH gradient (5.0-2.8) slightly improved the separation of LF and Ig as shown in Figure 45.

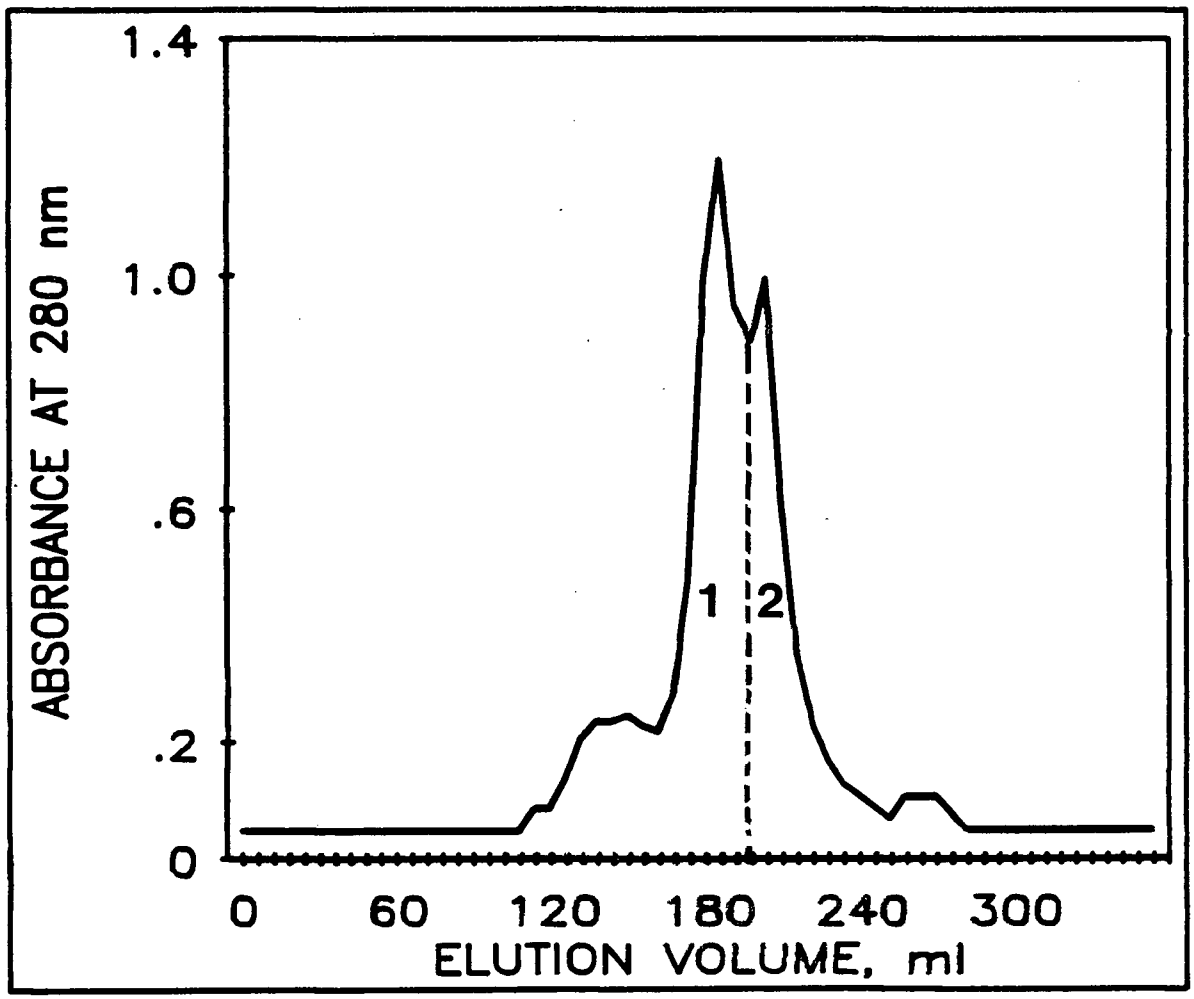


Figure 43. Sephacryl S-300 column chromatography of lactoferrin rich fraction obtained by MCIC of acid whey. 100 mg sample was applied to Sephacryl column (83 x 2.5 cm) and eluted with 0.05 M potassium phosphate buffer, pH 7.4 containing 0.01 M NaCl. 1 and 2, are fractions obtained. The flow rate was 30 mL/hr.

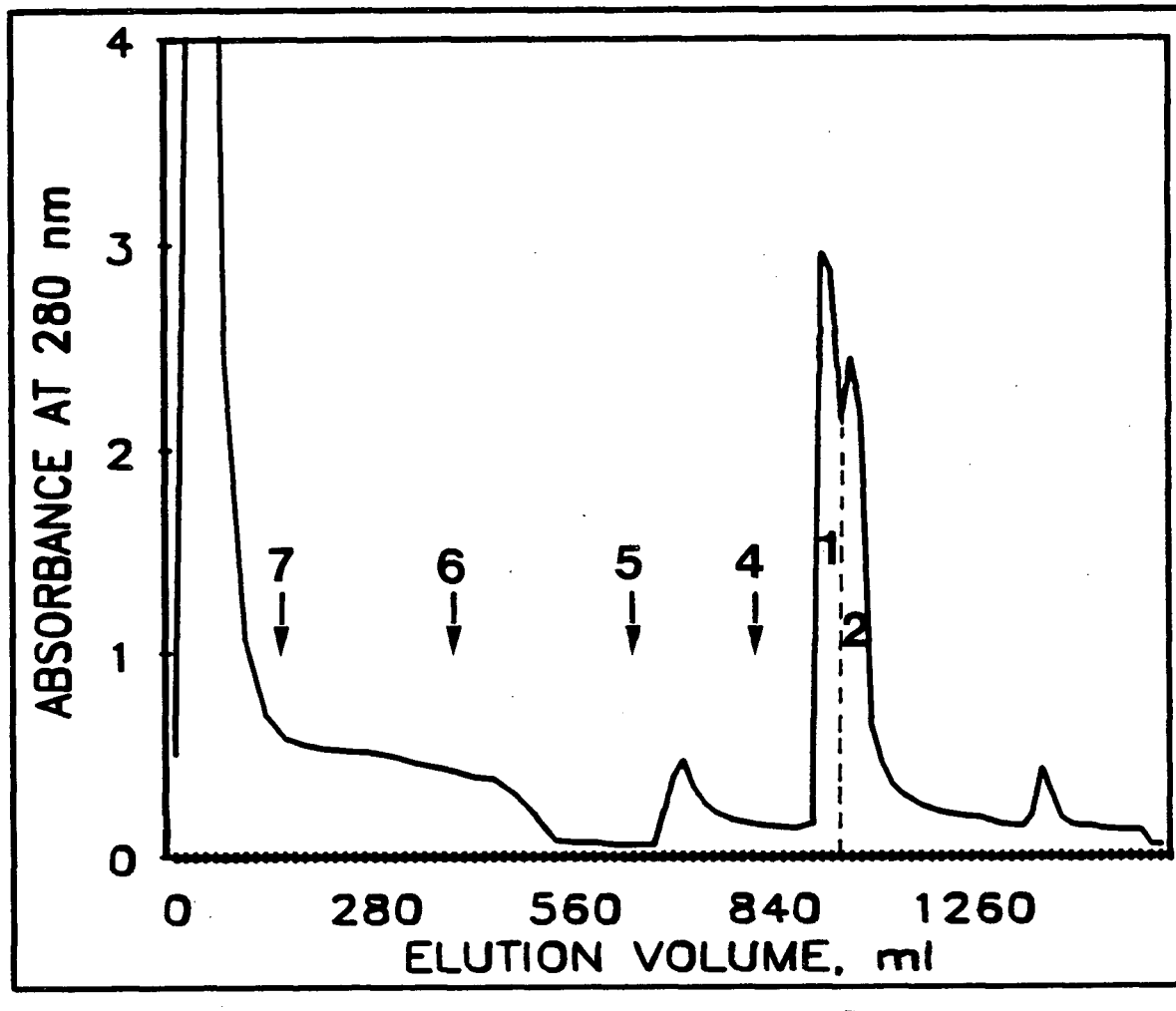


Figure 44. Stepwise elution profile of acid whey on MCIC eluted by decreasing pH values. Arrows indicate pHs 7, 6, 5 and 4 of 0.05 M Tris-acetate containing 0.5 M NaCl. 1 and 2 are fractions obtained.

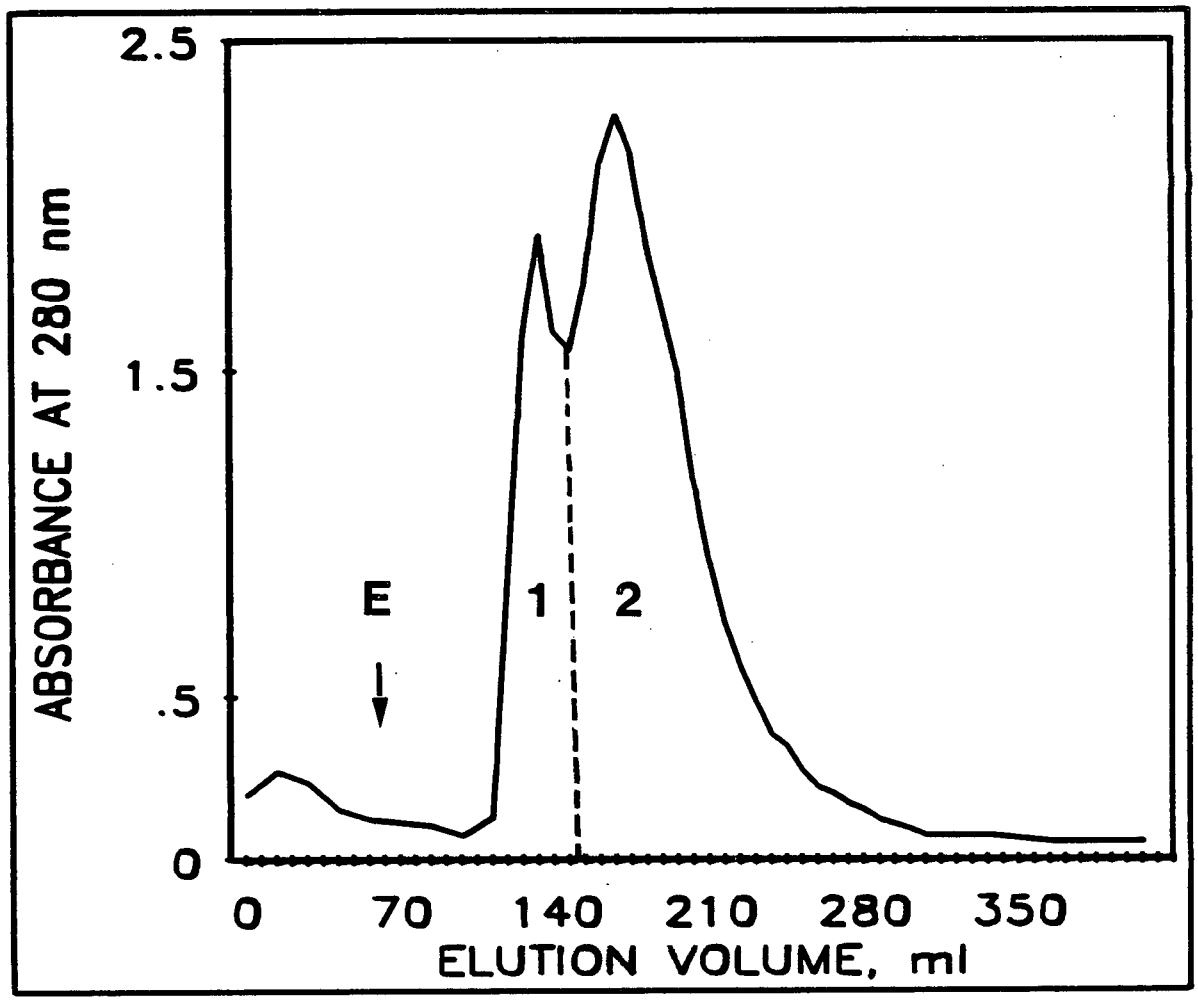


Figure 45. Elution profile of bound proteins of acid whey on MCIC column, eluted (E) by using pH gradient (5-2.8) of 0.05 M Tris-acetate containing 0.5 M NaCl. 1 and 2 are fractions obtained.

3. Imidazole gradient elution

Fraction 1 obtained by MCIC process contained Ig and BSA in addition to LF. This fraction was pooled and rechromatographed on MCIC column and eluted with a 0-0.01 M imidazole gradient. After washing off the unbound proteins two well-separated peaks were obtained (Figure 46). The first peak contained mainly Ig, while the second peak was mainly lactoperoxidase as indicated by SDS-PAGE (Figure 47). However, subsequent elution with the starting buffer at pH 2.8 gave an extra peak which was highly purified lactoferrin according to SDS-PAGE analysis. It is, therefore, possible to separate these three biologically active proteins.

The results of this study demonstrate that cheese whey can be a reliable source for extracting immunoglobulins and lactoferrin. Of the absorption chromatography techniques investigated, metal chelate-interaction chromatography is the best method as it is simple in operation for separating biologically important immunoglobulins, lactoferrin and lactoperoxidase. Furthermore, this method has advantages of high capacity, quantitative recovery without detectable damage to the immunological activity of the proteins and easy regeneration. Based on antilipopolysaccharide activity of isolated immunoglobulins and the well known bacteriostatic activity of lactoferrin, (Packard, 1982) the separated bioactive proteins may, therefore, be useful in fortification of infant formulae or infant feeding.

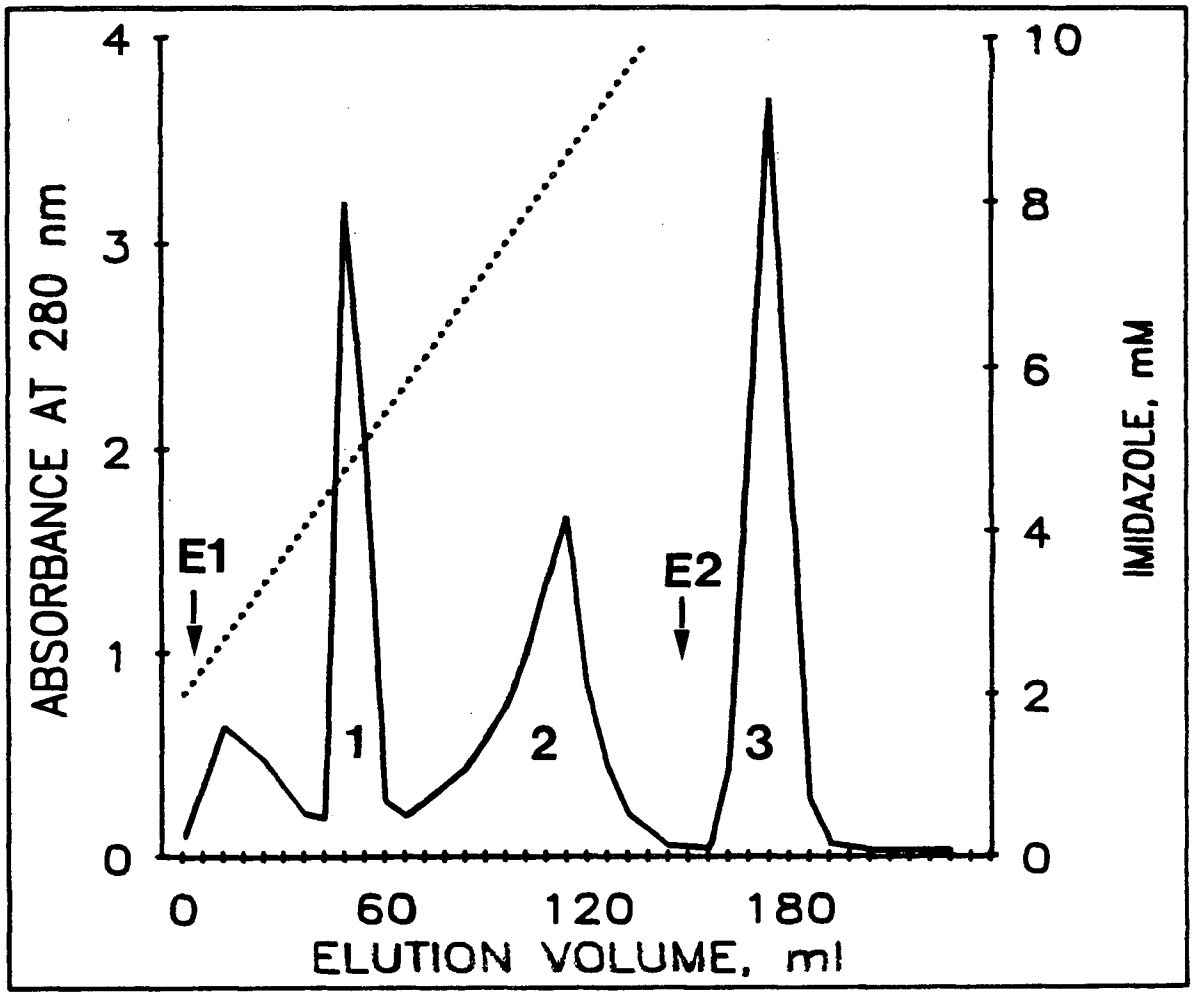


Figure 46. Elution profile of lactoferrin rich fraction on MCIC column. E1, elution with linear gradient of 0-10 mM imidazole solution (....); E2, elution with 0.05 M Tris-acetate containing 0.5 M NaCl, pH 2.8. 1, 2 and 3 are fractions obtained.

