

Isolation of immunoglobulins from cheese whey using ultrafiltration and immobilized metal affinity chromatography

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A method was developed to isolate immunoglobulins (Ig) from cheese whey using ultrafiltration (UF) and immobilized metal affinity chromatography (IMAC). Cheddar cheese whey was concentrated up to 30 times using UF prior to applying to an IMAC column. A 50-ml IMAC column was loaded with 17.3 ml of 50 mM CuCl_2 for 2/3 Cu saturation of the column bed volume. Using 20 times concentrated whey, 1240 mg of IgG could be isolated with 52% purity using glycine in the eluting buffer. Glycine was found to be a better competing ligand in the eluting buffer than NH_4Cl . Approximately 152 mg of lactoferrin and 90 mg of lactoperoxidase were isolated with the IgG. The concentration factor, IMAC matrix support, UF conditions and diafiltration did not affect the recovery or purity of IgG obtained by IMAC. By using concentrated whey rather than unconcentrated whey, an 80% reduction in time for IMAC could be achieved. IMAC was found to give higher recovery and purity of IgG from concentrated whey than ion exchange chromatography. A method was also developed to obtain IgG from raw skim milk using UF and IMAC.

Keywords: immobilized metal affinity chromatography, immunoglobulins, ultrafiltration, whey.

INTRODUCTION

The ingestion of immunoglobulins (Ig) from the colostrum of immunized cows can prevent enterotoxigenic *Escherichia coli* and rotavirus infections in humans (Hilpert *et al.*, 1977; Ebina *et al.*, 1985; Brüssow *et al.*, 1987; Hilpert *et al.*, 1987; Tacket *et al.*, 1988). Immunoglobulins from cow's milk can also prevent infections in animals such as calves, rats, lambs and foals (Bridger & Brown, 1981; Saif *et al.*, 1983; Michalek *et al.*, 1987). Researchers have suggested adding immunoglobulins to infant formulas to provide passive immunity against various pathogens (Goldman, 1989; Nakai, 1991).

To obtain immunoglobulins with specificity, cows are immunized against the pathogen of interest. Immunization is expensive, time critical and

may not be ethically acceptable. Studies have suggested immunization may not be necessary, though. Yolken *et al.* (1985) found anti-rotavirus activity in raw and pasteurized milk from non-immunized cows. Cow's milk immunoglobulins have also been found to cross-react with similar pathogens in other species (Brüssow *et al.*, 1987).

Bovine colostrum is often used as the source of immunoglobulins in immunosupplementation studies. Colostrum contains 50 mg/ml of immunoglobulins whereas milk only has 0.6 mg/ml (Jenness, 1988). About 80% of immunoglobulins in milk or colostrum is of the IgG class. Colostrum is not available in large quantities, which makes it an unreliable source of immunoglobulins. Milk or whey would be better sources of immunoglobulins since these are available in large volumes. Stott & Lucas (1989) suggested that immunoglobulins from pooled milk have activity against a greater variety of pathogens than colostrum from any one cow. Since the immunoglobulin concentration in milk or whey is low, a simple method to

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isolate immunoglobulins for immunosupplementation purposes is needed. In this study, a method to isolate immunoglobulins was developed using ultrafiltration (UF) and immobilized metal affinity chromatography (IMAC).

UF is a pressure-driven membrane process that can be used in the separation and concentration of substances having molecular weights between 10^3 and 10^6 daltons (Renner & Abd El-Salam, 1991). UF is used in the dairy industry for the pre-concentration of milk before cheesemaking and in the manufacture of whey protein concentrates. UF has been used to concentrate immunoglobulin in whey (Hilpert, 1984; Kothe *et al.*, 1987; Abraham, 1988; Stott & Lucas, 1989). Stott & Lucas (1989) obtained a 12% immunoglobulin solution using UF and diafiltration of whey with a 100 kDa molecular weight cut-off (MWCO) membrane. Depending on the purity of immunoglobulin required, UF and diafiltration may be adequate. Immunoglobulin of high purity would be better for fortification since more immunological activity could be obtained in a smaller dose. In addition, more abundant whey proteins like β -lactoglobulin are potential allergens for some individuals, so further purification would be beneficial.

IMAC or MCIC (metal chelate interaction chromatography) was first described as a separation technique in 1961 by Helfferich (1961). Porath *et al.* (1975) used IMAC to fractionate proteins in serum. The amino acid side chains of cysteine, histidine and arginine bind to metal ions immobilized on a solid matrix support which allows proteins with different amino acid compositions to be separated from one another (Sulkowski, 1985).

Al-Mashikhi *et al.* (1988) used IMAC to separate immunoglobulin and lactoferrin from cheese whey. From 1 litre of whey, they recovered about 200 mg of IgG/25 ml of Cu-loaded gel. Li-Chan *et al.* (1990) improved the IMAC method to isolate IgG from whey with high recovery and purity. A recovery of 100 mg of IgG/ml of Cu-loaded gel with 75–95% purity was obtained. Li-Chan *et al.* (1990) found that by continuously applying whey to an IMAC column, immunoglobulin would competitively displace other less tightly bound whey proteins. The order of displacement of proteins in cheese whey was β -lactoglobulin, α -lactalbumin, bovine serum albumin, lactoperoxidase-lactoferrin and immunoglobulin. One disadvantage of this IMAC method was that it was time-consuming. Large volumes of whey had to be applied due to the low concentration of immunoglobulin in whey.

To make the process more efficient, the feasibility of using whey concentrated by UF prior to IMAC was investigated.

MATERIALS AND METHODS

Materials

Cheddar cheese and mozzarella cheese wheys were obtained from Dairyland Foods (Burnaby, BC). The whey was stored at -25°C . The pH of the whey was adjusted to 6.0 with 1.0 N HCl or 1.0 N NaOH. Prior to use, the whey was centrifuged at $10\,000 \times g$ for 15 min at 5°C to remove insoluble matter. Raw milk was obtained from the University of British Columbia Dairy Unit. The milk was cooled to 5°C before centrifugation at $4000 \times g$ for 30 min at 5°C to remove fat. Whey concentrated commercially by UF was obtained from Olympia Cheese (Olympia, WA).

Ultrafiltration

A CPS-1 Cartridge Pumping System with HarpTM hollow fiber ultrafiltration membrane cartridges was purchased from Supelco Canada Ltd (Oakville, ON). The hollow fiber UF membrane cartridges were 20.3 cm in length with fiber inner diameters of 1.1 mm. The total membrane surface area was 0.03 m^2 . Three cartridges with molecular weight cut-offs of 50, 100 and 500 kDa were used. The processing solution was heated to $45\text{--}50^{\circ}\text{C}$. The UF processing conditions consisted of an average trans-membrane pressure of 100 kPa, recirculation rate of 1800 ml/min, and permeate flow rate of approximately 15 ml/min. Continuous diafiltration was used with distilled water as the diafiltration medium. Membranes were cleaned using 0.1 N HCl and were stored in 0.1% sodium benzoate at pH <7.0 .

Immobilized metal affinity chromatography (IMAC)

The IMAC method of Li-Chan *et al.* (1990) was used. Chelating Sepharose Fast Flow (CSFF) and Chelating Sepharose 6B (CS6B) from Pharmacia LKB Biotechnology AB (Uppsala, Sweden) were packed into columns with inner diameters of 1.4 cm and 2.5 cm for bed volumes of 10 ml and 50 ml, respectively. For a 10-ml column, 3.0 ml of 50 mM CuCl_2 was applied, resulting in $1/2\text{--}3/5$ copper loading of the total column bed volume. For a 50-ml column, 17.3 ml of 50 mM CuCl_2 was ap-

plied, resulting in 2/3 copper loading. The capacity of CSFF is 31–40 μmol of Cu^{2+} /ml of gel (Hutchens *et al.*, 1988).

