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Separation of Immunoglobulins and Lactoferrin Directly from Skim Milk by Metal Chelate Interaction Chromatography

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Abstract. Metal chelate interaction chromatograpy (MCIC) was utilized to separate immunoglobulins (Igs) and lactoferrin (Lf) directly from skim milk. Using trisacetate buffer pH 8.0 system, it was found that casein fractions were strongly bound to immobilized copper ions on MCIC column. This behaviour of casesins decreased the ability of Igs and Lf to bind to copper ions. Radial immunodiffusion indicated that Igs and Lf were found in the unbound and washed fractions of skim milk. However, when buffer system was changed to 0.02 M phosphate it was found that casein fractions could be first eluted while Igs and Lf were retained on the column as indicated by sodium dodecyl sulfate poly acrylamide gel electrophoresis (SDS-PAGE). Igs and Lf bound to the MCIC column could then be recovered by using tris-acetate buffer pH 4.0 or 0.01 M imidazole solution. The role of histidine and sulfhydryl groups in the interactions were investigated.

Introduction

Porath *et al.* [1] demonstrated that the ability of proteins to bind metal ions can be utilized to separate the proteins. These investigators attached metal ions to a chelate, covalently bound to an insoluble matrix. The chelating ligand, iminodiacetate, was immobilized to oxirane-activated agarose. This method, named "immobilized metal affinity chromatography" (IMAC) by Porath *et al.* [2], was later called "metal chelate interaction chromatography" (MCIC) by Rassi and Horvath [3].

As explained by Lonnerdal and Keen [4], the binding of proteins is believed to be the result of the ability of electronrich ligands, such as histidine, cysteine, and tryptophan, to substitute weakly bounded 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, this protein can bind strongly by multipoint attachment. This binding is stable even in IM 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 an iron binding protein is in Fe-saturated form or in an apo-form. Fe-saturated lactoferrin can bind to copper-loaded gel as strongly as the apo-form of lactoferrin [4,5].

The application of MCIC has been reported for separation of human serum proteins [2,6,7], human lactoferrin [5], lysozyme [8], human fibroblast interferron [9] and human serum albumin [10]. In our laboratory, we succeeded in isolation of immunoglobulins and trasferrin from bovine serum [11], ovotransferrin from egg white [12], and immunoglobulins and lactoferrin from cheddar cheese whey [13]. In this paper, the feasibility of using this method to isolate immunoglobulins and lactoferrin directly from skim milk is investigated, and the mechanism of metal-casein interaction is discussed.

Materials and Methods

Cow's skim milk was obtained from the local market. Alpha-casein, IgG and diethyl pyrocarbonate (DEP) were purchased from Sigma Chemical Company (St. Louis, MO). Beta-casein was purchased from Chemlog (South Plainfield, NJ). K-casein was prepared according to the method of Zittle and Custer [14]. Alpha-S1-casein was a gift from Dr. Yada and blocked sulfhydryl group K-casein (sss-k-casein) was prepared by the method of Liu [15] with sodium tetrathionate.

Synthesis of chelating gel

Sepharose 6B (Pharmacia Fine Chemical) was activated and cross-linked using 1,4-butanediol diglycidyl ether (BGE, purchased from Eastern Kodak Company, Rochester, NY) according to Sunderg and Porath [16]; iminodiacetic acid (IDA, from Sigma Chemical Co., St. Louis, MO) was coupled to the activated gel by the procedure of Porath and Olin [17] for preparation of IDA-Sepharose 6B.

Chromatograpy

IDA-BGE Sepharose 6B was packed into a column (1.4 × 7.0 cm). The upper one-half of the column was saturated with copper ions by passing 0.05 M $CuCl_2$ through the column as indicated by the blue colour, followed by washing with at least two bed volumes of starting buffer (0.05 M Trisacetate/0.5 M NaCl pH 8.2 or 0.02 M phosphate buffer containing 0.5 M NaCl, pH 7.0). After sample application, the unbound material was washed using the starting buffer; the bound proteins were then eluted with 0.05 M Trisacetate /0.5 M NaCl, pH 3.0 and/or 0.01 M imidazole (see Figures for details).

Histidine modification of caseins

Histidine residues of casein fractions were modified according to the method of Rogers *et al.* [18] with modifications. An aliquot of DEP was added directly to a stirring protein solution (25 ml containing 4-8 mg protein per ml solution in 0.05 M phos-

phate buffer, pH 6.6 containing 8 M urea). The reagent was added to make a final concentration of 20 mM. After 20 min. the ethoxyformyl histidine formed was determined from an increase in absorbance at 240 nm of the reaction solution (E 240 = 5900 L/M cm as reported by Roosemont [19]. The purity of DEP was determined according to Holbrook and Ingram [20].