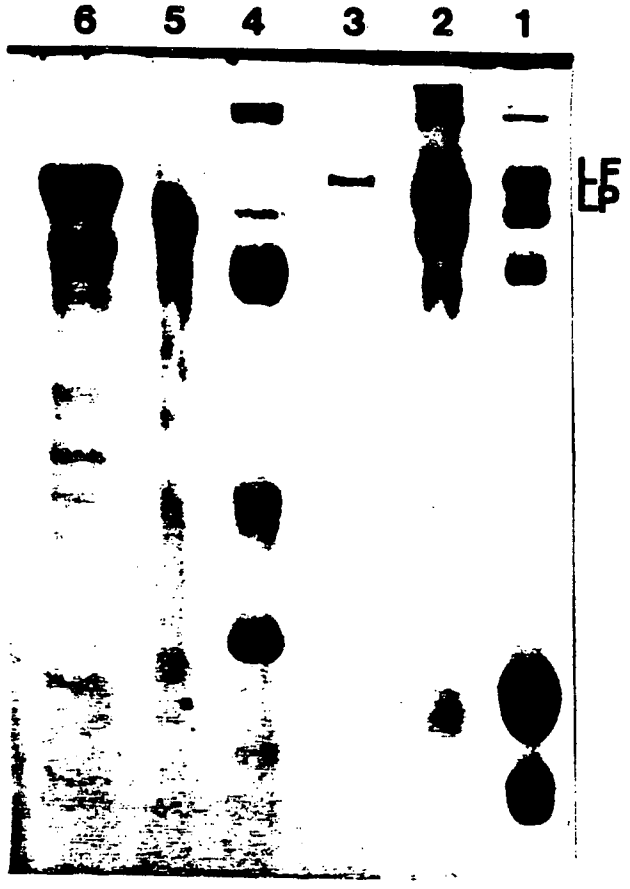


Figure 47. SDS-PAGE profiles of fractions obtained from Figure 46. Lane 1, whey proteins; Lane 2, control F1-MCIC; Lane 3, unbound fraction; Lanes 4, 5 and 6 are peak 1, 2 and 3 of Figure 46; LF, lactoferrin, LP, lactoperoxidase.

PART IV

METAL CHELATE INTERACTION CHROMATOGRAPHY OF SKIMMILK

In skimmilk there are two major fractions of milk protein, caseins and whey or serum proteins, which are the pH 4.6 insoluble and soluble fractions, respectively. In Part III, immunoglobulins and lactoferrin were isolated quite easily and efficiently from whey proteins by Cu-chelate chromatographic supports; however, if skimmilk can be used as a starting material, broader utilization of these anti-microbial compounds may be feasible. Two types of buffer were used for MCIC column chromatography in this study.

A. MCIC WITH TRIS-ACETATE BUFFER

The possibility of utilizing skimmilk for directly recovering immunoglobulins and lactoferrin was investigated. Figure 48 shows the elution profile of skimmilk before and after 50% dilution with 0.05 M Tris-acetate/0.5 M NaCl, pH 8.2. After washing the unbound proteins with the starting buffer, the adsorbed proteins were eluted with 0.05 M acetate-Tris/0.5 M NaCl pH 4.0. However, the amount of proteins eluted under acidic conditions (peak 1) was small, and the flow rate became quite slow indicating precipitation of casein resulting in clogging of the column. This behaviour was observed regardless of whether skimmilk was diluted or undiluted. Subsequent elution with 0.01 M imidazole recovered the bound proteins which appeared as the main fraction (peak 2) in the profiles. SDS-PAGE analysis (Figure 49) indicated that proteins eluted in the washing step were immunoglobulin, lactoferrin, α-lactalbumin, β-lactoglobulin and casein, while proteins eluted under acidic conditions were mainly immunoglobulin and lactoferrin. The fraction eluted with 0.01 M imidazole, however, was mainly casein.

Table 12 shows the IgG distribution of fractions obtained at different stages of the isolation process of IgG from skimmilk. Immunochemical analysis showed that the majority of active IgG was present in the fraction eluted at

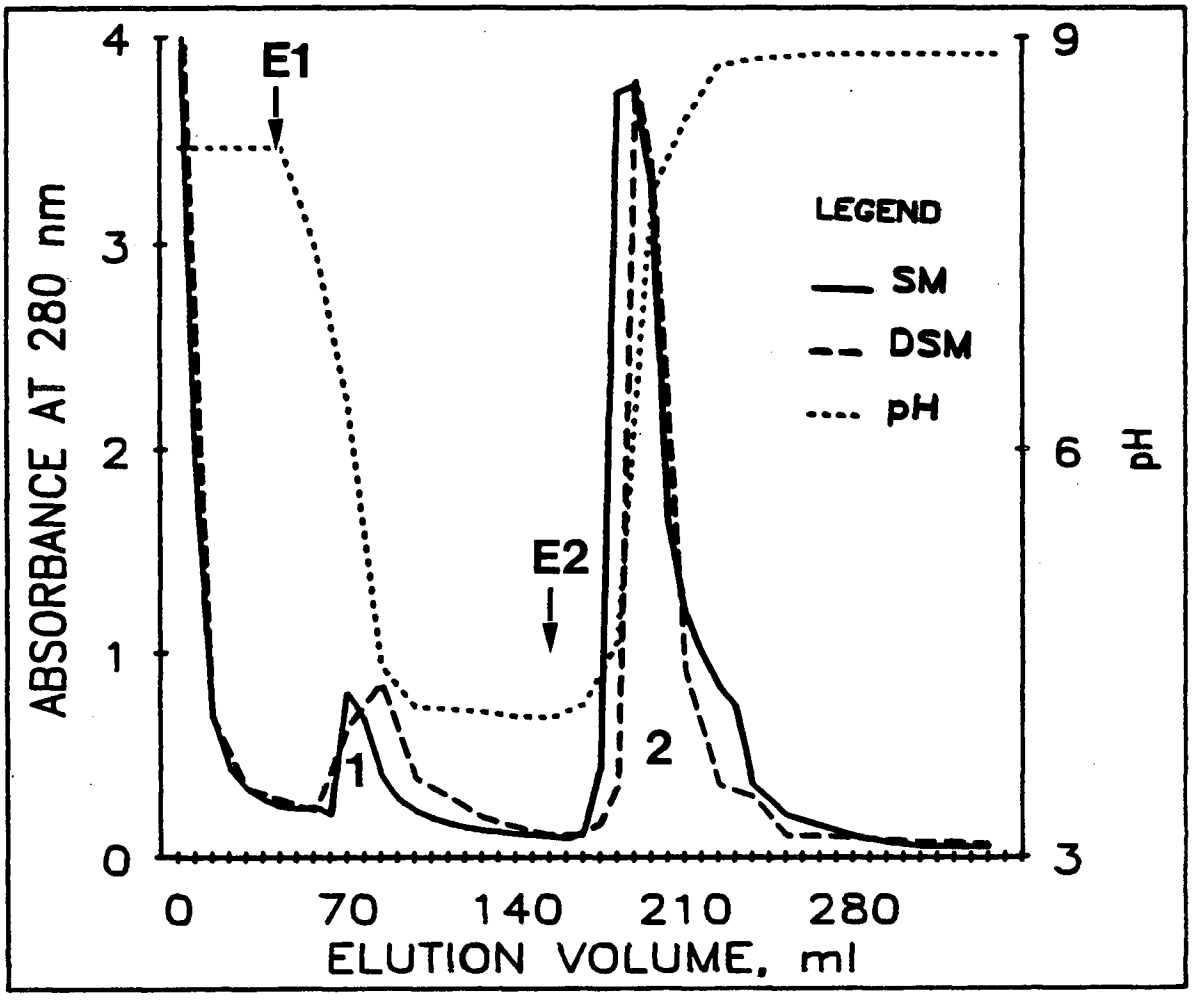


Figure 48. Elution profile of skimmilk on MCIC column. 100 mL skimmilk undiluted (SM) or 50% diluted (DSM) with 0.05 M Tris-acetate/0.5 M NaCl) was passed through Cu-loaded Sepharose 6B (1.4 x 7.0 cm), and washed (W) with same buffer. E1, elution with the same buffer at pH 4.0; E2, elution with 0.01 M imidazole solution. 1 and 2 are eluted fractions. The flow rate was 21 mL/hr.

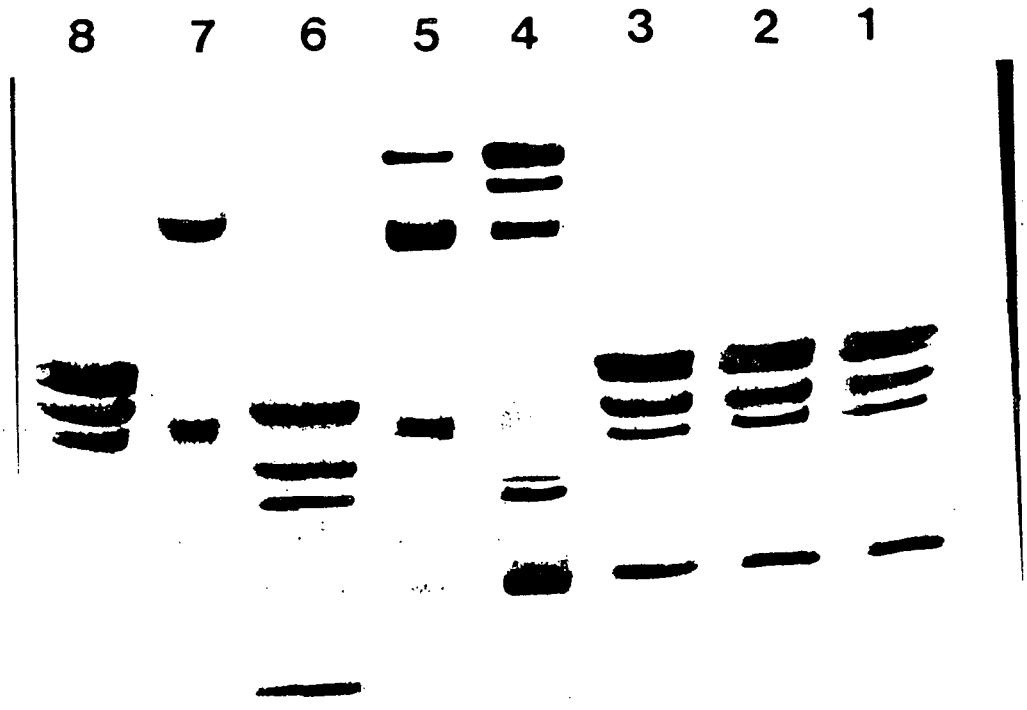


Figure 49. SDS-PAGE of fractions obtained in Figure 48. Lanes 1 and 2, skimmilk; Lane 3, unbound skimmilk to MCIC column; Lane 4, washing fraction; Lanes 5 and 6, are peak 1 and 2, respectively; Lane 7, standard IgG; Lane 8, α-casein.

Table 12. IgG content of different stages of the isolation of IgG from skimmilk on MCIC column.

Sample	Protein[a] Conc. of selected fraction mg/mL	IgG[b] Conc. mg/mL	IgG Purity %
Skimmilk (control)	37.8	0.562	1.49
Unbound skimmilk	18.0	0.246	1.36
Washing fraction	4.0	0.828	20.70
Peak 1 (Figure 48)	1.08	0.911	84.35
Peak 2 (Figure 48)	19.6	0.272	1.38

[a] Determined by Bio-Rad Protein Assay Kit (Bio-Rad Laboratories, Mississauga, Ont.)
[b] Determined by R.I.D.

acidic pH (pH 4.0). This fraction was more than 84% pure. However, the amount of IgG bound to the column was quite small (less than 10%) as compared to the unbound fraction of skimmilk. More than 20% pure IgG was detected in the washing fraction indicating that IgG of skimmilk bound rather weakly to the column. By eluting the strongly bound material with a strongly competing electron-donor solution (0.01 M imidazole) the fraction obtained contained almost no IgG but was rich in casein fractions (Figure 49). These results suggest that there was a competition between IgG and caseins to bind copper ions linked to agarose, and the casein fraction bound more strongly than immunoglobulins to the column under these conditions.

B. MCIC WITH PHOSPHATE BUFFER

An attempt was made to find conditions under which casein could be eluted while retaining immunoglobulins and lactoferrin on the column. Figure 50 shows the elution profile of a mixture of skimmilk and immunoglobulins on copper loaded column of MCIC after equilibration with 0.02 M phosphate buffer containing 0.5 M NaCl, pH 7.0. After washing with the starting phosphate buffer, the unbound proteins were removed. The bound proteins were then eluted with 0.01 M imidazole and 0.05 M Tris-acetate/0.5 M NaCl, pH 3.8 to obtain F1 and F2, respectively. Electrophoretic analysis (Figure 51) indicated that the unbound proteins (turbid fraction) (lane 3) were casein fractions, while proteins eluted with 0.01 M imidazole (lane 4) were immunoglobulins. Peak 2 eluted with Tris-acetate containing 0.5 M NaCl, pH 3.0 was too small to detect by SDS-PAGE.

Figure 50 shows the elution profile of a mixture of Ig and lactoferrin in the presence of caseins in skimmilk, under the same conditions on MCIC column. A similar pattern to that of skimmilk-Ig mixture was obtained; however, the

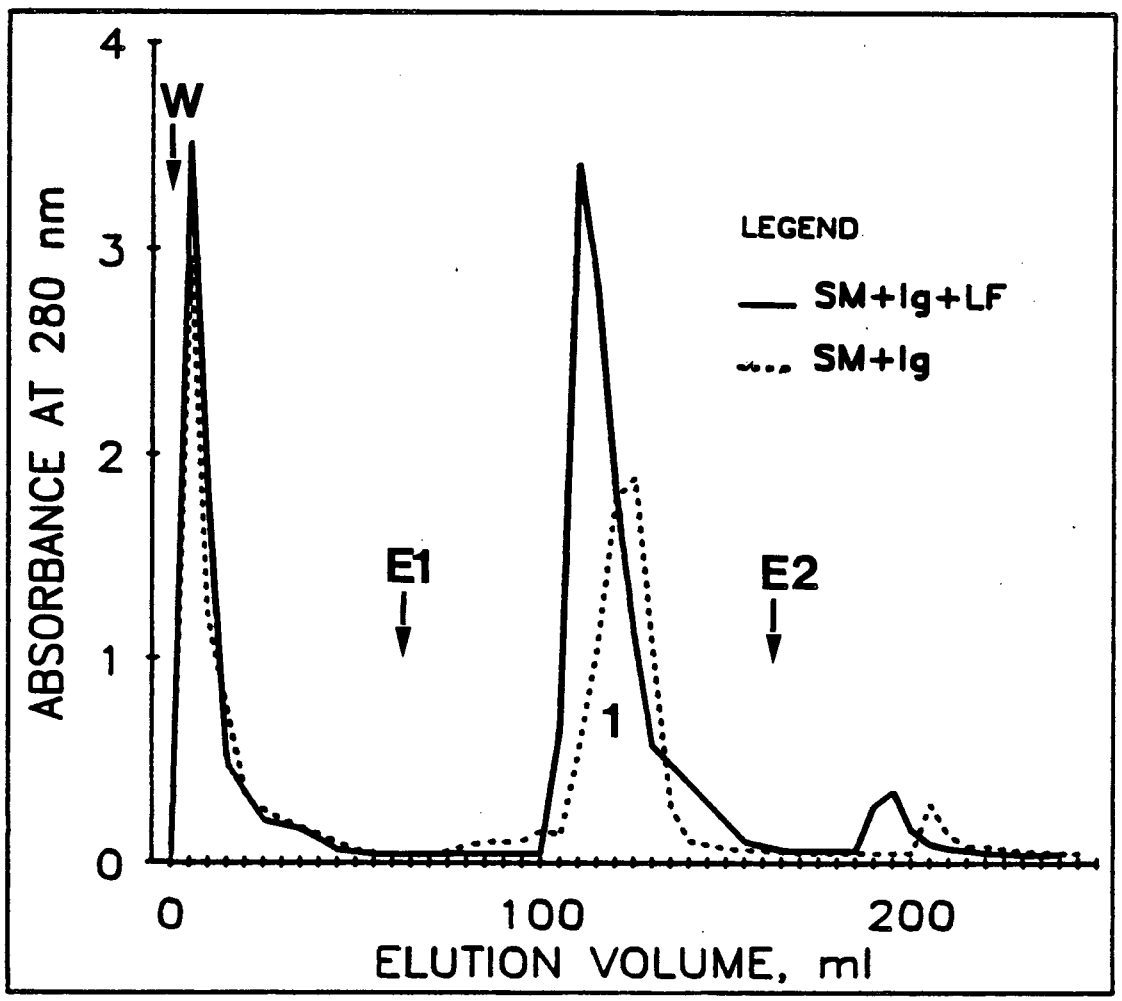


Figure 50. Elution profiles of skimmilk (SM), Ig and LF mixture on MCIC column. 60 mg of Ig and LF was mixed with 1 mL skimmilk (SM+Ig+LF) and 30 mg Ig was mixed with 1 mL skimmilk (SM+Ig) and passed through MCIC column (1.4 x 7.0 cm). W, washing with 0.02 M phosphate buffer containing 0.5 M NaCl, pH 7.0; E1, elution with 0.01 M imidazole; E2, elution with Tris-acetate containing 0.5 M NaCl, pH 3.0.