The IMAC procedure consisted of rinsing the column with distilled H_2O prior to applying CuCl_2 . Any unbound Cu was removed by washing with distilled H_2O . A flow rate of 0.4–0.5 ml/min was used for the 10-ml column, and a flow rate of 1.0–3.3 ml/min was used for the 50-ml column. IMAC was run at room temperature. The column was equilibrated with 5–6 column volumes of 50 mM sodium phosphate buffer at pH 6.0. Sample was then applied to the column. Sufficient sample was applied to saturate the column with IgG. Unbound proteins were removed by washing with buffer. The protein content of the effluent was monitored by measuring the absorbance at 280 nm with a spectrophotometer. When the A_{280} fell below 0.2, washing was stopped. The bound proteins were eluted with 50 mM glycine or 1 M NH_4Cl in 50 mM sodium phosphate buffer at pH 8.0. Elution was stopped when the A_{280} of eluted fractions dropped below 0.6. Elution was then repeated with distilled deionized water. Fractions eluted from the column were dialyzed against distilled water and then lyophilized. The column was cleaned with 0.05 N NaOH, then rinsed with distilled water, and stored in 20% ethanol.

Ion exchange chromatography

A 60-ml column with an inner diameter of 2.6 cm was packed with carboxymethyl cellulose grade 2 from Phoenix Chemicals Ltd (Christchurch, NZ). The column was equilibrated with 6-column volumes of 50 mM citric acid–sodium citrate buffer at pH 5.4 or pH 5.0. Concentrated whey was desalted by electrodialysis. The Micro Acilyzer G1 Desktop Desalinator (Asahi Chemical Industry Co. Ltd., Kawasaki, Japan) with an AC-230-10 cartridge was used for electrodialysis. Electrodialyzed concentrated whey was applied to the column at a flow rate of 2.0 ml/min at room temperature. After applying the whey, the column was washed with 10 mM buffer at pH 5.4 or pH 5.0. The protein content was monitored by measuring the A_{280} and washing was stopped when the absorbance dropped below 0.6. The bound protein was eluted with 250 mM NaCl in 10 mM buffer at pH 5.4 or pH 5.0. Elution was stopped when the absorbance dropped below 0.6. The elution procedure was repeated with 1.0 M NaCl in 10 mM buffer at pH 5.4 and pH 5.0. The column was cleaned with 10 mM

buffer at pH 5.4 or pH 5.0, and was stored in 20% ethanol. Fractions from the column were dialyzed against distilled water and lyophilized.

Conductivity

Conductivity was measured with a Model 31 conductivity bridge with a #3403 conductivity cell from Yellow Springs Instrument Co. Ltd. (Yellow Springs, OH). The conductivity was corrected for a temperature of 18°C. A 2% difference in conductivity for every degree difference from 18°C was assumed (Davis, 1951).

Lactose determination

The lactose content of whey samples was determined using the phenol–sulfuric acid method of Lawrence (1968).

Lactoperoxidase assay

The lactoperoxidase content of fractions was determined using the ABTS [2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt] assay as outlined in the Sigma Chemical Co. (St. Louis, MO) Bulletin No. 8-84.

Protein determination

Protein content was measured by the biuret method using Sigma Diagnostics Total Protein Reagent (Sigma Chemical Co., St. Louis, MO). The recommended protocol was modified, so readings could be made with an ELISA reader at 550 nm. To each well in an Immulon 2 flat-bottomed, polystyrene microtitre plate (Dynatech Laboratories Inc., Chantilly, VA), 50 μl of sample and 200 μl of Total Protein Reagent were added in triplicate. The plate was allowed to incubate at room temperature for 10 min before reading. Bovine serum albumin (BSA) was used as a standard.

SDS-PAGE

SDS-PAGE was done using the PhastSystem electrophoresis unit (Pharmacia LKB Biotechnology AB, Uppsala, Sweden). PhastGel gradient 10–15% acrylamide gels with 2% cross-linking and PhastGel SDS buffer strips (0.55% SDS) were obtained from Pharmacia LKB Biotechnology AB (Uppsala, Sweden). The gels were run at 250 V, 10

mA, 15°C for 63 Vh. The gels were Coomassie stained with 0.1% PhastGel Blue R solution in 30% methanol and 10% acetic acid. The gels were destained in 30% methanol and 10% acetic acid, and were preserved in 10% acetic acid and 5% glycerol.

Radial immunodiffusion techniques

RID for IgG concentration

RID plates were prepared according to Ingild (1983) with modifications. Each RID plate was prepared by using 0.06–0.18 ml of anti-bovine IgG (whole molecule) developed in rabbit (Sigma Chemical Co., St. Louis, MO or ICN Immuno-Biologicals, Lisle, IL) with PBS (0.15 M NaCl in 10 mM sodium phosphate buffer at pH 7.0) to make a total volume of 2.0 ml. Agarose Type IV (50 mg) (Sigma Chemical Co., St. Louis, MO) was mixed with 4.6 ml of PBS and 0.4 ml of 0.35% NaN₃ and boiled to dissolve the agarose. Both solutions were equilibrated in a 55°C waterbath and then mixed together before pouring into an RID plate (ICN ImmunoBiologicals, Lisle, IL). Plates were allowed to solidify at room temperature for 15 min and were stored in a moist chamber at 4°C. The plates were prepared by cutting 3.0-mm diameter holes and 3.0 µl of sample was added to each well. Bovine IgG from serum (Sigma Chemical Co., St. Louis, MO) was used as a standard. Standard solutions of IgG ranging in concentration from 0.1 to 1.5 mg/ml were prepared using an extinction coefficient of 13.7 for a 1% solution in a 1-cm cell at 280 nm (Fasman, 1976). RID plates were incubated at room temperature in a moist chamber until there was no further increase in precipitin ring diameter, which took about 48 h. A standard curve was plotted with the square of the diameter of precipitin ring versus IgG concentration.

RID for lactoferrin concentration

Antibodies against bovine lactoferrin were developed in chickens. Three laying white leghorn hens from the University of British Columbia Poultry Unit were obtained. The chickens were housed and fed under normal conditions. The chickens received an initial injection of bovine lactoferrin solution in Freund's complete adjuvant (FCA). A 5.0 mg/ml solution of bovine lactoferrin from milk (Sigma Chemical Co., St. Louis, MO) in PBS was prepared. Using an equal volume of FCA, the lactoferrin solution was added dropwise to the

adjuvant while vortexing the adjuvant to form a water-in-oil emulsion. Each chicken was injected intramuscularly with 0.25 ml of the emulsion at four sites on the breast muscle to give a total injection of 1.0 ml. After 2 weeks, the chickens were given a second injection using lactoferrin solution in Freund's incomplete adjuvant (FIA) (Difco, Detroit, MI) following the same protocol. A third booster of lactoferrin in FIA was given after 3 months to maintain anti-bovine lactoferrin IgY levels.

IgY was isolated from egg yolk using the method of Akita & Nakai (1992). The method consisted of separating the yolk from the white. The egg yolk contents were then separated from the vitelline membrane and diluted with 6–10 vols of water acidified to pH 2.7–2.8 with 0.1 N HCl. The pH of the mixture was adjusted to 5.0–5.2 using 0.1 N HCl or 0.1 N NaOH. The mixture was held at 4°C for at least 2 h and then centrifuged at 10 000 × g for 45 min at 5°C. To the supernatant, 33% (w/v) (NH₄)₂SO₄ was added. The solution was allowed to stand overnight before being centrifuged at 10 000 × g for 30 min at 5°C. The precipitate containing IgY was collected. The precipitate was reconstituted with water, dialyzed against water and then lyophilized.