SDS-Polyacrylamide gel electrophoresis

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out in a vertical slab unit (electrophoresis apparatus SJ–1060 SDH, Atto Co., Tokyo, Japan) according to the method of Laemmli [21]. Stacking gel and separating gel were 3% (in 0.125 M Tris-HCl buffer, pH 6.8 and 10% (in 0.375 M Tris-HCl buffer, pH 8.8) polyacrylamide, respectively; both gel containing 0.2% SDS. The electrophoresis buffer was 0.025 M Tris-0.192 M glycine, pH 8.3 containing 0.1% SDS. Samples were dissolved in stacking gel buffer containing 5% SDS and 2% mercaptoethanol,heated for 1.5 min in a boiling water bath and subjected to electrophoresis. The electrophoresis was performed at 100 V for 4–5 hr. Bromophenol blue was used as tracking dye to monitor sample migration. The staining solution of Weber and Osborn [22] consisting of 0.25% Coomassie Brilliant blue R–250 in acetic acid/methanol/water (1:5:5) was used. The destaining solution was acetic acid/methanol/water (7:5:88).

Immunochemical analysis

Immunochemical quantitation of immunoglobulin G (IgG) was carried out by radial immunodiffusion (R.I.D.) with a Kit (Miles Laboratories, Elkhart, IN). Protein content was determined using Bio-Rad Protein Assay Kit (Bio-Rad Laboratories, Mississauga, Ontario).

Results and Discussion

MCIC of skim milk

Immunogluobulins and lactoferrin were isolated quite easily and efficiently from whey proteins by Cu-chelate agarose media [13]. In an attempt to separate immunoglobulins directly from skim milk, two different elution methods, using Cu-chelate agarose column, were applied.

a – Elution with Tris-acetic acid buffer

Fig. 1 shows the elution profile of skim milk 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 absorbed proteins were eluted with 0.05 M Trisacetate/0.5 M NaCl at pH 4.0. However, the amount of proteins eluted under acidic conditions (F1) were small, and the flow rate became quite slow, indicating precipitation of casein thereby clogging the column. This phenomenon was observed whether skim milk was diluted or undiluted. Sebsequent elution with 0.01 M imidazole recovered the

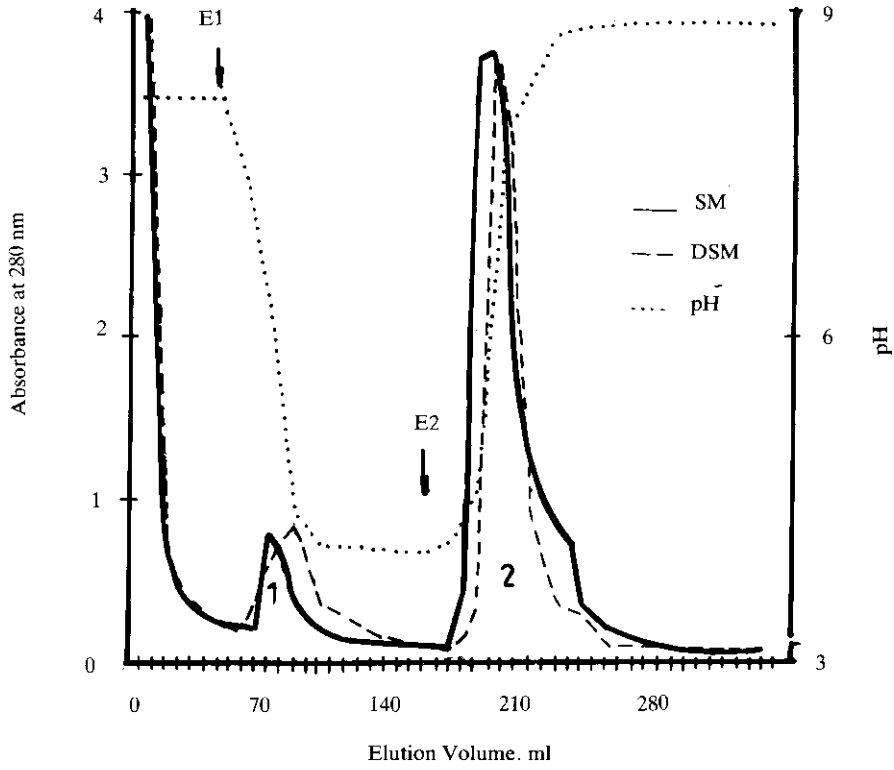

Fig. 1. Elution profile of skim milk on MCIC column. One hundred milliliters skim milk (undiluted or 50% diluted with 0.5 M Trisacetate/0.5M NaCl) were passed through Cu-loaded sepharose 6B, and washed (W) with same buffer E1, elution with the same buffer at pH 4.0; E2, elution with 0.01 M imidazole solution. The flow rate was 21 ml/hr.

majority of bound proteins (F2). SDS-PAGE analysis (Fig. 2) indicated that proteins eluted in the washing step were immunoglobulins (Ig) and lactoferrin (Lf) in addition to a-lactalbumin, B-lactoglobulin and casein, while proteins eluted under acidic conditions were mainly Ig and Lf. However, the fraction eluted with 0.01 M imidazole was mainly casein.