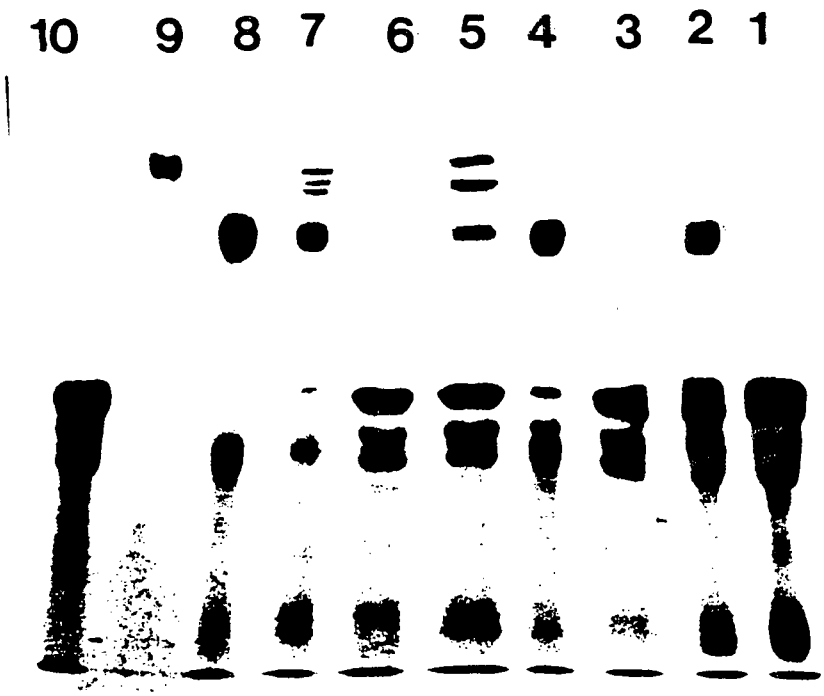


Figure 51. SDS-PAGE of fractions obtained in Figure 50. Lane 1, skimmilk; Lane 2, skimmilk and Ig mixture; Lanes 3 and 4 are unbound and peak 1 of SM-Ig mixture application, respectively; Lane 5, SM-Ig-LF mixture; Lanes 6 and 7 are unbound and peak 1 of SM-Ig-LF mixture application, respectively; Lane 8, immunoglobulins; Lane 9, lactoferrin and Lane 10, α-casein.

amount of protein bound to the column was increased. SDS-PAGE profile (Figure 51) indicated that the unbound fraction (lane 6) was basically casein while proteins eluted with 0.01 M imidazole were a mixture of Ig and lactoferrin (lane 7). The bound protein mixture (Ig and LF) may be separated by using gel filtration or 0-0.01 M imidazole gradient.

Results suggest that the type of ions in the buffer used for initial column equilibration and washing had a great influence on whether or not proteins were unbound or bound to the MCIC column. Since caseins are classified as phosphoproteins (Whitney et al., 1976), it is believed that using phosphate buffer in the equilibration step of the column can form a complex with the copper ion (with different color from that formed with Tris-acetic acid buffer) which may prevent the phosphoproteins from binding to the column. These results also suggest the involvement of phosphoserine groups in the interaction with the copper ions.

C. MECHANISM OF CASEIN-METAL INTERACTION

The principle of protein separation by MCIC process lies in the different affinities of proteins to bind to immobilized metal ions. It is suggested that this binding is dependent on the availability of histidine, cysteine and tryptophan residues of the proteins to form stable coordination complexes with metal ions (Sulkowski, 1985). In general, adsorption of protein to MCIC is performed at a slightly alkaline pH with high ionic strength solutions to decrease non-specific electrostatic interactions. Elution is commonly accomplished by lowering the pH, which reverses protein coordination to the metal-chelate and results in protein displacement. Alternatively, a competing electron donor as a mild chelating agent (e.g., imidazole) or a strong chelating agent (e.g. EDTA) may be used to purge bound proteins. Since histidine has been

suggested to exhibit the strongest retention factor on a copper chelate column at pH 6.0, (Rassi and Horvath, 1986), the elution profile of histidine-modified casein fractions was investigated.

1. α-Casein

α-Casein represents more than 62.5% of total casein fractions in cow's milk and is composed of α_S and κ-casein in ratio of 4:1 (Whitney et al., 1976).

Figure 52 shows the elution profile of control α-casein and histidine-modified α-casein. After loading control α-casein on Cu-chelate gel and washing with the starting alkaline pH buffer, only 10.7% of total applied protein could be eluted under these conditions. The rest of protein (89.2%) was displaced by using a mild chelating agent i.e. 0.01M imidazole (Table 13). However, blocking 3.7 histidine residues by diethyl pyrocarbonate out of the total of four histidines per mole α-casein (Webb et al, 1974) increased the amount of unbound protein from 10.7% to 82.2% (Table 13) indicating the involvement of histidine groups in the interaction with the copper ion. Less than 18% of the protein was bound and could be eluted with 0.01 M imidazole, which might indicate the involvement of other amino acids i.e., Trp, Cys, Tyr in the interaction.

2. α_{S1}- and β-casein

α_{S1}-Casein is a subfraction of the whole casein which represents a slightly less than 50% of the total casein. Figure 53 indicates the elution profiles of α_{S1}-casein and histidine blocked α_{S1}-casein. Without modification of histidine residues of α_{S1}-casein, only a small amount (6.1%) of the protein applied was eluted off the column in the washing step and 93.9% of protein interacted with the copper under alkaline pH and was subsequently

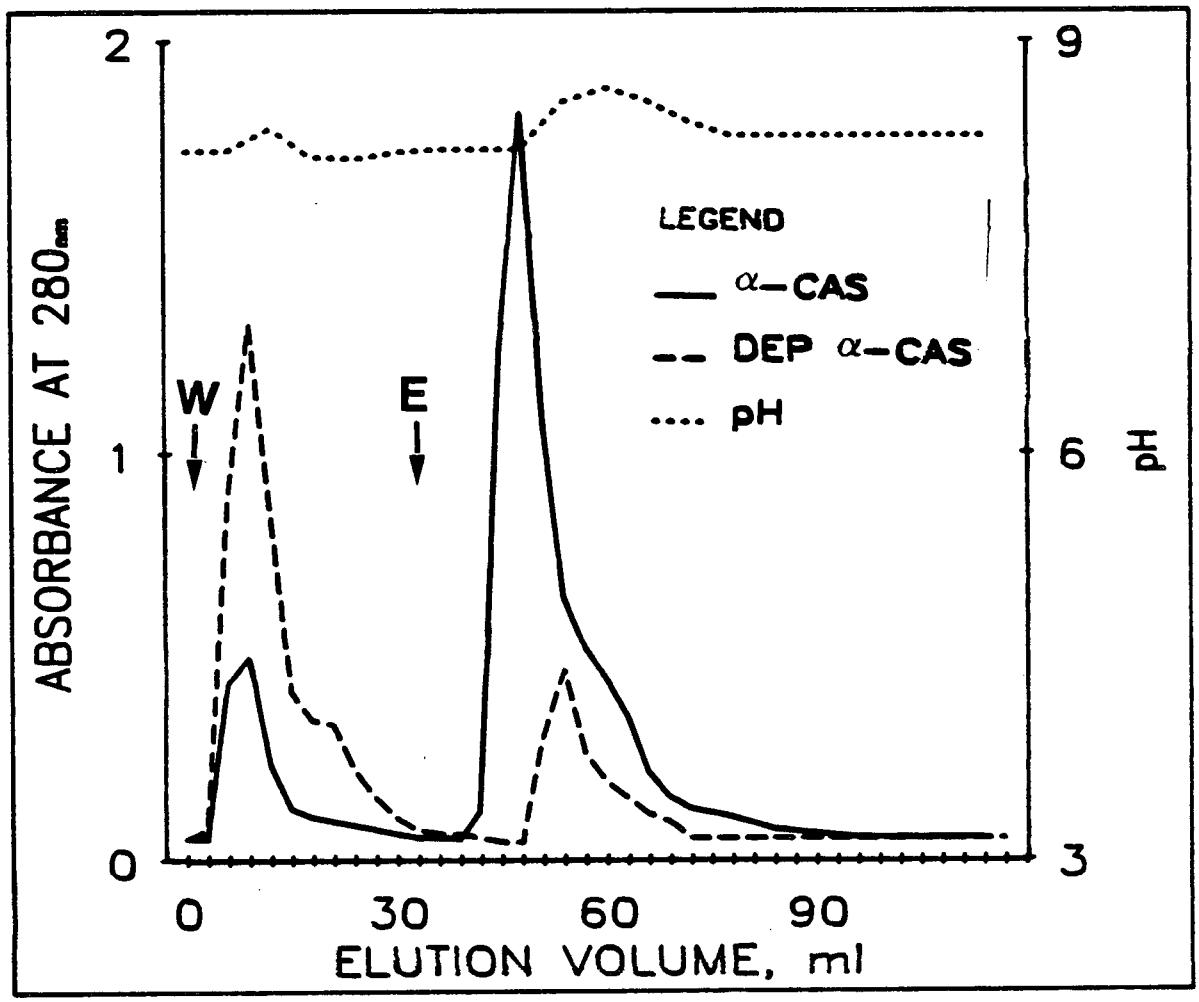


Figure 52. Metal chelate interaction chromatography of α-casein. 3 mL of protein (10 mg/mL) before (α-CAS) and after diethylpyrocarborate modification (DEP-α-CAS) equilibrated with 0.05 M Tris-acetate/0.5 M NaCl, pH 8.2 and applied to copper chelate Sepharose 6B (1.4 x 7.0 cm). W, washing with the same equilibrating buffer; E, elution with 0.01 M imidazole. Flow rate was 30 mL/hr.

Table 13. Binding of casein fractions[a] to MCIC column before and after modification of histidine groups.

Proteins[b]	Washing step (W) %	Eluting step (E) %
Control α-casein	10.7	89.2
DEP-α-casein	82.2	17.8
Control α_{s1}-casein	6.1	93.9
DEP-α_{s1}-casein	94.3	5.7
Control β-casein	13.6	86.4
DEP-β-casein	54.7	45.3
Control polymer κ-casein	87.9	12.1
DEP-Polymer-κ-casein	94.3	5.7
SSS-κ-Casein	38.5	61.5
DEP-SSS-κ-Casein	98.9	1.1

[a] Calculated based on total absorbance units

[b] See Figures 52, 53, 54 and 55 for abbreviation identity

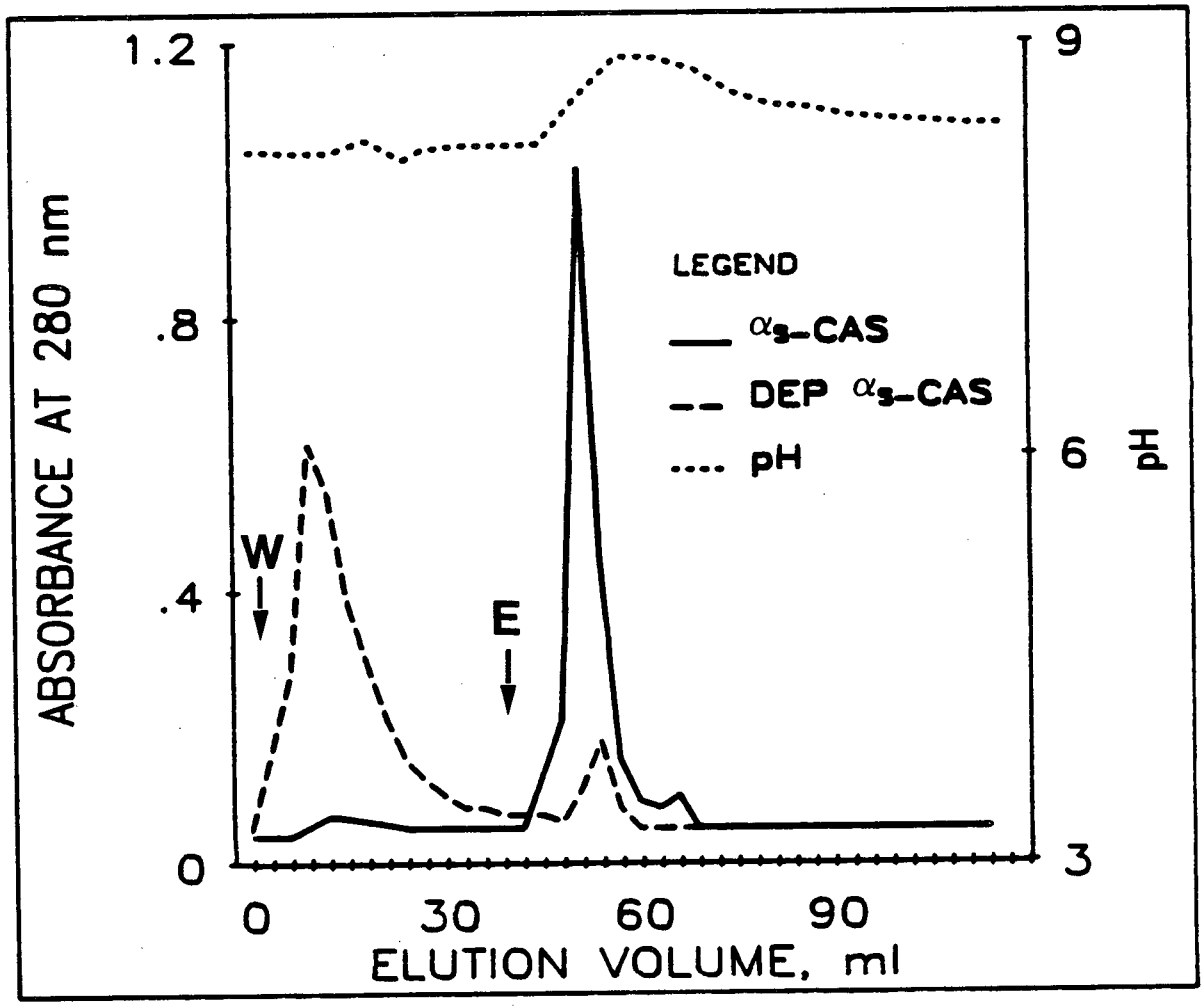


Figure 53. Metal chelate interaction chromatography of α_{s1}-casein before (α_{s}-CAS) and after diethylpyrocarbonate modification (DEP α_{s}-CAS). See Figure 52 for conditions of separation.

eluted with the eluting solution (Table 13). However, blocking 4.2 histidine residues by DEP out of a total of 5 histidine residues per mole α_{s1}-casein (Whitney et al, 1976) destroyed the protein's ability to bind to copper ion immobilized on agarose. Based on the calculation of total UV absorbance, 94.3% of applied protein was recovered in the washing step and a small amount of the applied protein (5.7%) was bound and subsequently eluted with 0.01 M imidazole.

The second major protein of bovine casein is β-casein which represents 30% of total casein fraction (Whitney et al., 1976). Figure 54 shows the elution profiles of β-casein before and after histidine modification with DEP. Compared to 13.6% of unbound control β-casein, 54.7% was unbound after histidine modification (Table 13). This indicates that even though 4.4 histidine residues were modified out of the total 5 histidine residues per mole β-casein (Whitney et al, 1976), 45.3% of protein applied was bound to the copper ion and subsequently eluted with 0.01 M imidazole solution. The reason for this high binding rate after histidine modification compared to other casein fractions (Table 13) may be due to the temperature-dependent association-dissociation properties of β-casein.