To prepare anti-bovine lactoferrin IgY for RID, the lyophilized IgY was solubilized with a vol. of PBS equal to 1/10 the initial egg yolk volume. This volume was chosen based on the results of Meisel (1988). Plates were prepared in a similar manner to plates for IgG except the PBS composition was changed to 1.5 M NaCl in 10 mM sodium phosphate buffer at pH 8.0. Each plate contained 0.20 ml of anti-bovine lactoferrin IgY solution. Bovine lactoferrin from milk (Sigma Chemical Co., St. Louis, MO) was used as a standard. The concentration of lactoferrin was determined using an extinction coefficient at 1% and 1 cm of 15.1 (Fasman, 1976).

RESULTS AND DISCUSSION

Effect of matrix support and concentration factor of whey

UF is used to concentrate whey up to 20–30 times in the manufacture of whey protein concentrates (Glover, 1985). To retain most of the protein in the whey, an UF membrane with a molecular

Table 1. Change in the composition of cheddar cheese whey following ultrafiltration

Concentration factor		pH	Conductivity ^a (μ mho)	Lactose concentration ^b (mg/ml)	IgG concentration ^c (mg/ml)	Protein concentration ^d (mg/ml)
Unconcentrated	Whey	5.98	6667	47.8 $\pm$ 1.3	0.35	5.6 $\pm$ 0.1
	Concentrate	—	—	—	—	—
10 $\times$	Whey	5.94	6491	50.7 $\pm$ 2.0	0.44	5.3 $\pm$ 0.4
	Concentrate	5.68	6250	51.3 $\pm$ 1.1	4.16	37.8 $\pm$ 3.5
20 $\times$	Whey	6.05	6542	50.5 $\pm$ 1.0	0.38	6.8 $\pm$ 0.5
	Concentrate	5.63	5818	51.3 $\pm$ 0.4	7.06	77.4 $\pm$ 4.3
30 $\times$	Whey	5.99	6786	53.9 $\pm$ 1.2	0.52	6.8 $\pm$ 0.3
	Concentrate	5.37	5714	47.4 $\pm$ 0.5	15.58	100.2 $\pm$ 3.1

^aCorrected for 18°C.^bMeasured using the sulfuric acid and phenol colorimetric method of Lawrence (1968). Means $\pm$ S.D.; $n = 4$.^cMeasured by radial immunodiffusion. Values are the average of duplicates. The differences between duplicate measurements were less than 10%.^dMeasured using the biuret method. Means $\pm$ S.D.; $n = 3$.

weight cut-off (MWCO) of 10 kDa or larger is often used. The main proteins in whey are β -lactoglobulin (18 277 Da) and α -lactalbumin (14 174 Da), with smaller amounts of bovine serum albumin (66 267 Da), lactoferrin (77 000 Da), lactoperoxidase (89 000 Da), and IgG (150 000 Da) (Glover, 1985; Whitney, 1988; Yoshida, 1988). Cheddar cheese whey (CCW) was therefore concentrated 10, 20 and 30 times using a 50 kDa MWCO membrane before applying to an IMAC column. Table 1 shows the changes in whey composition after UF. Conductivity and pH decreased during UF due to the loss of ions. Lactose concentration remained relatively constant in the concentrated whey, although there was a net loss of lactose. Based on the IgG concentration, IgG was not lost during UF. Some protein loss occurred due to the permeability of α -lactalbumin and β -lactoglobulin.

Tables 2 and 3 show the recovery of IgG from 10-ml IMAC columns filled with Chelating Sepharose Fast Flow and Chelating Sepharose 6B. More sample was applied to the CS6B column than to the CSFF column since previous work suggested a greater recovery of IgG with CS6B. Based on the recovery of IgG using unconcentrated whey, CS6B had 15% greater recovery of IgG than CSFF. CSFF differs from CS6B in having extra crosslinking which makes it more stable. This difference could have resulted in slightly different recoveries. It could also explain the observed compression of CS6B but not CSFF during sample application. Since CSFF was more stable with only slightly lower recovery, it appeared to be the better support.

Li-Chan *et al.* (1990) obtained up to 100 mg of IgG/ml of Cu-loaded bed volume with CS6B using

Table 2. IgG recovery and purity of fractions obtained by immobilized metal affinity chromatography of unconcentrated and concentrated cheddar cheese whey using 10 ml of Chelating Sepharose Fast Flow loaded with 3.0 ml of 50 mM CuCl₂

Concentration factor	Fraction	IgG recovered ^a (mg)	Purity of IgG ^b (g/100 g protein)
Unconcentrated	Applied whey (922 ml)	320	4.9
	Unbound (900 ml)	108	2.1
	Wash (44 ml)	5	35.7
	Elution (NH ₄ Cl) (79 ml)	160	58.3
	Elution (dH ₂ O) (33 ml)	30	59.3
	EDTA wash (9 ml)	4	90.7
10 $\times$	Applied 10 $\times$ concentrate (93 ml)	380	10.1
	Unbound (97 ml)	120	4.7
	Wash (68 ml)	10	6.2
	Elution (NH ₄ Cl) (94 ml)	120	53.3
	Elution (dH ₂ O) (34 ml)	21	57.1
	EDTA wash (10 ml)	10	100.0
20 $\times$	Applied 20 $\times$ concentrate (47 ml)	330	9.1
	Unbound (46 ml)	70	2.9
	Wash (85 ml)	21	5.2
	Elution (NH ₄ Cl) (88 ml)	130	59.0
	Elution (dH ₂ O) (37 ml)	43	84.0
	EDTA wash (20 ml)	20	100.0
30 $\times$	Applied 30 $\times$ concentrate (32 ml)	424	11.8
	Unbound (31 ml)	170	7.1
	Wash (93 ml)	44	6.6
	Elution (NH ₄ Cl) (87 ml)	110	62.0
	Elution (dH ₂ O) (59 ml)	40	89.1
	EDTA wash (14 ml)	14	100.0

^aRecovered IgG calculated as IgG activity (mg/ml) by radial immunodiffusion $\times$ volume (ml) of fraction. Values are the average of duplicates. The differences between duplicate measurements were less than 10%.^bPurity of IgG (g/100 g) calculated as IgG activity by radial immunodiffusion/total protein in fraction $\times$ 100. Protein content was calculated as means $\pm$ S.D.; $n = 3$. Values are the average of duplicates. The differences between duplicate measurements were less than 10%.

Table 3. IgG recovery and purity of fractions obtained by immobilized metal affinity chromatography of unconcentrated and concentrated cheddar cheese whey using 10 ml of Chelating Sepharose 6B loaded with 3.0 ml of 50 mM CuCl₂

Concentration factor	Fraction	IgG recovered ^a (mg)	Purity of IgG ^b (g/100 g protein)
Unconcentrated	Applied whey (1510 ml)	530	4.9
	Unbound (1510 ml)	201	2.1
	Wash (52 ml)	8	13.0
	Elution (NH ₄ Cl) (90 ml)	210	63.0
	Elution (dH ₂ O) (47 ml)	14	63.6
	EDTA wash (14 ml)	18	18.2
10 ×	Applied 10 × concentrate (152 ml)	620	10.1
	Unbound (154 ml)	300	6.1
	Wash (111 ml)	17	8.2
	Elution (NH ₄ Cl) (99 ml)	110	46.9
	Elution (dH ₂ O) (29 ml)	11	41.7
	EDTA wash (16 ml)	33	100.0
20 ×	Applied 20 × concentrate (77 ml)	544	9.1
	Unbound (74 ml)	180	3.8
	Wash (109 ml)	21	7.0
	Elution (NH ₄ Cl) (100 ml)	150	62.9
	Elution (dH ₂ O) (31 ml)	10	40.9
	EDTA wash (18 ml)	60	100.0
30 ×	Applied 30 × concentrate (52 ml)	690	11.8
	Unbound (52 ml)	340	9.2
	Wash (130 ml)	34	6.5
	Elution (NH ₄ Cl) (110 ml)	110	53.1
	Elution (dH ₂ O) (101 ml)	20	43.2
	EDTA wash (14 ml)	83	100.0

^aRecovered IgG calculated as IgG activity (mg/ml) by radial immunodiffusion × volume (ml) of fraction. Values are the average of duplicates. The differences between duplicate measurements were less than 10%.

^bPurity of IgG (g/100 g) calculated as IgG activity by radial immunodiffusion/total protein in fraction × 100. Protein content was calculated as means ± S.D.; *n* = 3. Values are the average of duplicates. The differences between duplicate measurements were less than 10%.

CCW. By visually estimating the volumes of the columns that were blue due to copper loading, the calculated capacity of both CSFF and CS6B columns in this study was about 40 mg/ml. The differences in capacity between studies could be accounted for by the crude measurement of Cu-loaded bed volume, and slight variations depending on the batch of whey used. Based on the total bed volume of the columns, the recovery of IgG between studies was similar. For a 10-ml column, the recovery was approximately 200 mg of IgG.

The capacity based on the total bed volume depends upon the amount of 50 mM CuCl₂ applied. Columns were not fully Cu-loaded to prevent Cu

contamination of the isolated IgG. In the studies, at least 1/3 of the column was not Cu-loaded since the Cu front migrated during application of sample and elution. Li-Chan *et al.* (1990) found a slight increase in Cu concentration of fractions eluted with pH 8.0 phosphate-buffered glycine, but the increase did not exceed recommended daily intakes of Cu based on reported dosages of IgG given to infants or adults. The degree of Cu-loading that would keep Cu contamination to a minimum should be established before using this process to isolate IgG for supplementation.

The amount of IgG recovered in the elution with NH₄Cl decreased when concentrated whey was used, as seen in Tables 2 and 3. However, more IgG was recovered in the elution with distilled water or the EDTA wash. The loss of IgG from concentrated whey in the elution with NH₄Cl was more pronounced in the larger 50-ml column, and could be corrected by changing the eluting buffer, as discussed later. IgG binding more tightly to the column when concentrated whey was used may have caused these results. UF may have caused slight changes in IgG conformation increasing binding efficiency without causing loss in IgG concentration as measured by RID. Chojnowski & Dzuiba (1982) noted UF resulted in a positive differential spectrum of protein solutions obtained from whey ultrafiltrates. The positive differential spectrum indicated the number of chromophobic groups, such as hydrophobic groups of aromatic amino acids or disulfide groups, increased with UF. These changes could cause IgG to bind more efficiently.

The difference in pH or ionic composition of the concentrated whey should not have affected IgG binding. Li-Chan *et al.* (1990) found IgG could be bound at pH 5.0, 6.0 or 8.0. To quench non-specific electrostatic interactions, salt (1 M NaCl) is usually added to the equilibrating buffer (Sulkowski, 1985). The decrease in conductivity of concentrated whey should therefore have reduced the binding efficiency of IgG and decreased the purity.

Whey concentrated 10, 20 or 30 times resulted in similar recoveries of IgG. At 30 times concentration though, the whey was more viscous which caused some problems in applying it to the column. Whey concentrated around 20 times appeared to be more suitable for use with IMAC.

The purity of IgG obtained was around 50–60% for unconcentrated whey. The purity remained similar for concentrated whey, but tended to fluctuate for CS6B, possibly due to the instability of

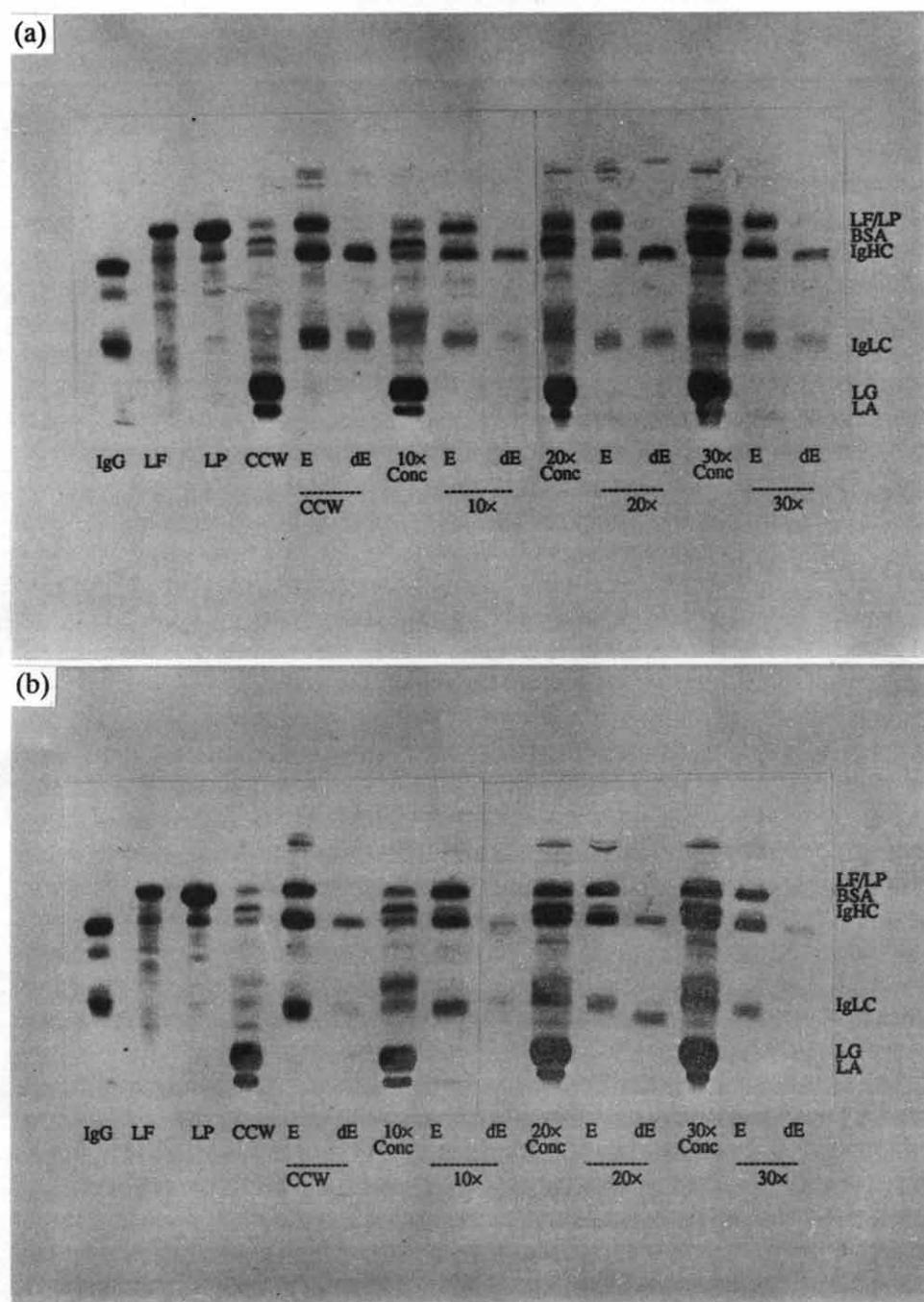


Fig. 1. Reduced SDS-PAGE profiles of fractions eluted with 1 M NH_4Cl and distilled H_2O after Cu-IMAC treatment of un-concentrated and concentrated whey: (a) using Chelating Sepharose Fast Flow as the matrix support, (b) using Chelating Sepharose 6B as the matrix support. Abbreviations for fractions: IgG, IgG standard; LF, lactoferrin; LP, lactoperoxidase; CCW, cheddar cheese whey; Conc, concentrate (retentate); E, peak eluted with 1 M NH_4Cl ; and dE, peak eluted with distilled H_2O . Abbreviations for bands: LP, lactoperoxidase; LF, lactoferrin; BSA, bovine serum albumin; IgHC, IgG heavy and light chains; LG, β -lactoglobulin; and LA, α -lactalbumin.