3. κ-casein

κ-casein which represents 12.5% of the total casein in cow's milk occurs in the form of a mixture of aggregates of κ-caseins held together by intermolecular disulfide bonds (Whitney et al, 1976). κ-Casein prepared by the method of Zittle and Custer (1963) was considered to assume an aggregated form. Figure 55A shows the elution behaviour on Cu-chelate agarose of this preparation with and without histidine modification. The amount of unbound κ-casein for control and histidine modified κ-casein were 87.9% and 94.3% respectively (Table 13). This indicated that the amount of κ-casein adsorbed on Cu-chelate and recovered

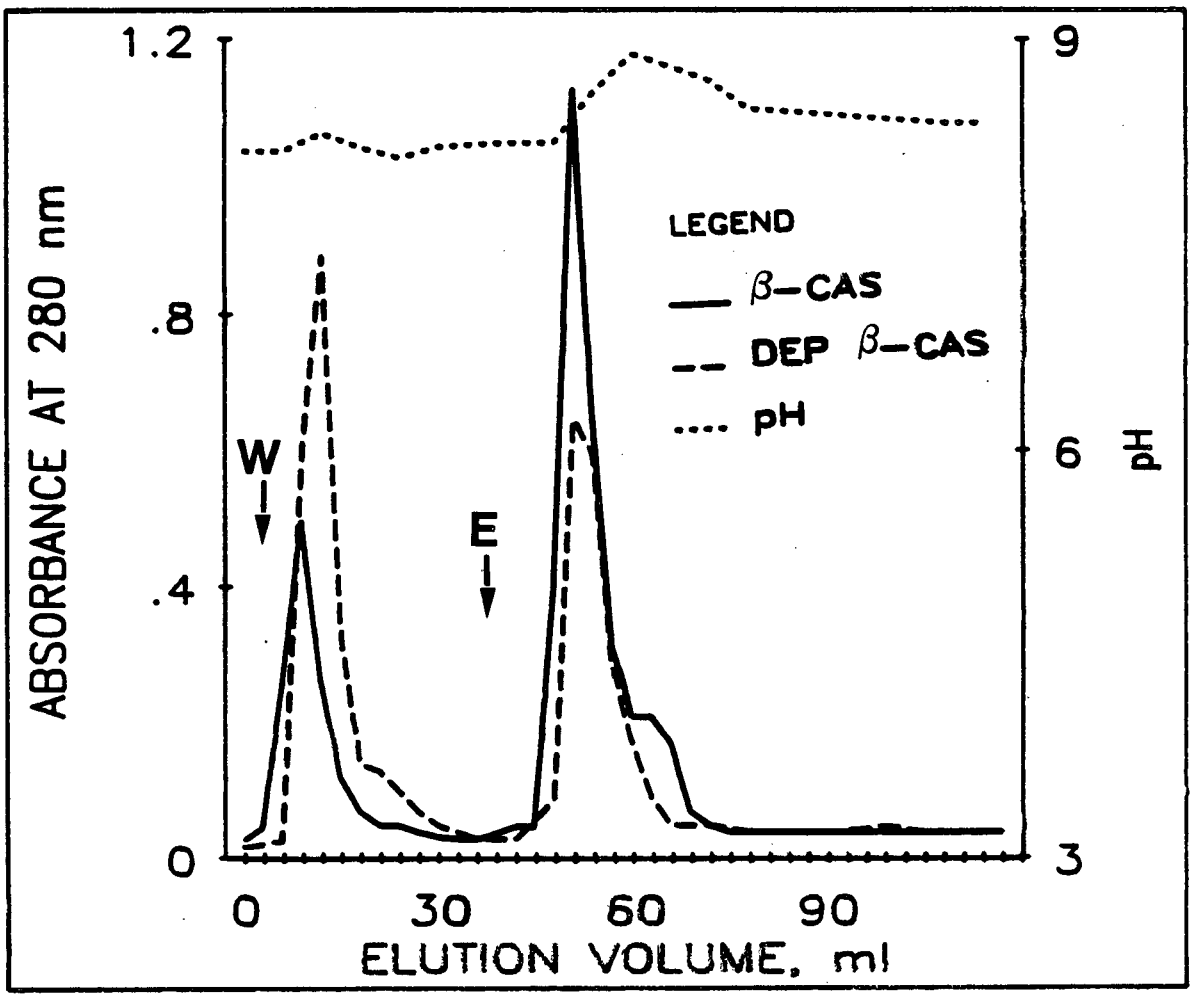


Figure 54. Metal chelate interaction chromatography of β-casein before (β-CAS) and after diethylpyrocarbonate modification (DEP β-CAS). See Figure 52 for conditions of separation.

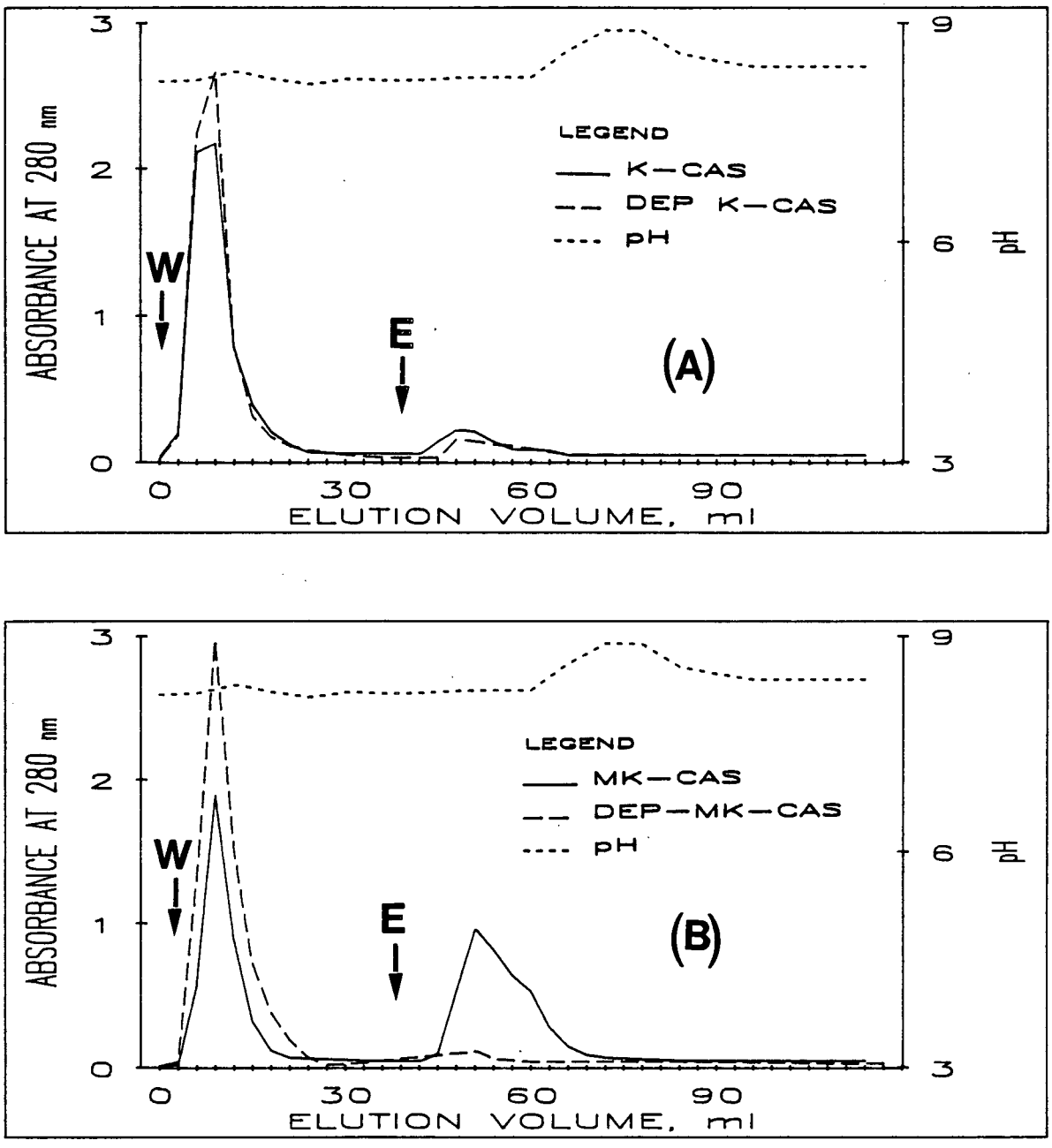


Figure 55. Metal chelate interaction chromatography of (A) aggregated κ-casein (κ-CAS) (B) monomer κ-caseins (MK-CAS) before and after diethylpyrocarbonate modification (DEP κ-CAS). See Figure 52 for separation conditions.

by 0.01 M imidazole was quite low for both control κ-casein and histidine modified κ-casein. This behavior of aggregated κ-casein on the MCIC column may be due to its aggregated structure which probably restricted the access to metal ions. Whether the aggregated structure of κ-casein blocked the copper-κ-casein interaction was determined. Figure 55B shows the elution profiles of κ-casein monomers formed by reducing the disulfide bonds and blocking them with sodium tetrathionate. The amount of bound protein for reduced κ-casein (MK-CAS) was more than 61% of the total protein applied compared to 12.1% of aggregated κ-casein adsorbed onto the same column (Figure 55B). Modification of 2.7 histidine residues out of 3 histidine residues per mole monomer κ-casein (Whitney et al., 1976) decreased the amount of bound modified κ-casein to 1.1% indicating the involvement of histidine residues in the interaction of κ-casein with copper ions. This suggests that disulfide bonds of the aggregated κ-casein had little or no effect on the interaction with metal ions.

In conclusion, use of skimmilk directly as a starting material for Ig and lactoferrin separation is feasible. Using Tris-acetate buffer as an equilibrating buffer induced competition between Ig and caseins to bind copper ions and decreased the capacity of the MCIC column in binding of immunoglobulins. However, using phosphate buffer as an equilibrating buffer prevented phosphoproteins from binding to the column and allowed them to be collected in the unbound fraction while immunoglobulins and lactoferrin are retained on the column.

Chemical modification studies with Tris-acetate buffer equilibrated MCIC columns, indicated the involvement of histidyl residues of some casein fractions in the interaction with copper ions.

PART V

SEPARATION OF IMMUNOGLOBULINS AND TRANSFERRIN FROM BLOOD SERUM AND PLASMA BY METAL CHELATE INTERACTION CHROMATOGRAPHY

It was reported that in 1982, 800,000 tonnes of blood were dumped into sewage systems throughout Europe. This is equivalent to 140,000 tonnes of potentially valuable protein that was literally flushed down the drain (Alexander, 1984). If one could extract these proteins they could be used for food processing and animal feeding. However, most of the available methods involve selective precipitation methods which are batch processes and thus difficult to mechanize. The possibility of using MCIC method to extract immunoglobulins and transferrin from blood plasma and serum was assessed in this part of the thesis.

A. METAL CHELATE INTERACTION CHROMATOGRAPHY

1. Blood serum on Cu-loaded MCIC

When blood serum (obtained by incubating blood samples overnight at 5°C) in 0.05 M Tris-acetate/0.5 M NaCl buffer, pH 8.2, was applied to a Cu-loaded IDA-BGE Sepharose 6B column (Figure 56), the major portion of the blood proteins (mainly albumin) did not bind and were recovered when the column was washed with the starting buffer. The bound proteins were eluted with the pH gradient buffer (F1) and identified to be mainly immunoglobulins. Subsequent elution with 10 mM imidazole (F2) recovered the major portion of transferrin (Figure 57; lanes 5, 6 and 7). Fraction 1 gave a long arc by immunoelectrophoresis which was similar to that of standard IgG, while fraction 2 yielded arcs around the well similar to that of standard transferrin and immunoglobulins (Figure 58).

2. Blood serum on MCIC columns loaded with other metal ions

Figure 59 represents the elution patterns of blood serum from columns packed with Zn-, Ni- and Co-loaded IDA-BGE Sepharose 6B. The capacity of Zn-loaded column to adsorb protein was found to be higher than that of Ni- and

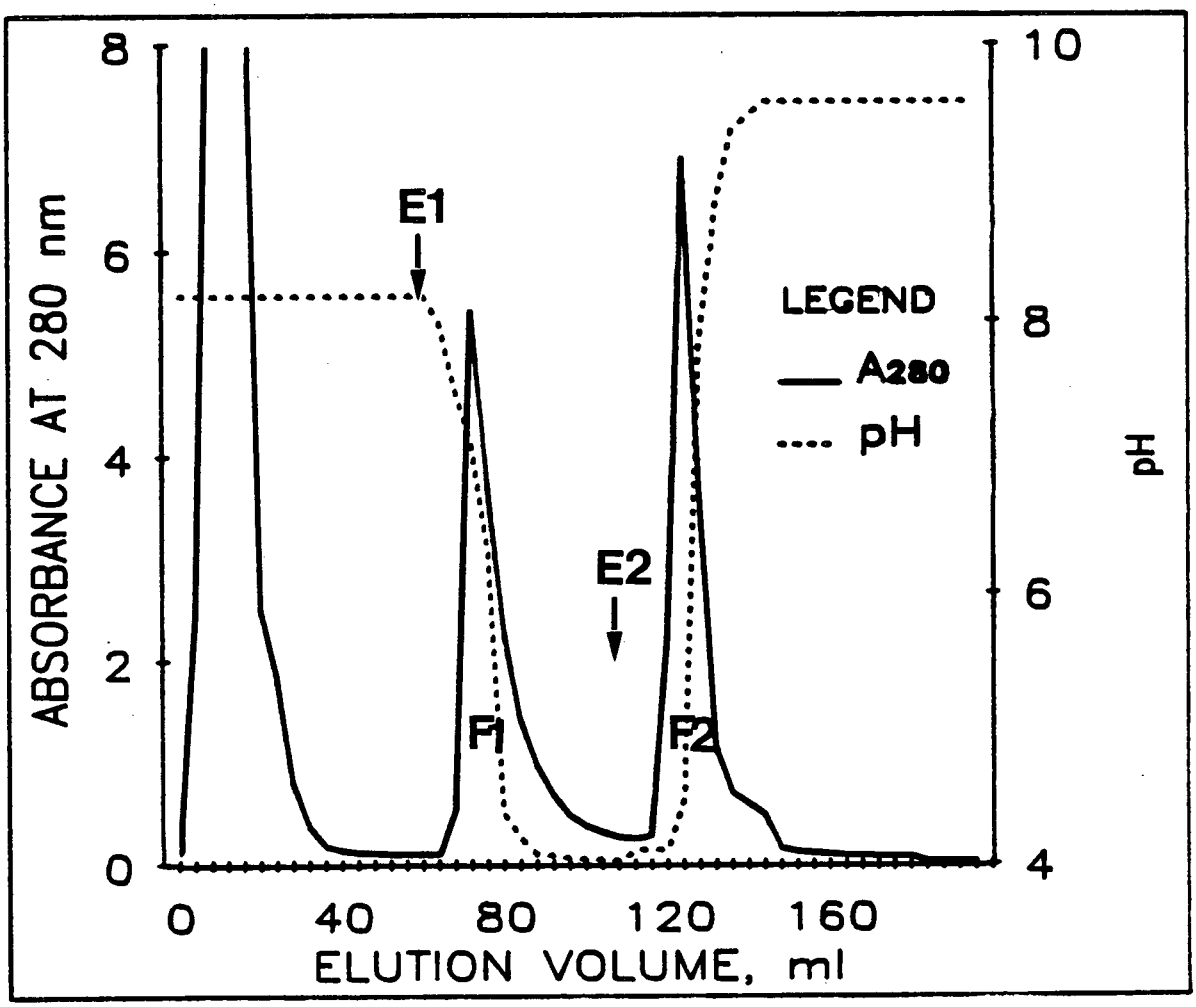


Figure 56. Immobilized copper affinity chromatography of blood serum. Blood serum (1 g in 10 mL 0.05 M Tris-acetate/0.5 M NaCl, pH 8.2) was applied to the column (1.4 x 7 cm). The column was washed with the starting buffer and then eluted with E1, 0.05 M Tris-acetate 0.5 M NaCl, pH 4.0, and with E2, 0.1 M imidazole. The flow rate was 30 mL/hr. Fl and Fz are fractions obtained.

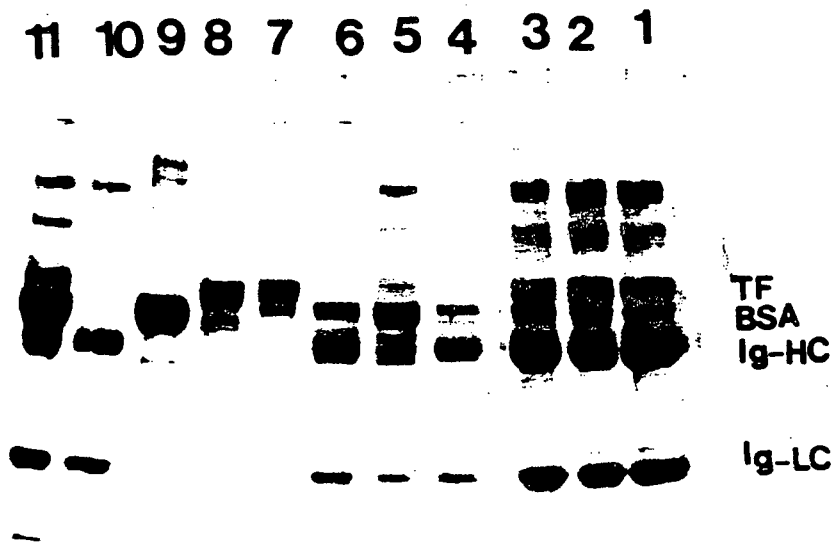


Figure 57. SDS-PAGE profiles of blood fractions from MCIC on Sepharose 6B column. Sample identification: Lanes 1, 2 and 3 are F1 of Figure 59 from Zn, Ni and Co loaded columns respectively; Lane 4, plasma protein eluted from Cu-loaded column with pH 4 buffer; Lanes 5, 6 and 7 are unbound, F1, and F2 in Figure 56, respectively; Lanes 8, 9 and 10 are standard transferrin(TF), bovine serum albumin (BSA), and immunoglobulins (Ig) respectively; Lane 11, blood plasma.

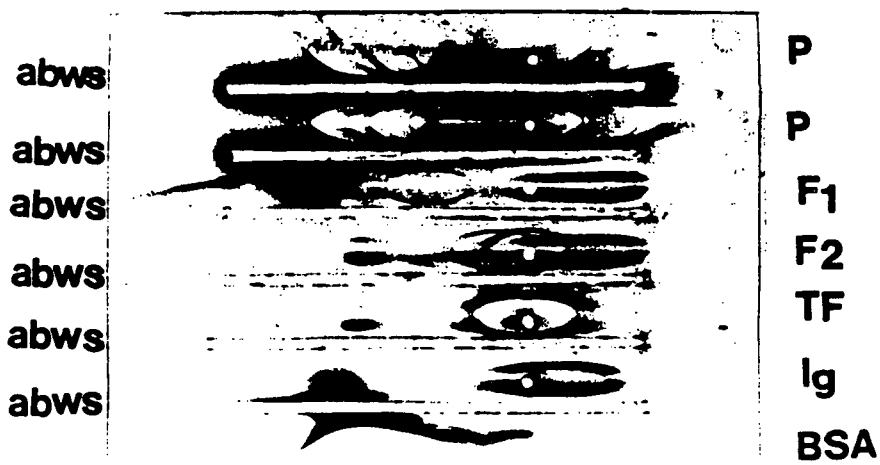


Figure 58. Immunoelectrophoresis of fractions obtained in Figure 56. P, blood plasma; F1 and F2 fractions obtained in Figure 56; TF, transferrin; Ig, immunoglobulins, BSA, bovine serum albumin; abws, rabbit antibovine whole serum.

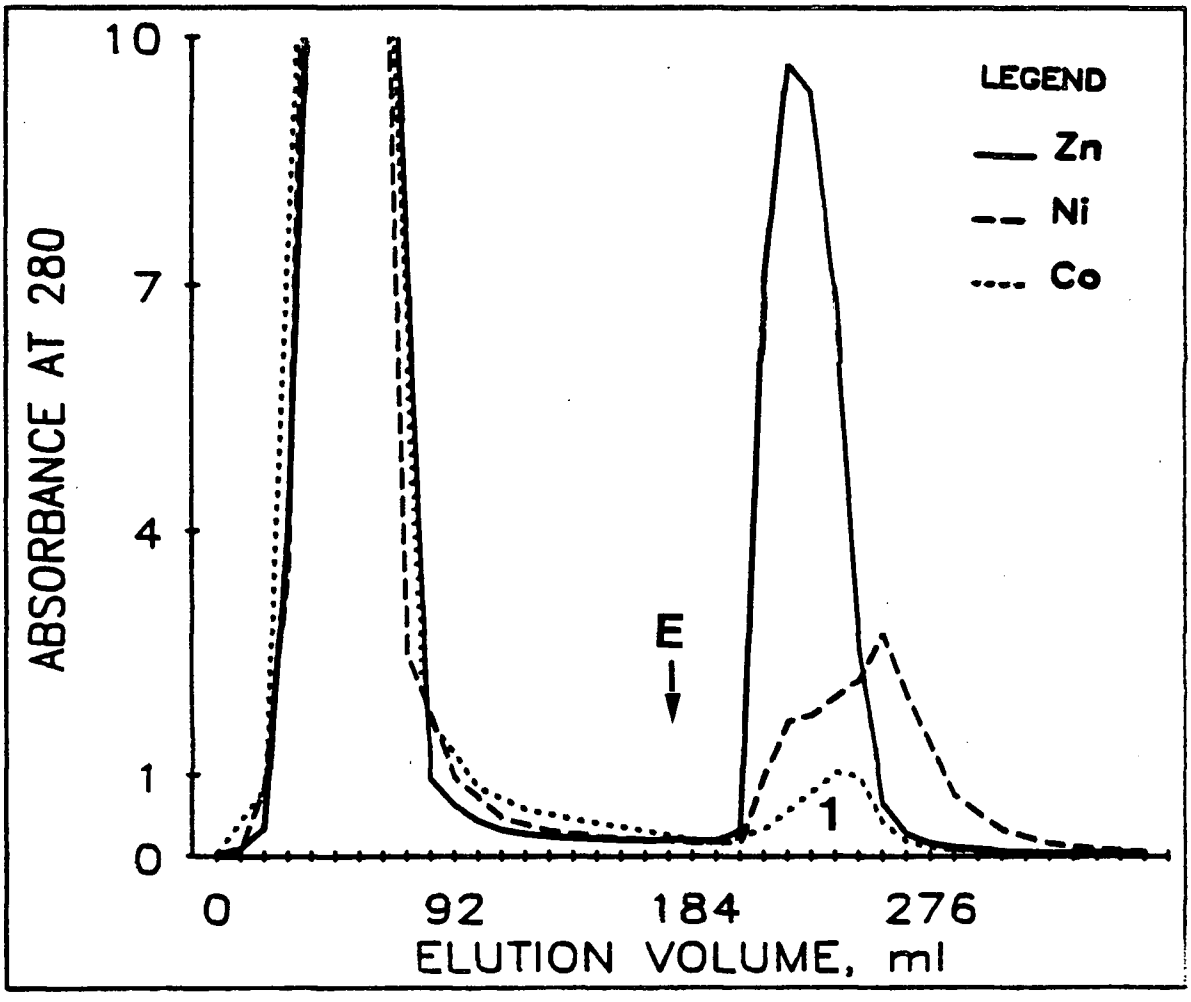


Figure 59. Immobilized Zn-, Ni- and Co- affinity chromatography of blood serum. Blood serum (2 g in 20 mL 0.05 M Tris-HCl/0.15 M NaCl, pH 8.0) was applied to the column (2.8 x 8.5 cm). The column was washed with the starting buffer then eluted (E) with 0.1 M Na-acetate/0.8 M NaCl, pH 4.6. The flow rate was 30 mL/h. 1, is fraction obtained.

Co-loaded columns. F1 fraction obtained from these columns are compared in Figure 57 (lanes 1, 2 and 3). They are composed of Igs, TF and other high molecular weight proteins and appear to contain a small amount of albumin.

3. Blood plasma on MCIC column

The plasma supplied had a red hue which was probably due to the partial hemolysis of red blood cells during centrifugation of blood after addition of sodium citrate as anticoagulant. When blood plasma in 0.05 M Tris-acetate buffer, pH 8.2, was applied to a Cu-loaded IDA-BGE Sepharose 6B, the major portion of the proteins eluted with 0.05 M acetate-Tris buffer at pH 4.0 were Igs as indicated by SDS-PAGE, R.I.D. and ELISA analysis (with more than 95% biological activity). The upper part of the Cu-loaded column, however, became strongly reddish. To identify this colored material, standard bovine hemoglobin was applied to the column. Figure 60 shows the elution patterns of hemoglobin from Zn-, Ni-, Co- and Cu-loaded IDA-BGE Sepharose 6B columns. It was found that 0.1 M acetate/0.8 M NaCl buffer, pH 4.5, 0.1 M acetic acid/.5 M NaCl, pH 2.8, or 0.01 M imidazole were not effective for eluting the adsorbed hemoglobin from Cu-loaded IDA-BGE Sepharose 6B; however, elution with 50% ethanol was effective for hemoglobin removal. It is interesting to note that chicken hemoglobin bound to a copper-loaded column was eluted quite easily with acidic buffers (our unpublished data) which may indicate structural differences between chicken and bovine hemoglobins. Figure 60 also shows the behavior of hemoglobin towards other metal ions, zinc, cobalt and nickel ions. It was found that hemoglobin was readily eluted by using 0.1 M acetate/0.8 M NaCl buffer, pH 4.5, from the columns loaded with metals other than Cu. These results indicated that hemoglobin-metal interactions are dependent on the kind of metal ions and the source of hemoglobin.

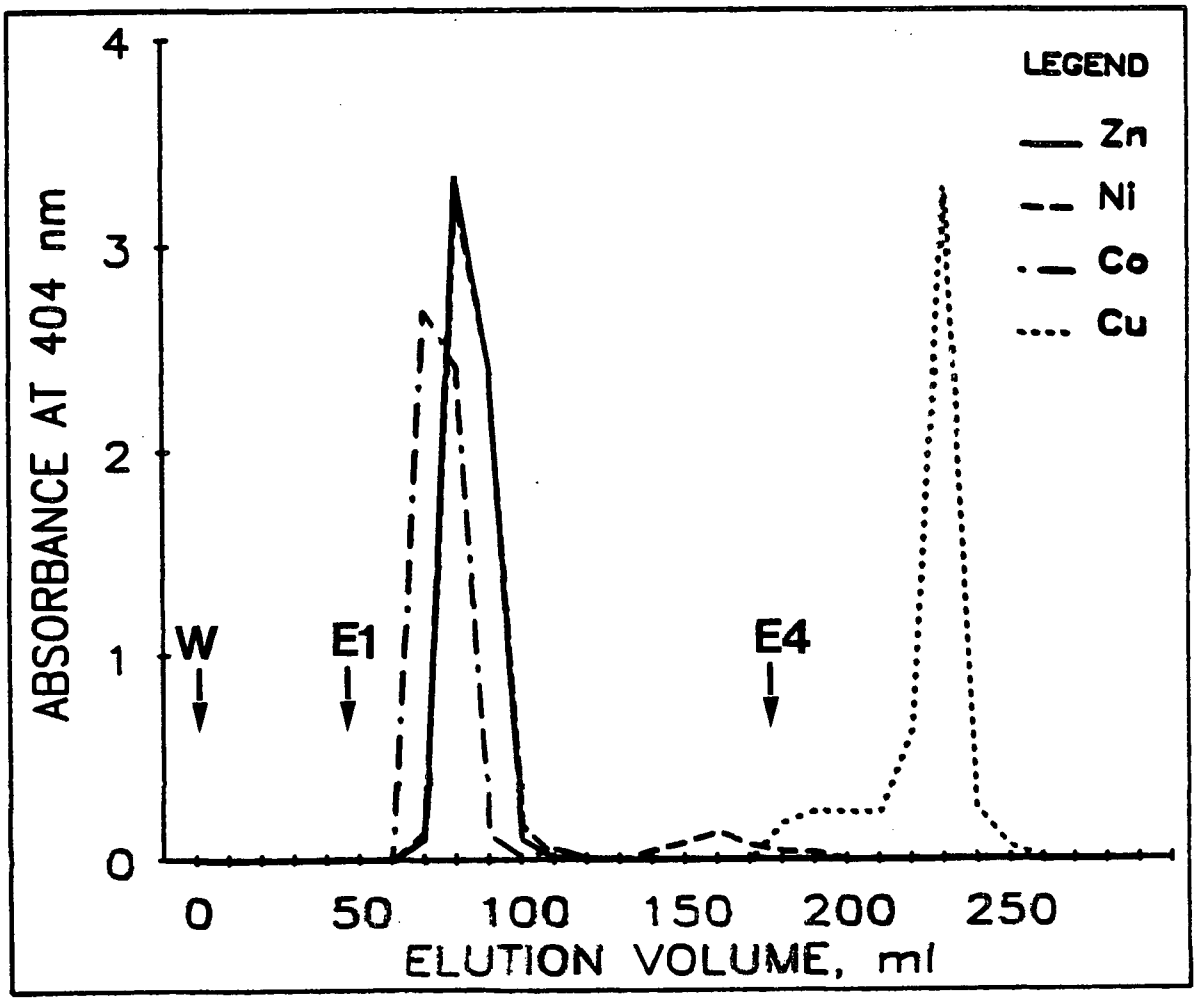


Figure 60. Elution profiles of adsorbed hemoglobin from MCIC columns (1.4 x 7.0 cm) loaded with Zn, Ni, Co and Cu. 2 mL of hemoglobin (3 mg/mL in 0.05 M Tris-HCl/0.15 M NH_4Cl, pH 8.0) was applied to the column and washed (W) with 2-3 times bed volumes of the starting buffer. E1, 0.1 M Na-acetate /0.8 M NaCl, pH 4.5; E4, 50% ethanol.

B. IMMUNOCHEMICAL ASSAYS

Table 14 compares the IgG contents of different fractions obtained from MCIC columns loaded with different metal ions. The copper-loaded column gave the highest IgG purity in F1 (Figure 56). However, F2 (Figure 56) also contained some IgG which was evident in the immunoelectrophoretogram (Figure 58). This indicated that not all IgG was eluted at pH 4.0 (Figure 56); some IgG subclasses (IgG_1, IgG_2) may bind more strongly than others. By comparing Zn-, Ni-, and Co-loaded columns, F1 obtained from the Ni-loaded column gave the highest IgG purity while the Zn-loaded column gave the lowest IgG content. Even though Co-loaded column gave high IgG purity it is not recommended for the isolation of Igs since its capacity to bind Igs was low and decreased with repeated use.

C. BACTERIOSTATIC ACTIVITY OF BLOOD IMMUNOGLOBULINS AND TRANSFERRIN

Figure 61 shows that immunoglobulins and transferrin isolated by MCIC method had inhibitory effects on the growth of E. coli during the first 3 hr as compared to the control. This results were in agreement with that reported by Stephens et al. (1980) who found that the bacteriostatic activity of IgG_1 against E. coli could be enhanced by addition of lactoferrin isolated from human milk. The inhibitory effect of transferrin alone was higher than that of Igs. However, mixing Igs with TF may have a synergistic effect on the inhibition of E. coli. Transferrin, by virtue of its high affinity for iron, can retard microbial growth by making this element relatively unavailable (Harrison, 1985).

D. ANTI-LIPOPOLYSACCHARIDE ACTIVITY OF BLOOD IMMUNOGLOBULINS

It is well known that antibodies are considered to be the architecture of the immune system (Packard, 1982). Human and animals are defenseless without

Table 14. IgG contents* of blood serum or plasma fractions obtained from MCIC column loaded with different metal ions.

Fraction***	Protein (mg/mL)**	IgG (mg/mL)	%IgG
Cu (Figure 56 F1)	26.5	26.0	98.1
Cu (Figure 56 F2)	23.3	10.2	43.8
Cu (plasma)	26.0	25.0	96.2
Zn (Figure 59 F1)	21.6	5.0	23.2
Ni (Figure 59 F1)	24.6	20.0	81.3
Co (Figure 59 F1)	25.2	20.0	79.4

* Radial immunodiffusion was used for the determination.

** Bio-Rad Protein Assay Kit (Bio-Rad Laboratories, Mississauga, Ont) was used for the determination.

*** Serum fraction unless otherwise noted.

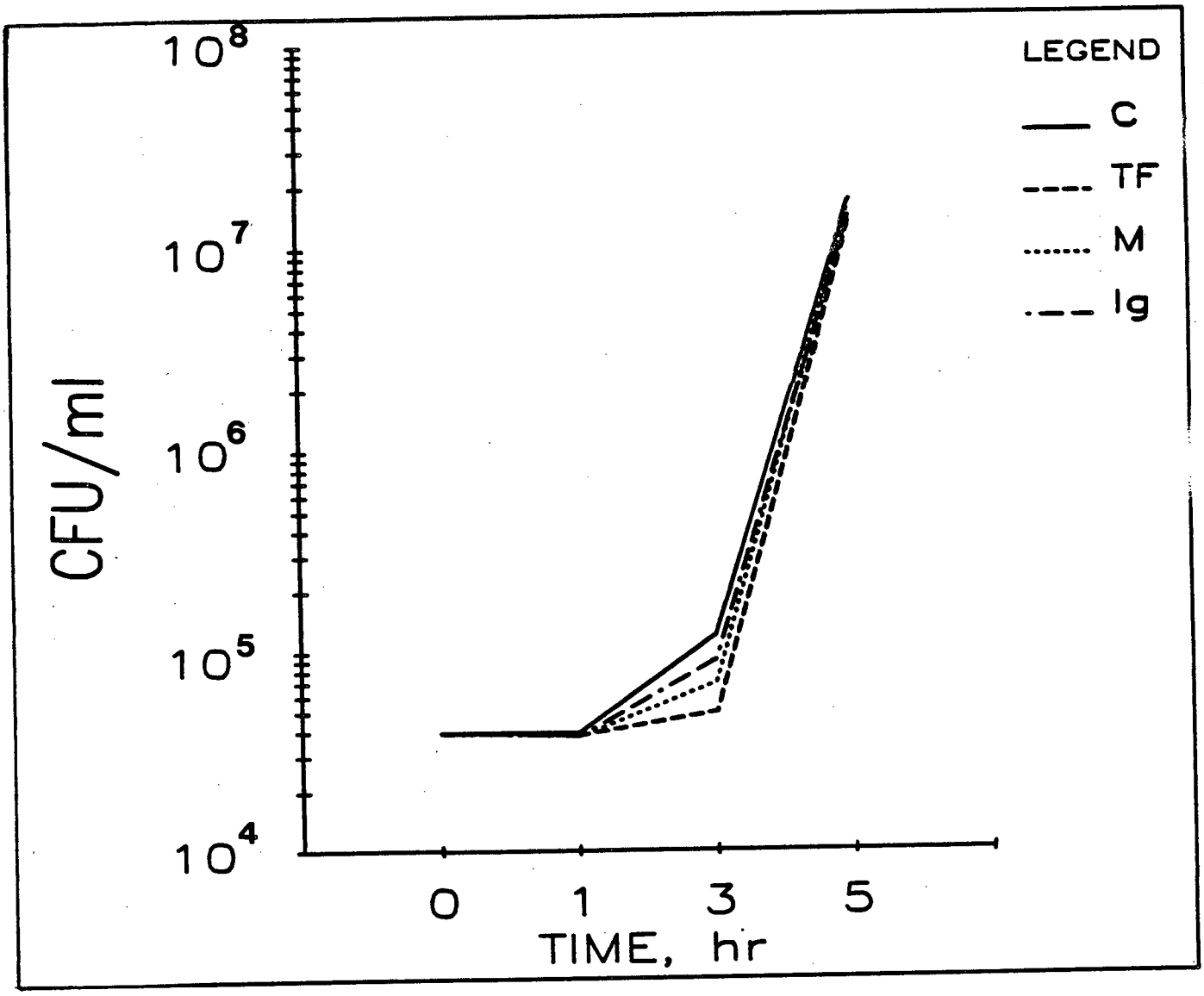


Figure 61. Bacteriostatic activity of isolated immunoglobulins and transferrin against E. coli. C, control; TF, transferrin (10 mg/mL); M, mixture of TF (5 mg/mL) and Ig (5 mg/mL); Ig, immunoglobulins (10 mg/mL); CFU, cell forming unit .

them. The key characteristics of the immune system are specificity, memory and ability to recognize foreign bodies. Figure 62 shows the activity of bovine IgG isolated from blood by MCIC method toward LPS extracted from E. coli, S. typhimurium and B. parapertussis. Blood IgG recognition and binding to LPS from S. typhimurium and E. coli may indicate that dairy cows had experienced these bacterial infections, however, IgG binding to B. parapertussis which causes whopping cough in infants may indicate similarities in the surface exposed antigens to those of Enterobacteriaceae family. The binding of blood IgG with these antigens may interfere with some of the physiological activity or may change the adhesion properties of these bacteria with the intestinal surface and thus prevent, to some extent, the infection caused by these bacteria (Packard, 1982).