the column. Figure 1 shows the major impurities in the isolated IgG were lactoferrin–lactoperoxidase and some α -lactalbumin and β -lactoglobulin. Lactoferrin and lactoperoxidase have antimicrobial activity which could be beneficial for supplementation purposes. Li-Chan *et al.* (1990) obtained IgG purities from 75–95%. The

particular batch of whey used was found to affect the purity of IgG to some extent. The purity could possibly be increased by using gradient elution.

The major advantage in using concentrated whey was the substantial reduction in time for IMAC. The time of application was reduced proportionally to the concentration factor. Applica-

Table 4. IgG recovery and purity of fractions obtained by IMAC of CCW concentrated by UF using a 50-ml Chelating Sepharose Fast Flow column loaded with 17.3 ml of 50 mM CuCl₂ and 1 M NH₄Cl in the eluting buffer

Fraction	IgG recovered ^a (mg)	Purity of IgG ^b (g/100 g protein)	Lactoferrin recovered ^c (mg)	Purity of lactoferrin ^d (g/100 g protein)	Lactoperoxidase recovered ^e (mg)	Purity of lactoperoxidase ^f (g/100 g protein)
Applied 15.3 × concentrate (248 ml)	1500	7.9	370	2.0	87 ± 0	0.5 ± 0.03
Unbound (250 ml)	260	1.9	130	0.9	ND	—
Wash (480 ml)	35	1.8	50	2.6	ND	—
Elution (NH ₄ Cl) (319 ml)	280	33.1	160	18.0	43 ± 5.0	4.9 ± 0.6
Elution (dH ₂ O) (188 ml)	390	75.5	ND	—	2 ± 0.4	0.4 ± 0.1
EDTA wash (90 ml)	50	100.0	ND	—	<1	<0.1
0.05 N NaOH wash (115 ml)	580	55.4	ND	—	12 ± 1	1.1 ± 0.1
Equivalent CCW (3794 ml)	1540	5.6	380	1.4	29 ± 0	<0.1
Permeate (3546 ml)	ND ^g	—	ND	—	1 ± 0.2	<0.1

^aRecovered IgG calculated as IgG activity (mg/ml) by radial immunodiffusion × volume (ml) of fraction. Values are the average of duplicates. The differences between duplicate measurements were less than 10%.

^bPurity of IgG (g/100 g) calculated as IgG activity by radial immunodiffusion/total protein in fraction × 100. Protein content measured by the biuret method (Means ± S.D.; *n* = 3). Values are the average of duplicates. The differences between duplicate measurements were less than 10%.

^cRecovered lactoferrin calculated as lactoferrin activity (mg/ml) by radial immunodiffusion × volume (ml) of fraction. Values are the average of duplicates. The differences between duplicate measurements were less than 10%.

^dPurity of lactoferrin (g/100 g) calculated as lactoferrin activity by radial immunodiffusion/total protein in fraction × 100. Protein content measured by the biuret method (Means ± S.D.; *n* = 3). Values are the average of duplicates. The differences between duplicate measurements were less than 10%.

^eRecovered lactoperoxidase calculated as lactoperoxidase activity (mg/ml) by ABTS assay for lactoperoxidase activity × volume of fraction. Means ± S.D.; *n* = 4.

^fPurity of lactoperoxidase (g/100 g) calculated as lactoperoxidase activity by ABTS assay for lactoperoxidase/total protein in fraction × 100. Protein content measured by the biuret method (Means ± S.D.; *n* = 3). Values are means ± S.D.

^gNot detectable.

Table 5. IgG recovery and purity of fractions obtained by IMAC of CCW concentrated by UF using a 50-ml Chelating Sepharose Fast Flow column loaded with 17.3 ml of 50 mM CuCl₂ and 50 mM glycine in the eluting buffer

Fraction	IgG recovered ^a (mg)	Purity of IgG ^b (g/100 g protein)	Lactoferrin recovered ^c (mg)	Purity of lactoferrin ^d (g/100 g protein)	Lactoperoxidase recovered ^e (mg)	Purity of lactoperoxidase ^f (g/100 g protein)
Applied 13.6 × concentrate (280 ml)	1600	7.8	410	2.2	81 ± 9	0.4 ± 0.1
Unbound (285 ml)	250	1.6	150	1.0	ND	—
Wash (580 ml)	90	4.7	ND	—	ND	—
Elution (gly) (251 ml)	1240	52.3	152	5.8	88 ± 5	3.3 ± 0.2
Elution (dH ₂ O) (90 ml)	30	62.5	ND	—	1 ± 0.1	2.0 ± 0.3
EDTA wash (80 ml)	ND ^g	—	ND	—	<1	<0.1
0.05 N NaOH wash (92 ml)	40	40.0	60	60.0	1 ± 0.1	1.0 ± 0.1
Equivalent CCW (3808 ml)	1600	5.5	400	1.4	6 ± 1	<0.1
Permeate (3528 ml)	ND	—	ND	—	1 ± 0.1	<0.1

^aRecovered IgG calculated as IgG activity (mg/ml) by radial immunodiffusion × volume (ml) of fraction. Values are the average of duplicates. The differences between duplicate measurements were less than 10%.

^bPurity of IgG (g/100 g) calculated as IgG activity by radial immunodiffusion/total protein in fraction × 100. Protein content measured by the biuret method (Means ± S.D.; *n* = 3). Values are the average of duplicates. The differences between duplicate measurements were less than 10%.

^cRecovered lactoferrin calculated as lactoferrin activity (mg/ml) by radial immunodiffusion × volume (ml) of fraction. Values are the average of duplicates. The differences between duplicate measurements were less than 10%.

^dPurity of lactoferrin (g/100 g) calculated as lactoferrin activity by radial immunodiffusion/total protein in fraction × 100. Protein content measured by the biuret method (Means ± S.D.; *n* = 3). Values are the average of duplicates. The differences between duplicate measurements were less than 10%.

^eRecovered lactoperoxidase calculated as lactoperoxidase activity (mg/ml) by ABTS assay for lactoperoxidase activity × volume of fraction. Means ± S.D.; *n* = 4.

^fPurity of lactoperoxidase (g/100 g) calculated as lactoperoxidase activity by ABTS assay for lactoperoxidase/total protein in fraction × 100. Protein content measured by the biuret method (Means ± S.D.; *n* = 3). Values are means ± S.D.

^gNot detectable.

tion of sample is the most time-consuming aspect of IMAC and can take several hours or days depending on the size of the column. A 70–80% reduction in time was possible by using concentrated whey, even with a 2- to 3-fold increase in time for washing. Considering whey is routinely concentrated in the making of whey protein concentrates, this method could be easily adapted for use to isolate IgG. Another advantage in using concentrated whey was the decrease in Cu front migration during application of sample. This reduces the problem of Cu contaminating the isolated IgG.

Effect of UF conditions of whey on the isolation of IgG using IMAC

Whey was concentrated under three different UF conditions: 50 kDa MWCO membrane at 4°C, 50 kDa MWCO membrane at 45°C and 100 kDa MWCO membrane at 4°C. At the lower temperature, UF took 1.5–2.0 times longer due to the increased viscosity. There was also a 15–23% loss of IgG at the lower temperature. The cause of the loss was not determined. The recovery and purity of IgG from the various concentrates using IMAC were similar. Diafiltration of 10 times concentrated whey with 8 times the volume in distilled water also did not affect IgG recovery or purity. UF conditions used commercially for concentrating whey should therefore not affect the isolation of IgG using IMAC.

Effect of elution buffer on the isolation of IgG using IMAC

Tables 4 and 5 show the recovery and purity of IgG obtained from 20 times concentrated CCW using 1 M NH_4Cl or 50 mM glycine, respectively, in the elution buffer for a 50-ml CSFF column loaded with 17.3 ml of 50 mM CuCl_2 . Table 4 shows that IgG was removed from the IMAC column only after cleaning the column with 0.05 N NaOH. NH_4Cl was a poorer competing ligand than glycine. Glycine gave a 47% higher recovery of IgG. Figure 2 shows the elution profiles for the two elution buffers. The profiles demonstrate that glycine was a stronger competing ligand, resulting in greater removal of bound proteins including IgG. Imidazole is also a strong competing ligand, but is not food grade, unlike glycine or NH_4Cl . As a control, CCW was applied to the 50-ml column loaded with 13.5 ml of 50 mM CuCl_2 and eluted

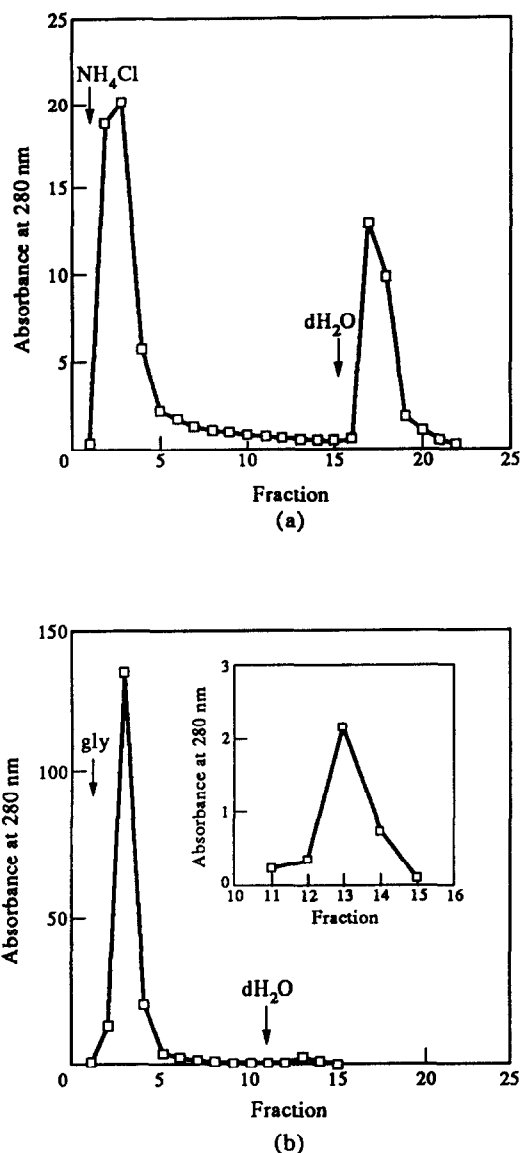


Fig. 2. Elution profile of adsorbed proteins from a 50-ml CSFF column loaded with 17.3 ml of 50 mM CuCl_2 : (a) using 1 M NH_4Cl in buffer and distilled water; (b) using 50 mM glycine in buffer and distilled water. The flow rate was 2.4 ml/min. Fraction volumes were 25 ml.

with glycine. Less CuCl_2 was applied since the Cu front migrated more when using whey than when using concentrated whey. The elution with glycine recovered 1090 mg of IgG, which was within the expected range using concentrated whey. The purities were also similar.

Tables 4 and 5 indicate that the purity of IgG recovered was similar for the two elution buffers. Figure 3, showing the SDS-PAGE profiles obtained for each elution buffer, verifies the purities. The tables also show that most of the lactoferrin and lactoperoxidase in the applied concentrated whey could be isolated with the IgG.