E. CAPACITY OF MCIC COLUMN FOR TRANSFERRIN

Figure 63 shows the A_{280} elution profile from the application of 0.2% transferrin solution to a copper loaded chelating Sepharose 6B column. Saturation of the column was reached at an elution volume of about 184 mL indicating no further protein was adsorbed. At this point, the capacity for TF was calculated to be approximately 167 mg/mL copper-loaded gel. Thus, the binding capacity for bovine TF appears to be higher than that reported by Lonnerdal et al. (1977) for human lactoferrin, which was 70 mg LF/mL gel containing 50 µmole copper ions. Subsequent elution with buffer at pH 4.0 recovered only 15% of the applied protein; however, the residual TF was recovered by using 0.01 M imidazole as an eluent. This behavior of TF on MCIC column may indicate the biphasic nature of TF. One phase was eluted simply by protonation (acidic pH) and the second phase was eluted by using a stronger eluent.

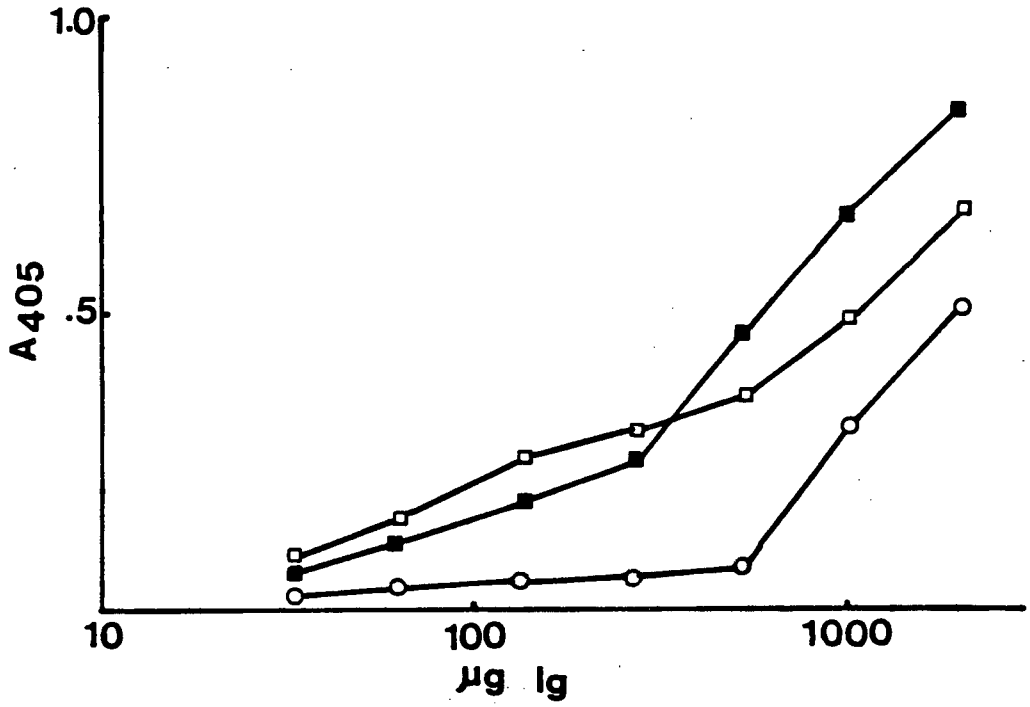


Figure 62. Anti-lipopolysaccharide activity of blood IgG isolated by metal chelate interaction chromatography method. ■—■, E. coli LPS; □—□, S. typhimurium LPS; ○—○, B. parapertussis LPS.

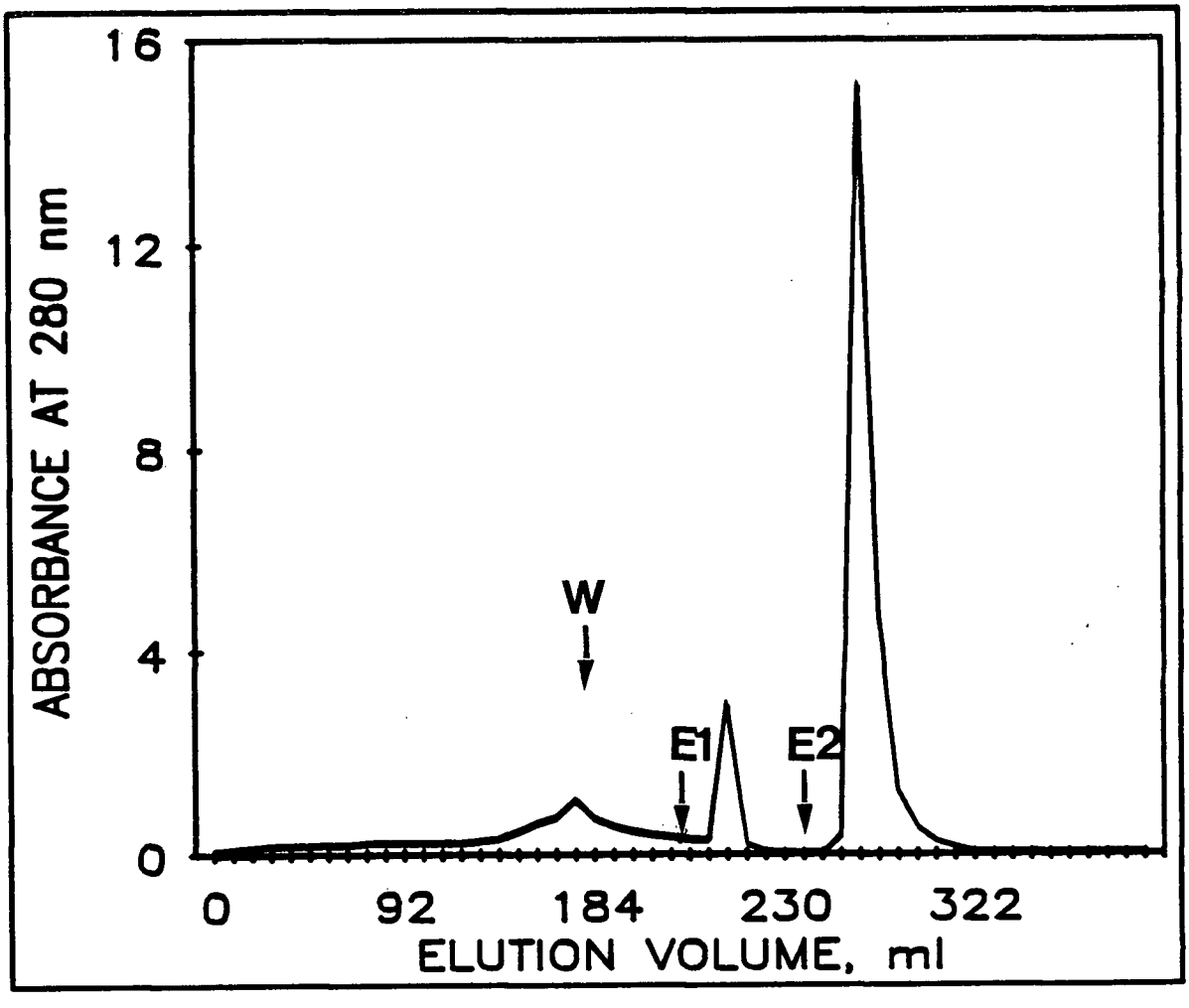


Figure 63. Saturation point of adsorption of standard TF on Cu-loaded IDA-BGE Sepharose 6B. 0.2% TF was passed through 10 mL column (7 x 1.4 cm) equilibrated with 0.05 M Tris-acetate containing 0.5 M NaCl, pH 8.2, W, wash with starting buffer, E1, elution with 0.05M Tris-acetate containing 0.5M Nacl, pH 4.0; E2, elution with 0.01 M imidazole.

F. MECHANISM OF PROTEIN-METAL INTERACTION

Porath et al., in their pioneering work (1975), postulated that histidine, cysteine and tryptophan residues of a protein were most likely to form stable coordination bonds with metal ions at a neutral or alkaline pH. However, there have been no detailed data published on the role of these amino acid residues in the interaction with metal ions. Figure 64 shows the elution profile of standard bovine transferrin on Cu-loaded column before and after modification of histidine groups with DEP. By washing the column, with three bed volumes of the starting alkaline buffer, no TF bound to the column was eluted; the bound TF was eluted with 0.01 M imidazole. Modification of 16.6 histidine residues out of the total 18 residues per mole TF (Sutton and Jamieson, 1972) almost completely inhibited the interaction of the protein with metal ion. Almost all of the protein introduced into the column was restored in the washing buffer without binding to the column. The decreased binding of histidine-modified TF with copper ion would clearly indicate the importance of histidine residues in the coordination binding with the metal ions. Similar behavior was observed when histidine-modified immunoglobulins were passed through Cu-chelate gel. These results would support the theory of Porath et al. (1975).

These studies demonstrate that immunoglobulins can be isolated from blood serum and plasma. Radial immunodiffusion analysis indicated that Cu-loaded column yielded the highest IgG activity (higher than 95%), indicating the mildness of this method for isolation of Igs. However, it is recommended to regenerate the Cu-loaded column with 50% ethanol when blood plasma is used as starting material.

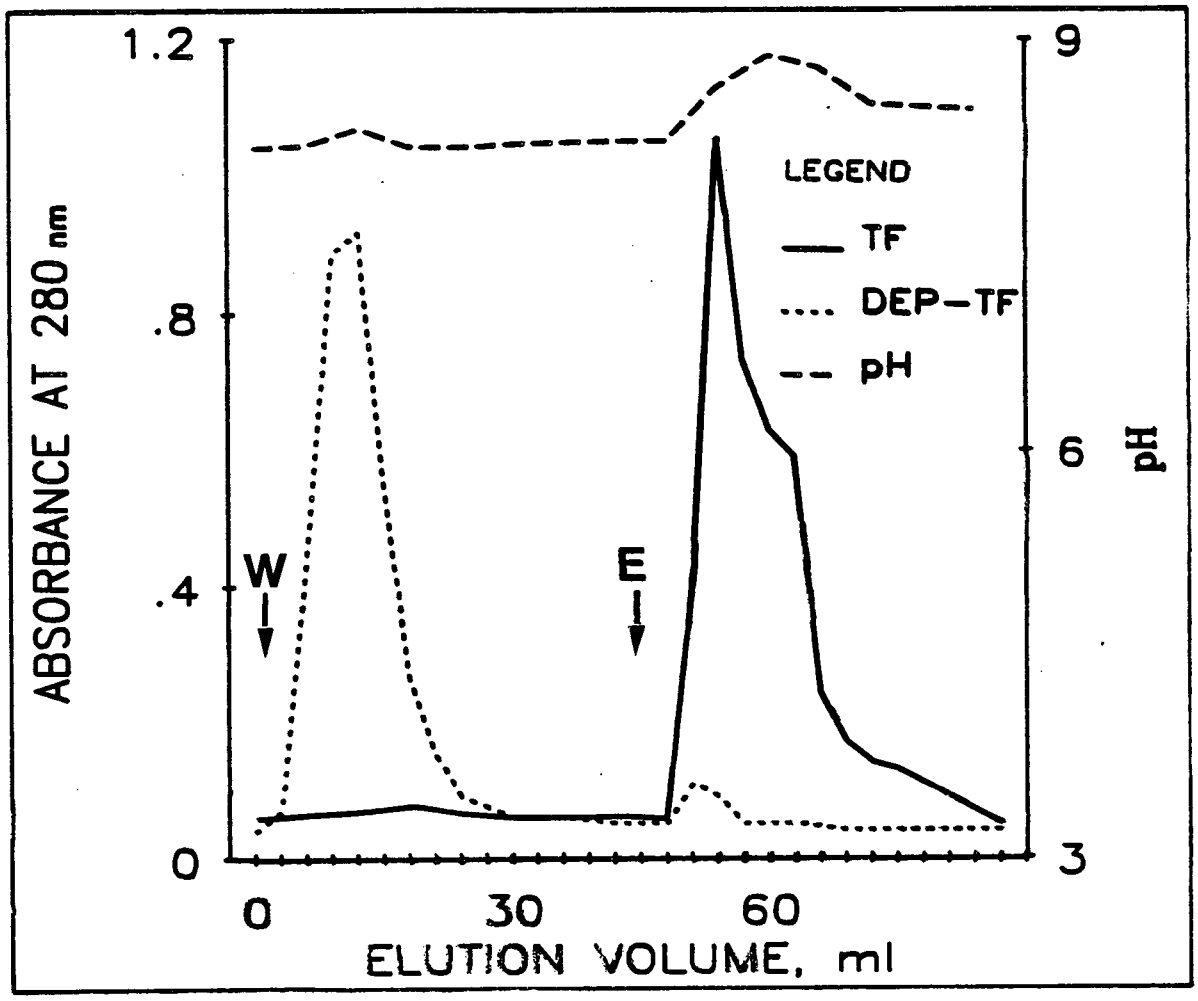


Figure 64. Elution profiles of control (TF) and diethyl pyrocarbonate treated transferrin (DEP-TF). Samples (30 mg/5 mL 0.05 M Tris-acetate/0.5 M NaCl, pH 8.2) were applied to the column (1.4 x 7.0 cm) and washed (W) with the starting buffer then eluted (E) with 0.01 M imidazole. The flow rate was 30 mL/hr.

PART VI

SEPARATION OF OVOTRANSFERRIN FROM EGG WHITE BY METAL CHELATE INTERACTION CHROMATOGRAPHY

The greater resistance against enterobacterial infection of human infants fed with breast milk than those fed with artificial formula has been attributed, to a great extent, to the presence of a large quantity of lactoferrin in human milk compared to cow's milk (Packard, 1982). The similarity in structure and biological activity between ovotransferrin and lactoferrin justify the antimicrobial effect of ovotransferrin being added to infant formula (Valenti et al., 1983; Giacco-Del et al., 1985). In addition, it has been found that ovotransferrin does not sensitize infants (Giacco-Del et al., 1985). This part ofthe thesis deals with the separation and the mechanism of the binding of ovotransferrin by using MCIC method.

A. METAL CHELATE-INTERACTION CHROMATOGRAPHY OF EGG WHITE

It is known that the transition metals can form a complex with compounds rich in electrons (Porath et al., 1975). These compounds may be aromatic or heterocyclic, including proteins due to their contents of Cys, His, and Tyr. However, the binding of these groups to metal ions depends on the availability of these groups or the topography of the protein molecule (Sulkowski, 1985).

Figure 65 represents the elution profile of undiluted, blended egg white on copper-loaded Sepharose 6B. As indicated by SDS-PAGE (Figure 66), at an early stage the unbound material was mainly ovalbumin, however, at the later stage, most of egg white proteins passed through suggesting the column had reached its saturation point. During the washing step, another peak (FW) appeared in the eluant which, based on electrophoretic pattern, could be a mixture of ovotransferrin and lysozyme . The bound material eluted at pH 4.0 (peak 1) appeared to be pure ovotransferrin, purer than the commercial ovotransferrin. The protein eluted with 0.01M imidazole (peak 2), was likely to be ovotransferrin. Immunoelectrophoresis conducted against anti-egg white proteins antiserum

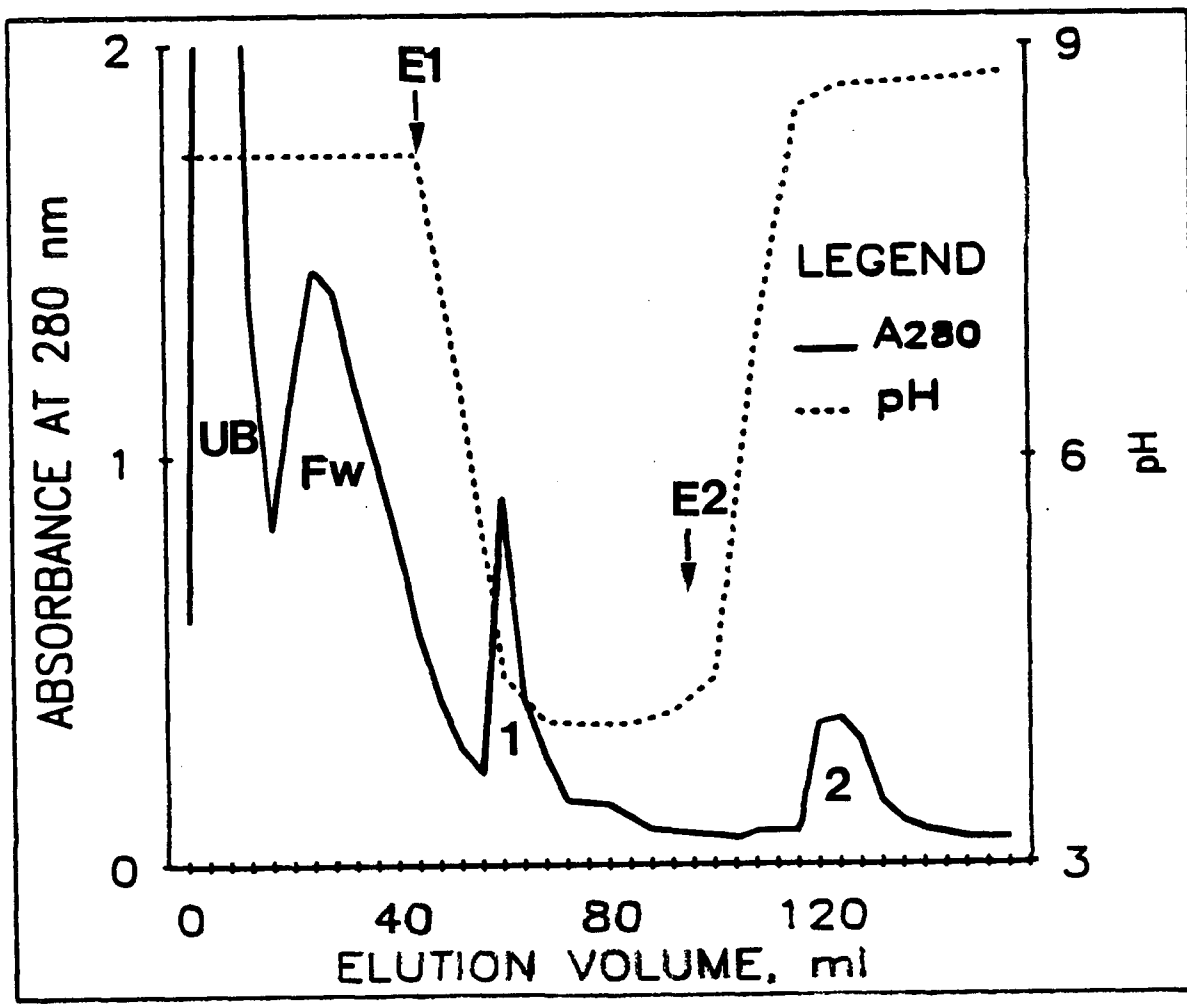


Figure 65. Metal chelate-interaction chromatography of egg white. 2 mL of undiluted blended egg white was passed through Cu-loaded Sepharose 6B MCIC column (7 x 1.4 cm). UB, unbound proteins; FW, fraction eluted with washing step; E1, elution with starting buffer, pH 4.0; 1, fraction eluted with E1; E2, elution with 0.01M imidazole; 2, fraction eluted with E2.

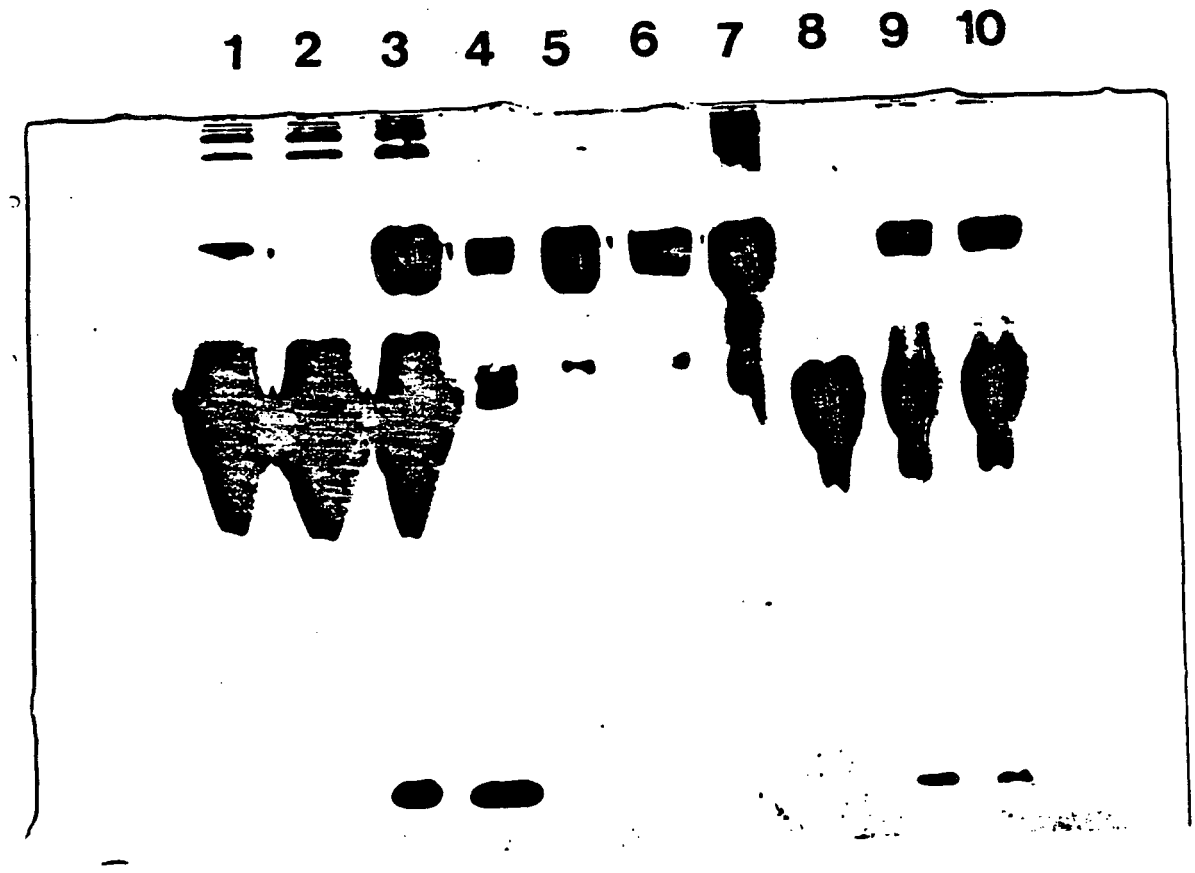


Figure 66. SDS-PAGE of fractions of egg white obtained by MCIC column shown in Figure 65. Lanes 1, 2 and 3, are unbound fractions; Lane 4, washing fraction; Lane 5, peak 1; Lane 6, peak 2; Lanes 7 and 8, standard ovotransferrin and ovalbumin, respectively; Lanes 9 and 10, control egg white.

indicated that the ovotransferrin fraction (peak 1) obtained by MCIC was fairly pure being free from contaminants which were observed in the commercial ovotransferrin (Figure 67).

B. CAPACITY OF MCIC COLUMN FOR OVOTRANSFERRIN

Figure 68 shows the A_{280} elution profile when 0.2% commercial OVT solution (A_{280} = 1.94) was applied to a copper-loaded chelating Sepharose 6B. The column was saturated at elution volume of about 60 mL when A_{280} of the eluted fraction reached 1.94. At this point, the capacity for OVT was calculated to be approximately 20 mg OVT/mL copper-loaded gel. Subsequent elution with buffer at pH 4.0 (E1) and with 0.01 M imidazole (E2) recovered 65% and 30% of the bound OVT, respectively. Thus, the binding capacity of OVT appeared to be lower than that reported by Lonnerdal et al. (1977) for human lactoferrin (LF), which was 70 mg LF/mL gel containing 50 µmol copper ions.

The desorption of OVT in two steps may indicate the presence of at least two forms of OVT. One form can be eluted by lowering the pH. The acidity weakens the binding of proteins with the metal ions by protonation of the protein electron donor groups which are responsible for binding with the metal ions. The second form can be eluted by using a strong competitor, i.e. imidazole. Imidazole can efficiently compete with exposed His groups on the protein for metal binding. As reported by Sulkowski (1985), the presence of one histidine residue is sufficient for retention while the presence of 2 or 3 His results in multipoint attachment to IDA-Cu gel and a stronger retention. The presence of two forms of conalbumin reported by Clark et al.(1963) and Feeney et al. (1963) was discussed by Powrie and Nakai (1986). Rogers et al. (1977) reported two forms of histidine in OVT, one was reactive and the other was DEP-non-reactive.

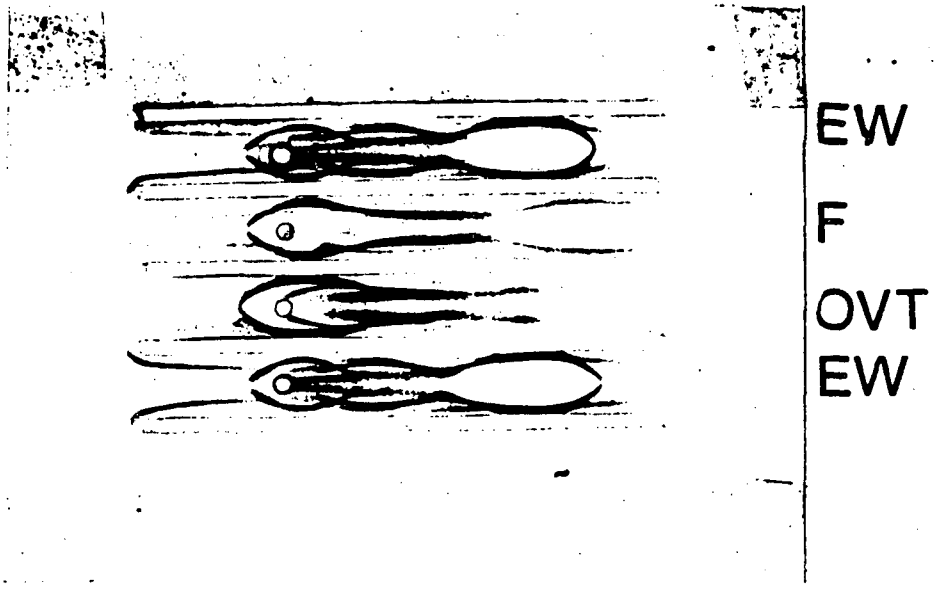


Figure 67. Immunoelectrophoresis against anti whole egg white antiserum of ovotransferrin fraction (F) prepared by the MCIC method as compared to commercial ovotransferrin (OVT), and egg white (EW).

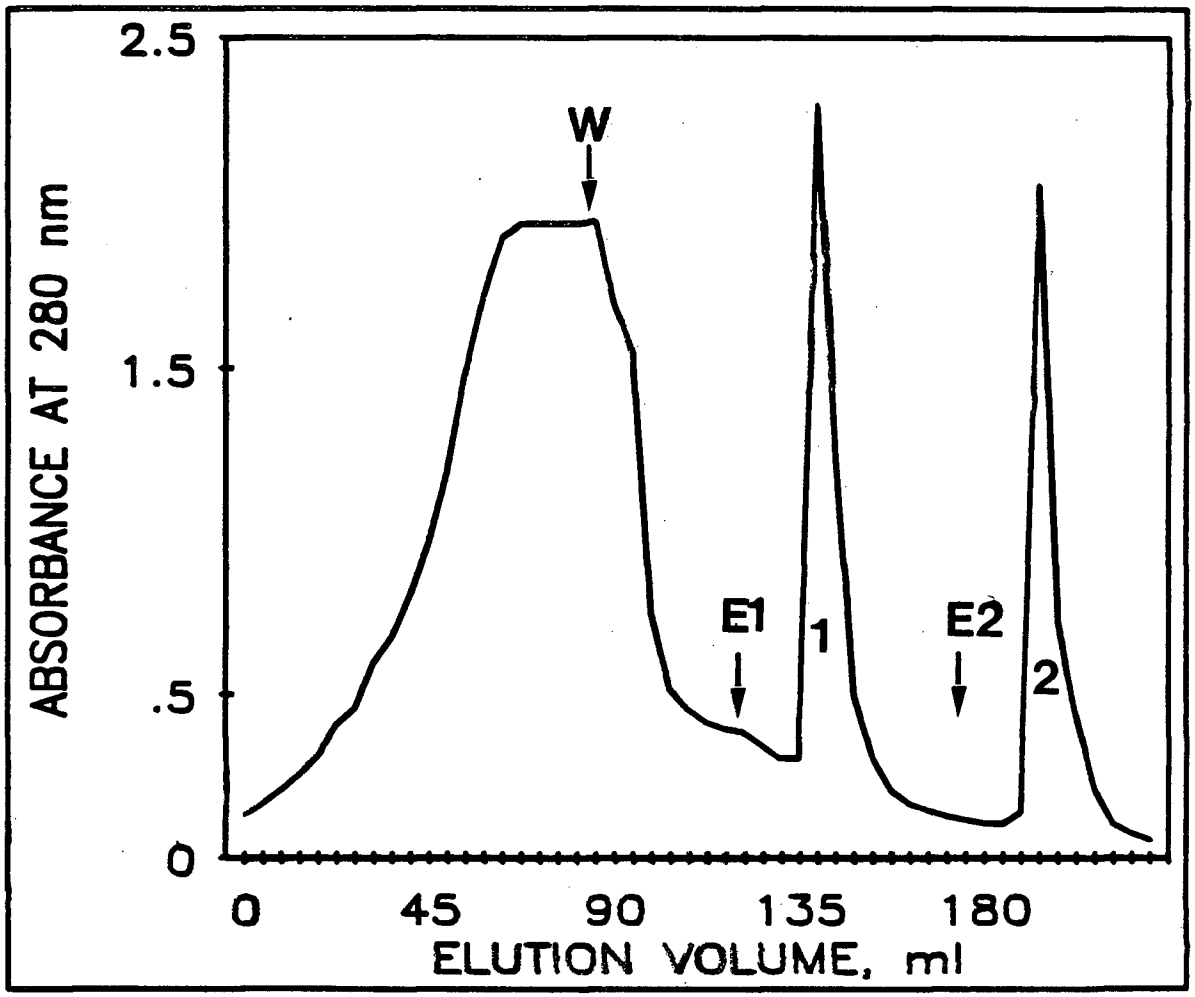


Figure 68. Saturation profile of commercial ovotransferrin on Cu-loaded Sepharose 6B column. 0.2% ovotransferrin was passed through 3 mL of a Cu-loaded column (7 x 1.4 cm). E1, elution with 0.05 M Tris-acetate /0.5 M NaCl, pH 4.0; E2, elution with 0.01 M imidazole. 1 and 2 are eluted ovotransferrin.

C. MECHANISM OF OVOTRANSFERRIN SEPARATION BY MCIC

The binding of OVT to metal-chelate gel is believed to be the result of the ability of electron-rich groups such as histidine and tryptophan to substitute weakly bonded water or buffer in the metal complex (Lonnerdal and Keen, 1982). The stability of the binding even in 1M NaCl would rule out the possibility of ionic interaction being the principal force in the interaction. To demonstrate whether the metal binding ability of OVT has any role in the mechanism of OVT separated by MCIC, metal bound OVT was applied to the MCIC column. Figure 69 represents the elution profile of metal free OVT (apo-OVT), ferric- and copper-saturated OVT from the MCIC column. Evidently, an iron- or copper- containing OVT was adsorbed on a Cu-chelate gel and subsequently eluted. Figure 69 also shows that metal-saturated OVT bound with MCIC column as strongly as the apo-form of OVT. Before and after chromatography of Fe-OVT and Cu-OVT, the protein contained two atoms of Fe^{3+} and Cu^{2+} per molecule, respectively (Lonnerdal and Keen, 1982). After addition of excess Fe^{3+} or Cu^{2+} and subsequent dialysis, OVT has two bound metal atoms per mole protein (Brock, 1985). However, on Cu-chelate gel, surface-exposed groups on OVT may have a tendency to bind copper ions.