Glycine is recommended to be used in the elut-

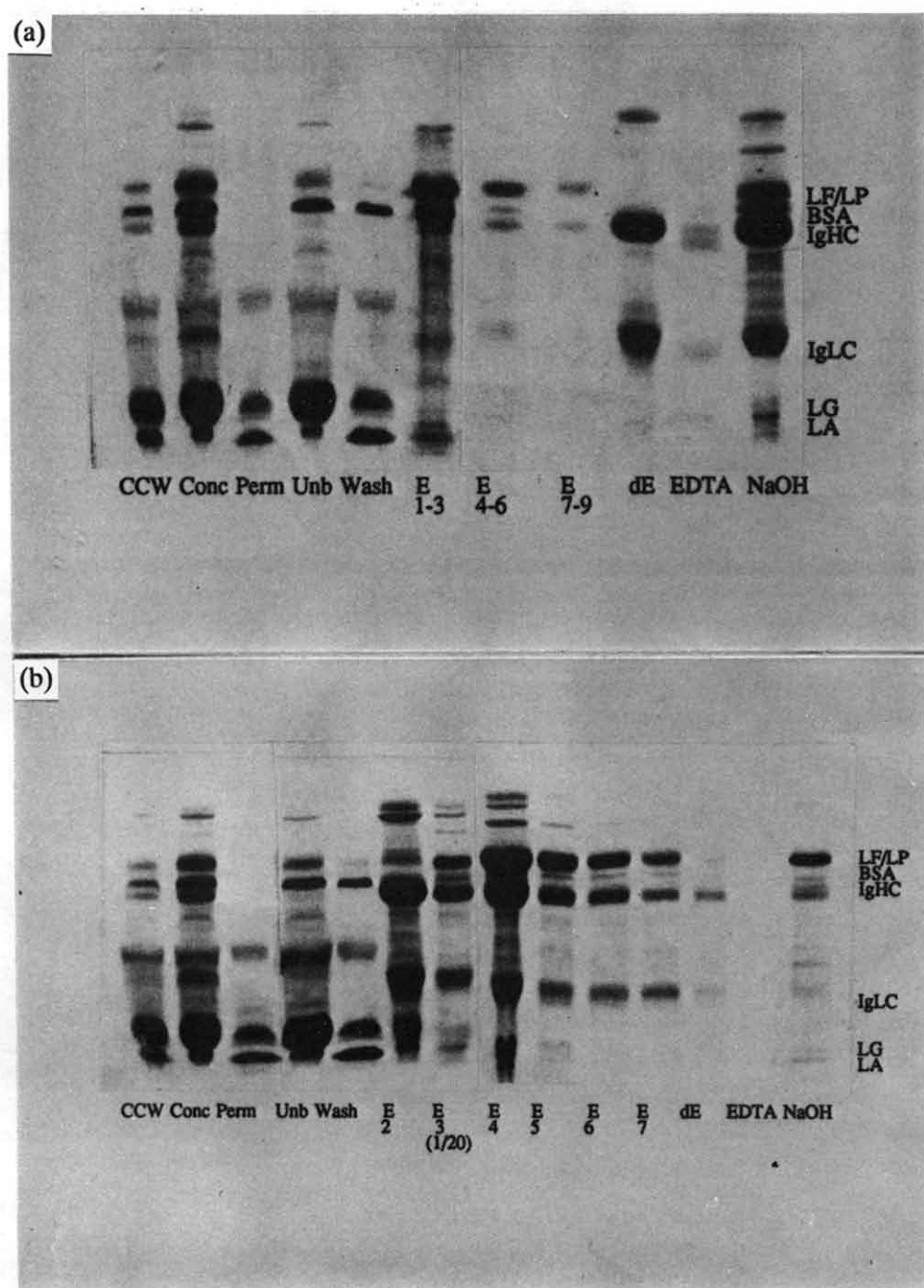


Fig. 3. Reduced SDS-PAGE profiles of fractions eluted (a) with 1 M NH_4Cl and distilled H_2O ; (b) with 50 mM glycine and distilled H_2O after Cu-IMAC treatment of concentrated whey. Abbreviations for fractions: CCW, cheddar cheese whey; Conc, concentrate (retentate); Perm, permeate; Unb, unbound; Wash, wash; E, eluted peak (numbers are fraction numbers, where fraction volume = 25 ml); dE, peak eluted with distilled H_2O ; EDTA, 0.033 M EDTA wash; and NaOH, 0.05 N NaOH cleaning. Abbreviations for bands: LP, lactoperoxidase; LF, lactoferrin; BSA, bovine serum albumin; IgHC, IgG heavy and light chains; LG, β -lactoglobulin; and LA, α -lactalbumin.

ing buffer rather than NH_4Cl , especially when using concentrated whey. By using glycine, isolated IgG would not have to be treated for salt removal and glycine could add to the nutritional value. A disadvantage of using glycine is that the Cu front tended to migrate during elution. The

possibility of Cu leaching could be minimized by adjusting the amount of copper loading, though.

Figure 4 illustrates the scheme recommended for IgG isolation using UF and IMAC. Using this scheme, an average of 1202 mg of IgG with a standard deviation of 85 mg was recovered in five

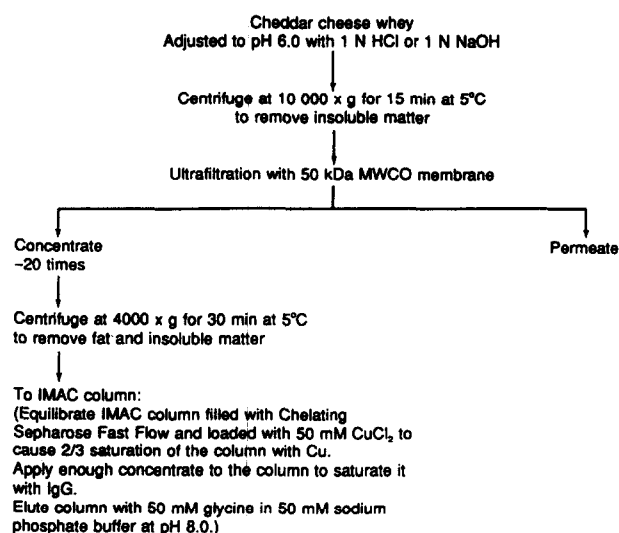


Fig. 4. Final method to isolate IgG from cheddar cheese whey using ultrafiltration and immobilized metal affinity chromatography.

trials using a 50-ml CSFF column loaded with 17.3 ml of 50 mM CuCl_2 . It should be noted that IMAC was run at room temperature, but it was also run at refrigeration temperature. The recovery and purity of the IgG obtained were similar, but there was a slight loss in IgG recovery when run at refrigeration temperature. It was also noted that the distribution of IgG in the eluted fractions varied. There was 8 times more IgG in the fraction eluted with distilled water and an increase in IgG in the NaOH cleaning effluent. This indicated that temperature can affect binding of IgG to IMAC columns.

Effect of whey on the isolation of IgG

The best recovery of IgG was obtained using concentrated cheddar cheese whey. Concentrated mozzarella whey and acid whey could also be used but with a 10% loss in IgG recovery. Commercially concentrated mozzarella whey was used for IMAC with similar results in IgG recovery as for mozzarella and acid whey. The purity of the recovered IgG from concentrated mozzarella and acid whey remained similar to that for concentrated cheddar cheese whey.

Isolation of IgG using ion exchange chromatography

Electrodialyzed CCW with a conductivity of 2.2 mmho was used for ion exchange chromatography. Table 6 shows the recovery and purity of IgG obtained using ion exchange chromatography. The ca-

Table 6. IgG recovery and purity of fractions by ion exchange chromatography of electrodialyzed concentrated CCW at pH 5.4 and pH 5.0

Fraction	IgG recovered ^a (mg)	Purity of IgG ^b (g/100 g protein)
pH 5.4		
Applied concentrate (42 ml)	340	9.2
Unbound (76 ml)	310	9.4
Wash (200 ml)	Not detectable	—
Elution (0.25 M NaCl) (110 ml)	30	21.4
Elution (1 M NaCl) (69 ml)	8	6.7
pH 5.0		
Applied concentrate (42 ml)	260	9.6
Unbound (50 ml)	200	13.3
Wash (465 ml)	<1	<0.1
Elution (0.25 M NaCl) (158 ml)	90	36.0
Elution (1 M NaCl) (173 ml)	<1	<0.1

^aRecovered IgG calculated as IgG activity (mg/ml) by radial immunodiffusion $\times$ volume (ml) of fraction. Values are the average of duplicates. The differences between duplicate measurements were less than 10%.

^bPurity of IgG (g/100 g) calculated as IgG activity by radial immunodiffusion/total protein in fraction $\times$ 100. Protein content was measured by the biuret method. (Means $\pm$ S.D.; $n = 3$. Values are the average of duplicates. The differences between duplicate measurements were less than 10%.

capacity of the ion exchange column was 1.47 mg of IgG/ml of gel with 35% purity at pH 5.0, and 0.47 mg of IgG/ml of gel with 20% purity at pH 5.4. The SDS-PAGE profiles for fractions eluted from the ion exchange column shown in Figure 5 indicates contamination of IgG by other whey proteins.

Although ion exchange chromatography had a lower capacity for IgG than IMAC, the cost of the ion exchange gel was about 30 times cheaper than the IMAC gel. Accounting for the differences in capacity of IMAC and ion exchange chromatography, IMAC was still about twice as expensive as ion exchange chromatography. The difference in cost may not be substantial after considering the need for electrodialysis of the whey using ion exchange chromatography, the smaller volumes of buffer required for IMAC compared to ion exchange chromatography, and less wear on pumps and savings in space of the columns for IMAC. By using glycine for elution in IMAC, there would also be no need to remove salt from IgG, whereas salt would have to be removed from eluted IgG using ion exchange chromatography. Considering IMAC gives a higher purity of IgG, the end product from IMAC would be more valuable. The same IMAC columns were used over 20 times without any problems, and could probably be reused even more, which would reduce costs.