To investigate if histidine groups were responsible for the copper-chelate gel and OVT interaction, DEP modified OVT was applied to the column. Figure 70 shows the elution profile of OVT before and after histidine group modification by DEP. Without modification, OVT was adsorbed strongly to the Cu-chelate gel and no OVT was eluted in the washing step at alkaline pH. Bound OVT could subsequently be recovered by 0.01M imidazole. Histidine modified OVT (DEP-OVT) did not bind to Cu-chelate gel and was almost completely washed out during the washing step at alkaline pH. This behavior of DEP-OVT on Cu-chelate gel clearly indicated that modification of 11.7 histidine residues out of 13 histidine

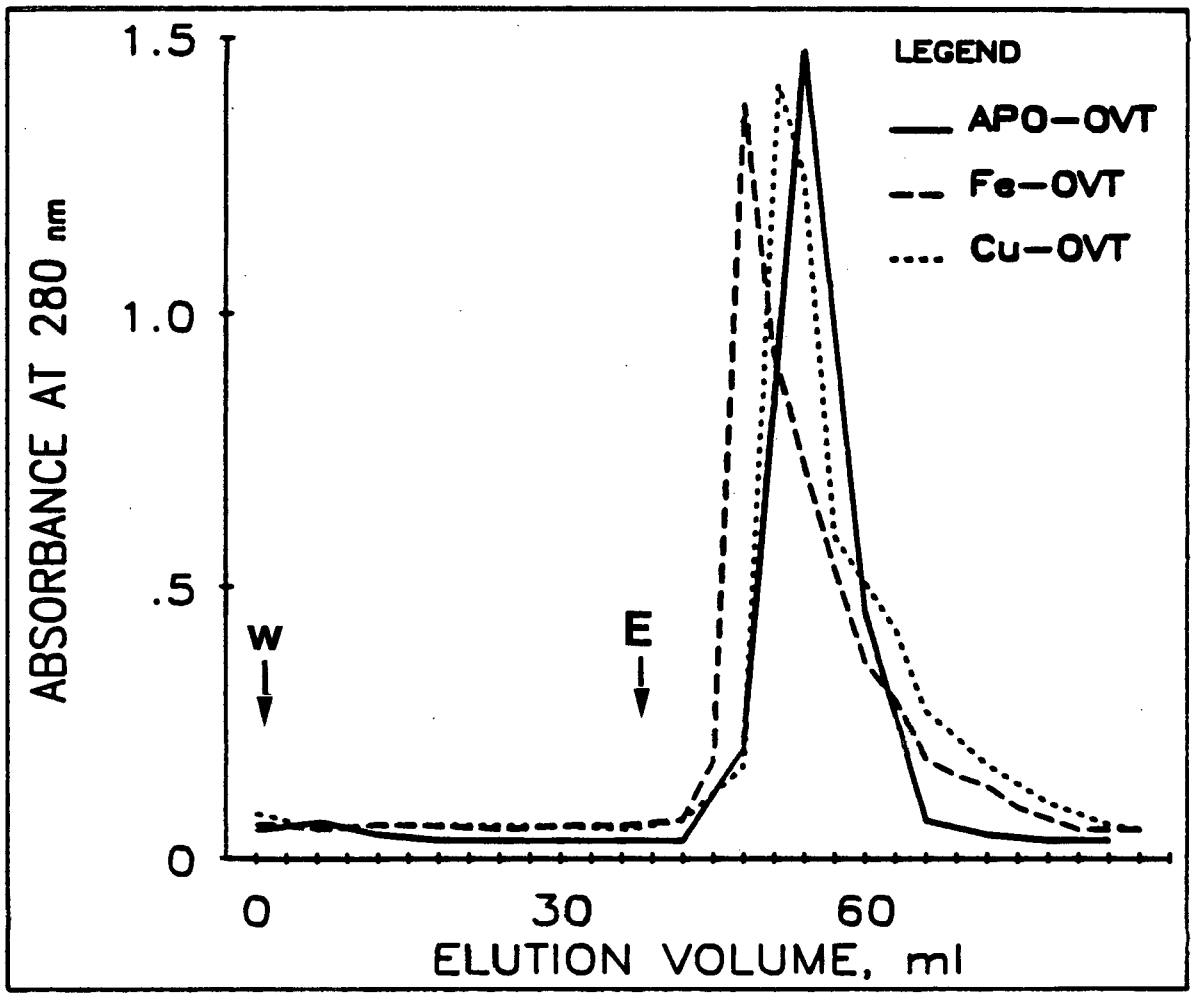


Figure 69. Metal chelate interaction chromatography of apo-ovotransferrin (APO-OVT), Fe-ovotransferrin (Fe-OVT) and Cu-ovotransferrin (Cu-OVT). 3 mL (8 mg/mL) was applied to Cu-loaded Sepharose 6B (7 x 1.4 cm) after equilibration with 0.05 M Tris-acetate/0.5 M NaCl, pH 8.2. W, Washing with the equilibrating buffer; E, elution with 0.01 M imidazole; flow rate was 30 mL/hr.

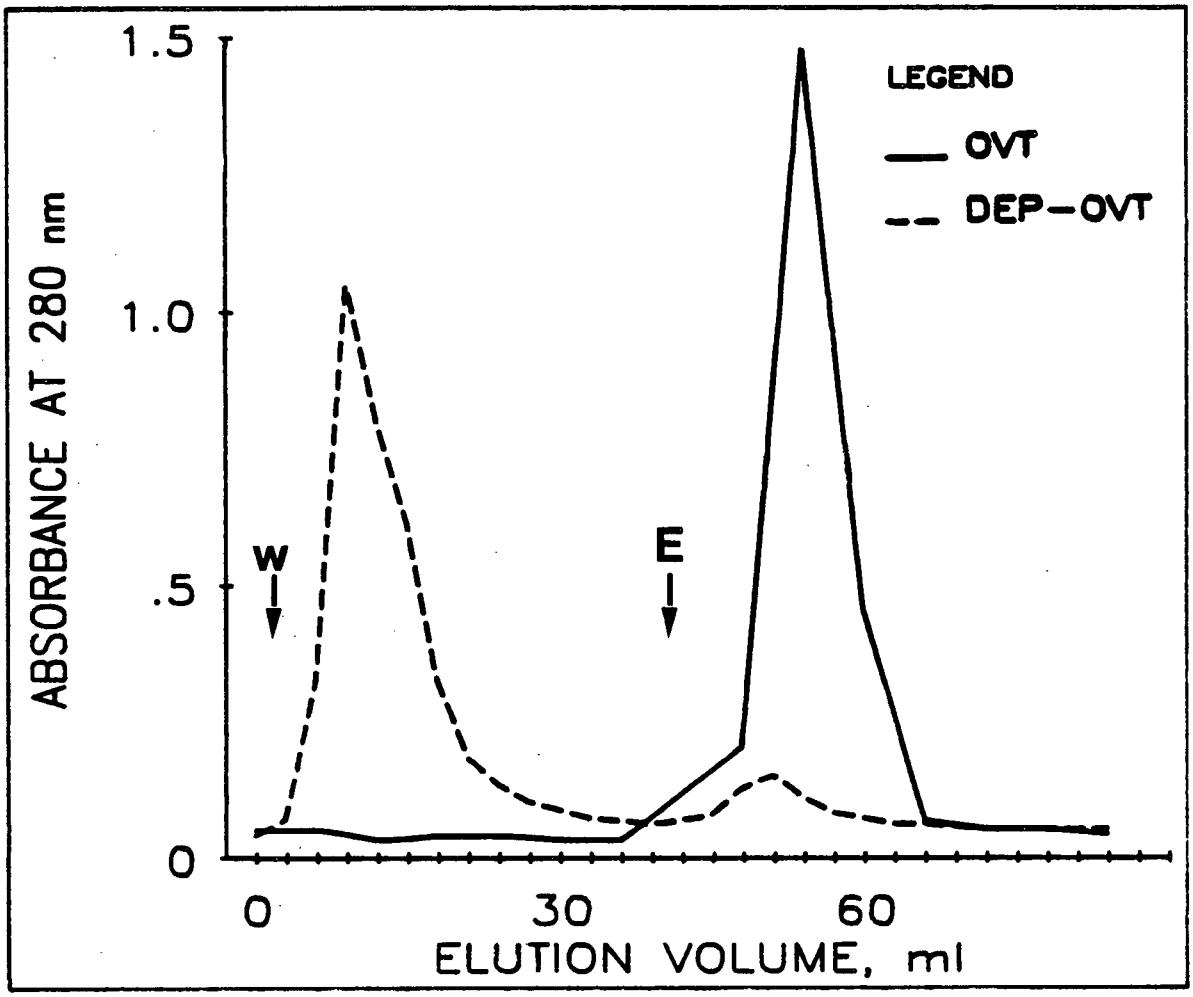


Figure 70. Metal chelate interaction chromatography of control ovotransferrin (OVT) and diethyl pyrocarbonate treated ovotransferrin (DEP-OVT). 3 mL (8 mg/mL) was applied to Cu-loaded Sepharose 6B (7 x 1.4 cm) after equilibration with 0.05 M Tris-acetate/0.5 M Nacl, pH 8.2. W, washing with the equilibrating buffer; E, elution with 0.01 M imidazole; flow rate was 30 mL/hr.

residue per mole OVT drastically inhibited the proteins' ability to bind Cu-chelate matrix. Considering two histidine residues are involved in the metal binding in the Fe- or Cu- saturated OVT, there are still 11 histidine groups left free to interact with immobilized metal on Sepharose 6B. However, blocking histidine groups would destroy the ability of OVT to bind MCIC column.

In conclusion, this part demonstrates for the first time a method to separate ovotransferrin from egg white by a single chromatographic step. The specificity and capacity of MCIC column for ovotransferrin are high, and it should be easy to adapt the method for isolation of ovotransferrin to a larger scale operation. The isolated OVT from egg white may be incorporated in infant formula, since, it has not had sensitizing effects on OVT-treated babies (Giacco-Del et al., 1985). Giacco-Del (1985) reported that when ovotransferrin-enriched milk was fed to 15 babies for 60 days the values of total IgE, as determined by the radioimmunoassay method, remained within the normal range.

CONCLUSIONS AND RECOMMENDATIONS

A hexametaphosphate method was developed for minimizing β-Lg and maximizing Ig in whey. The new method avoids saturation of iron binding proteins which abolishes their bacteriostatic activity and also avoids ultilization of non-food grade chemicals which would require regulatory approval, compared to SHMP which is already approved as a food grade chemical.

For isolation of bioactive proteins, MCIC treatment of cheese whey, skimmilk, blood plasma and serum and egg white is recommended, based on the following findings:

(1) Ig of almost 90% purity can be recovered from cheese whey using a simple process, with practically no pre-treatment of whey.

(2) The MCIC has high capacity for Ig isolation from cheese whey, at least 1 litre of whey per 25 ml copper-loaded gel.

(3) Lactoferrin and bovine serum albumin may also be separated from cheese whey.

(4) The treated whey or unbound fraction contains mainly β-lactoglobulin and α-lactalbumin in concentrations similar to the untreated wheys.

(5) This method can be used for extraction of Ig and TF from blood, and ovotransferrin from egg white. The preliminary experiments indicate the potential of this method to isolate Ig directly from skimmilk.

(6) The fact that chemical modification of histidine groups in Ig, TF, OVT, LF rich fraction and casein fractions, destroys coordinate binding to MCIC columns supports the idea of the involvement of histidine groups in the interaction with copper immobilized on gel; however, changing the elution conditions may activate the other mechanism of the interaction.

(7) The isolated immunoglobulins from colostrum, cheese whey and blood

recognize LPS isolated from E. coli, S. typhimurium and B. parapertussis. These results may encourage the addition of the bioactive proteins to infant formula to give similar performance as human milk.

(8) The MCIC column can be easily regenerated for re-use.

FUTURE WORK

In order to apply the MCIC process for large scale recovery of Ig, further studies are required,

(1) to search for cheaper and/or mechanically more stable material for the chelating support which would be more suitable for scale-up process;

(2) to determine the life-time of the column;

(3) to investigate the inhibition of pathogens with the separated Ig by animal assays;

(4) to study the structure-function relationship of Ig activity and improve the functionality of immunoglobulins.

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APPENDIX:

NON-FERRIC METHODS FOR β-LACTOGLOBULIN REMOVAL FROM CHEDDAR CHEESE WHEY

Different approaches were tried to selectively eliminate β-Lg from cheese whey.

1. Amundson and Watanawamchakorn approach

Amundson and Watanawamchakorn (1982) claimed that adjustment of the conductivity of 80% volume reduced electrodialyzed whey to 100-200 μMHOS and adjustment of the pH to 4.65 would result in 53.3% protein removal. However, when this method was tried on acid whey (prepared from raw milk by acidification) and cheese whey after extensive dialysis and adjustment of the conductivity (conductivity Bridge Model 31, Yellow Springs Instruments, Co., Inc., Ohio) by addition of 1 M NaCl, it did not work.

2. Solubility differences of β-Lg and Ig at different pHs

The pH of 1% β-Lg or Ig was adjusted with 0.1 N NaOH or 0.1 N HCl to the range 4-8, and the protein content of the supernatant was determined according to the method of Nakai and Le (1970) after centrifugation at 10,000 x g. The lowest solubility of β-Lg was found at pH 4.7 and it was 46% of the total β-Lg in the original solution. The lowest solubility of crude Ig was found around pH 5.8 and it was about 50% of the total Ig in the original solution.

To check the possibility of denaturation of β-Lg, two tests were performed. First, gel filtration of β-Lg on TSK HW-55 column (15 x 1.5 cm, flow rate 1 mL/min, eluted with 50 mM imidazole-KCl, pH 6.5) showed no evidence of aggregation in the elution patterns of standard β-Lg used, which suggested that β-Lg was native. The second denaturation test depended on the solubility of globular proteins at their isoelectric point in the presence of 0.2 M NaCl. It was found that more than 90% of β-Lg was soluble as shown by absorbance at 280nm under the above conditions after centrifugation at 10,000 X g.

To study the the effect of pH on the solubility, another attempt was made to dissolve 1% β-Lg and Ig at citrate buffer in different pH values 4.2, 4.6, 5.2, 5.6, and 6.0, with conductivity in the range of 4000 - 8000 μMHOS. However, the results indicated that no precipitation of β-Lg occurred whereas minor turbidity was noticed in the Ig solutions.

3. Combination of Amundson and Watanawamchakorn and Pearce methods

According to Amundson and Watanawamchakorn (1982) pH, ionic strength and the concentration of whey are the major factors influencing the precipitation of β-Lg, while according to Pearce (1983) the pH, temperature and the concentration of whey are the major factors influencing the preparation of enriched β-Lg fraction. In our study an attempt was made to combine these factors in addition to other factors i.e., cys, KI and $CaCl_2$. The ranges of the values of these factors were as follows:

Ionic strength (I) = 0.0-0.2, pH = 4.5-5.0, temperature (T) = 25-50°C, total solid (TS) = 6.5-20%, cysteine (Cys) = 0.0-0.2%, potassium iodide (KI) = 0.0-0.2%, calcium chloride ($CaCl_2$) = 0.0-0.2%.

According to Taguchi's scheme (1957) for fractional factorial analysis, the possible interactions were chosen to be IXpH, TXpH, IXT, KIXI, IXCa, CaXCys, IXCys.

Calcium free acid whey was prepared by the addition of potassium oxalate followed by dialysis for 48hr against distilled water. A set of experiments was performed (Taguchi, 1957, L_{16} for two levels). Experiments were carried out in random order and SDS-PAGE was used to evaluate the separation efficiency of β-Lg and α-La in each experiment.

ANOVA was performed (using a Monroe 1880 Calculator) for factors and interactions, and it was concluded that I, TS, pH, Cys, and Ca were important factors while KI and temperature were nonsignificant. Simplex optimization was then applied for the five factors with lower limits and upper limits as follows: I(0.0-1.00), TS (6.5-20%), pH (4.5-5.0) and (0.0-0.2 M) for Cys and Ca. Six vertices were experimented and SDS-PAGE showed that the optimization of these factors failed, therefore we could not selectively precipitate β-Lg.

In an attempt to remove Ca-phosphate from whey under alkaline condition, an Amberlite anion exchanger was used to adjust the pH of cheese whey to 8.0. However,the amount of Ca-phosphate removed by this process was much lower than that obtained by adjusting the pH using sodium hydroxide.

4. Precipitation of β-Lg by polyethylene glycol

The behaviour of solutions containing globular proteins and polyethylene glycol(PEG) has been investigated by many protein chemists (Haire et al., 1984). PEG provides an attractive alternative to other agents used for protein precipitation. It allows regulation of protein solubility without observable effects on protein structure and function. However the only noticable effect of PEG is the shift of the pKa of the ionizable groups at high PEG concentrations (Atha and Inghams, 1981). In some situations, the use of PEG has a definite advantage over the use of high salt concentrations, where salting in and salting out phenomena and specific interactions with both cation and anion complicate both the interpretation and the extrapolation to a well defined, biologically relevant state (Roth et al., 1979).

An aliquot of forty percent (W/V) PEG was added to 20 mL of cheese whey with good mixing to give final PEG concentrations of 6.7, 9.2, 11.4, 13.3 and 20%. Samples were then kept at room temperature for an hour and were then