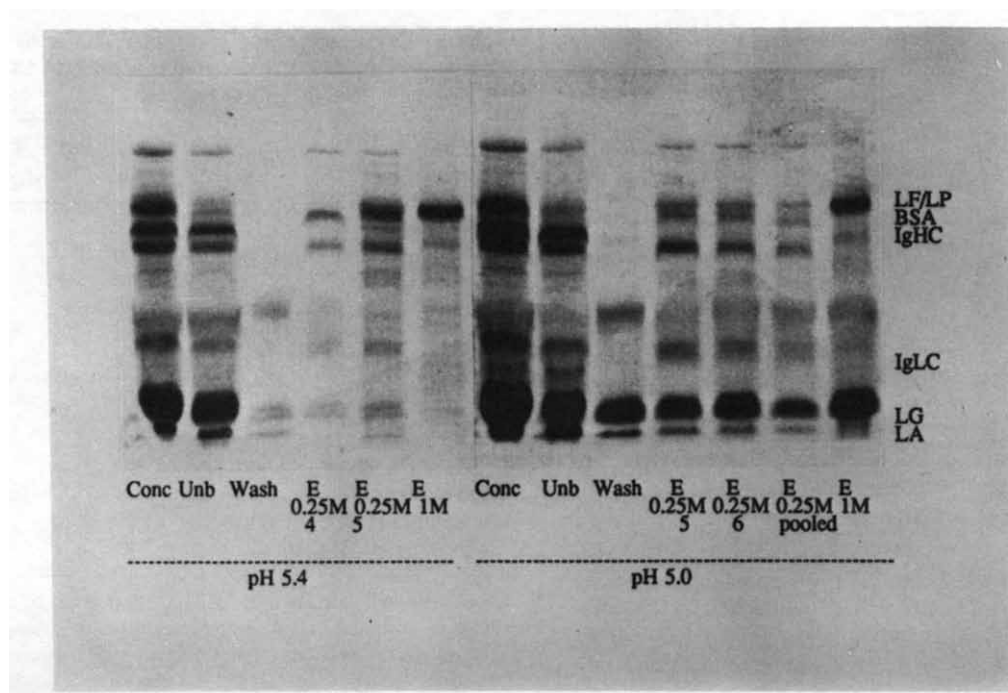


Fig. 5. Reduced SDS-PAGE profiles of fractions eluted from ion exchange chromatography at pH 5.4 and 5.0. Abbreviations for fractions: Conc, concentrate (retentate); Unb, unbound; Wash, wash; E 0.25 M, peak eluted with 250 mM NaCl in buffer (numbers are fraction numbers, where fraction volume = 12 ml); and E 1M, peak eluted with 1.0 M NaCl in buffer. Abbreviations for bands: LP, lactoperoxidase; LF, lactoferrin; BSA, bovine serum albumin; IgHC, IgG heavy and light chains; LG, β -lactoglobulin; and LA, α -lactalbumin.

Table 7. Composition of permeate and concentrate fractions obtained from the ultrafiltration of skim milk to isolate IgG

Fraction	pH	Conductivity ^a (μ mho)	Lactose concentration ^b (mg/ml)	IgG concentration ^c (mg/ml) (IgG(mg))	Protein concentration ^d (mg/ml) (Protein(mg))
Skim milk (5000 ml)	6.67	4630	55.9 $\pm$ 4.7	0.79 (3950)	21.2 $\pm$ 0.4 (106 000 $\pm$ 2000)
5 $\times$ concentrate of 500 kDa MWCO	6.44	3704	45.5 $\pm$ 1.5	2.94 (2940)	89.7 $\pm$ 6.2 (90 000 $\pm$ 6200)
Permeate of 500 kDa MWCO	6.52	5000	53.4 $\pm$ 1.4	0.17 (680)	1.1 $\pm$ 0.1 (4400 $\pm$ 400)
31 $\times$ concentrate of 50 kDa MWCO	5.54	5182	57.9 $\pm$ 1.8	5.14 (663)	18.5 $\pm$ 1.2 (2400 $\pm$ 160)
Permeate of 50 kDa MWCO	6.60	4907	53.5 $\pm$ 1.3	Not detectable	0.6 $\pm$ 0.2 (2300 $\pm$ 800)

^aCorrected for 18°C.

^bMeasured using the sulfuric acid and phenol colorimetric method of Lawrence (1968). Means $\pm$ S.D.; $n = 4$.

^cMeasured by radial immunodiffusion. Values are the average of duplicates. The differences between duplicate measurements were less than 10%.

^dMeasured using the biuret method. Means $\pm$ S.D.; $n = 3$.

Isolation of IgG from skim milk

Figure 6 outlines a scheme proposed to isolate IgG from skim milk. In countries where whey is not available, milk would be the preferred source of IgG. After extracting IgG using UF, the concentrate from the 500 kDa MWCO membrane and the permeate from the 50 kDa MWCO membrane could be recombined. This solution would have the same composition as skim milk minus some protein, lactose and minerals, and could be

used to make skim milk powder. The unbound fraction from the IMAC column could also be combined with the solution to add back more of the lost components.

Table 7 shows the composition of permeates and concentrates when using 5 litres of skim milk. Only about 20% of the IgG in the milk was recoverable in the final concentrate. However, if large volumes of milk are processed, sufficient IgG could still be available. The recovery of IgG was improved to about 40% by concentrating the milk

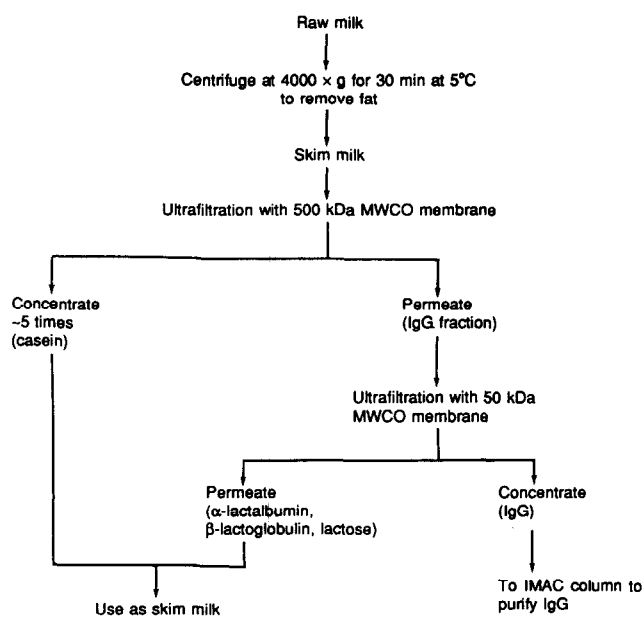


Fig. 6. Method to isolate IgG from skim milk using ultrafiltration and immobilized metal affinity chromatography.

7–8 times or by using diafiltration. It may be possible to use different UF membranes to improve recovery as well. Table 8 shows the recovery of IgG from the final concentrate obtained after IMAC. The recovery of IgG was 35% lower than that obtained with concentrated whey. The recovery may have been greater if glycine was used in the elution buffer since only 48% of IgG applied to the column was recovered using NH_4Cl . Glycine, being a stronger competing ligand than NH_4Cl , would displace more bound protein.

Table 8. IgG recovery and purity of fractions obtained by IMAC of skim milk processed by UF using a 10-ml Chelating Sepharose Fast Flow column loaded with 2.2 ml of 50 mM CuCl_2

Fraction	IgG recovered ^a (mg)	Purity of IgG ^b (g/100 g protein)
Applied 31 × concentrate of 50 kDa MWCO (51 ml)	260	27.6
Unbound (49 ml)	11	2.3
Wash (144 ml)	Not detectable	—
Elution (NH_4Cl) (57 ml)	40	54.1
Elution (dH_2O) (40 ml)	67	100.0
EDTA wash (17 ml)	13	86.7

^aRecovered IgG calculated as IgG activity (mg/ml) by radial immunodiffusion × volume (ml) of fraction. Values are the average of duplicates. The differences between duplicate measurements were less than 10%.

^bPurity of IgG (g/100 g) calculated as IgG activity by radial immunodiffusion/total protein × 100. Protein content measured by the biuret method. (Means ± S.D.; $n = 3$). Values are the average of duplicates. The differences between duplicate measurements were less than 10%.

In conclusion, UF combined with IMAC could be an efficient method to isolate immunoglobulins from whey and skim milk. The isolated immunoglobulins could be used for fortification of animal feeds, infant formulas, or products for older children or adults for prophylactic benefits.

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