Table 1 shows the IgG distribution of fractions obtained at different stages of the isolation process of IgG from skim milk. Immunological analysis showed that the majority of active IgG was present in the fraction eluted at acidic pH (pH 4.0) with more than 84% purity. The amount of IgG bound to the column was quite small (less than 10%) as compared to the unbound fraction of skim milk. In the washing fraction, more than 20% pure IgG was detected in this fraction which indicated that IgG

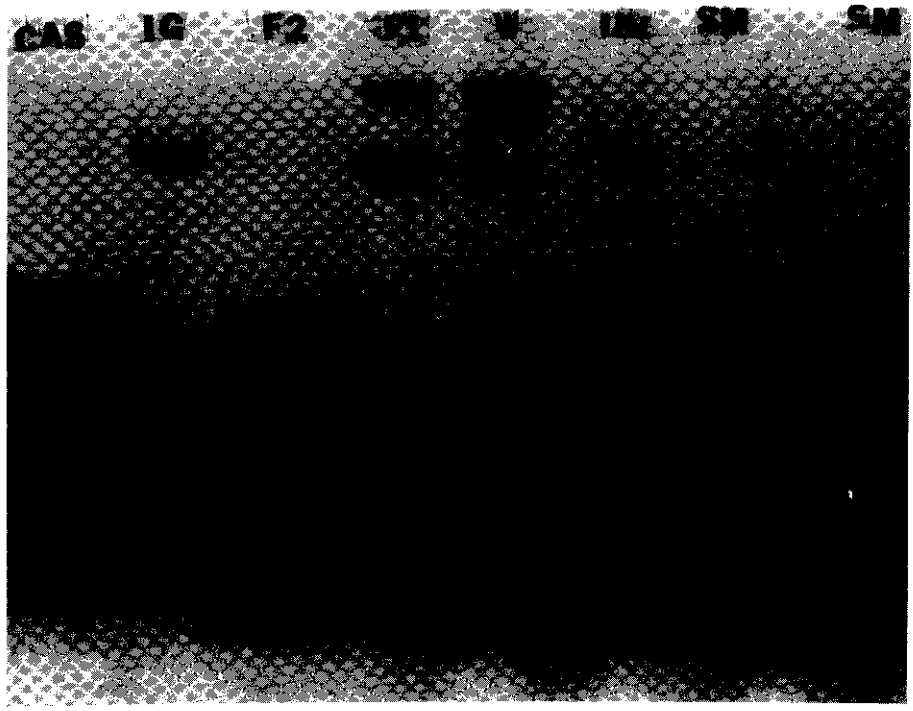

Fig. 2. SDS-PAGE of fractions obtained in Fig. 1. Sm, skim milk; Un, unbound skim milk to MCIC column; W, washing step fraction; F1, F2, fractions 1 and 2 in Fig. 1; IgG, standard immunoglobulin G, Cas, alpha-casein.

of skim milk bound rather weakly to the column. After eluting the strongly bound material with strongly competing electron-donor solution (0.01 M imidazole), this fraction appeared to contain almost no IgG, however, this fraction was rich in casein components (Fig. 2). These findings indicate that there is a competition between IgG and caseins to bind copper ions linked to agarose, and the casein fraction binds more strongly than immunoglobulins to the column under these conditions.

b – Elution with phosphate buffer

In an effort to establish conditions by which casein can be eluted while Ig and Lf stay on the column, Fig. 3 shows the elution profile of a mixture of skim milk and Ig 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, bound proteins were then eluted with 0.01 M imidazole and 0.05 M Trisacetate/0.5 M NaCl, pH 3.8 to obtain F1 and F2 respectively. Electrophoretic analysis (Fig. 4) indicated that under this condition, the

Table 1. IgG content of different stages of the isolation of IgG from skim milk on MCIC column

Sample	Protein[a] conc. of selected fraction mg/ml	IgG[b] conc. mg/ml	IgG purity %
Skim milk (control)	37.8	0.562	1.49
Unbound skim milk	18.0	0.246	1.36
Washing fraction	4.0	0.828	20.70
Peak 1 (Figure 8B)	1.08	0.911	84.35
Peak 2 (Figure 8B)	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.

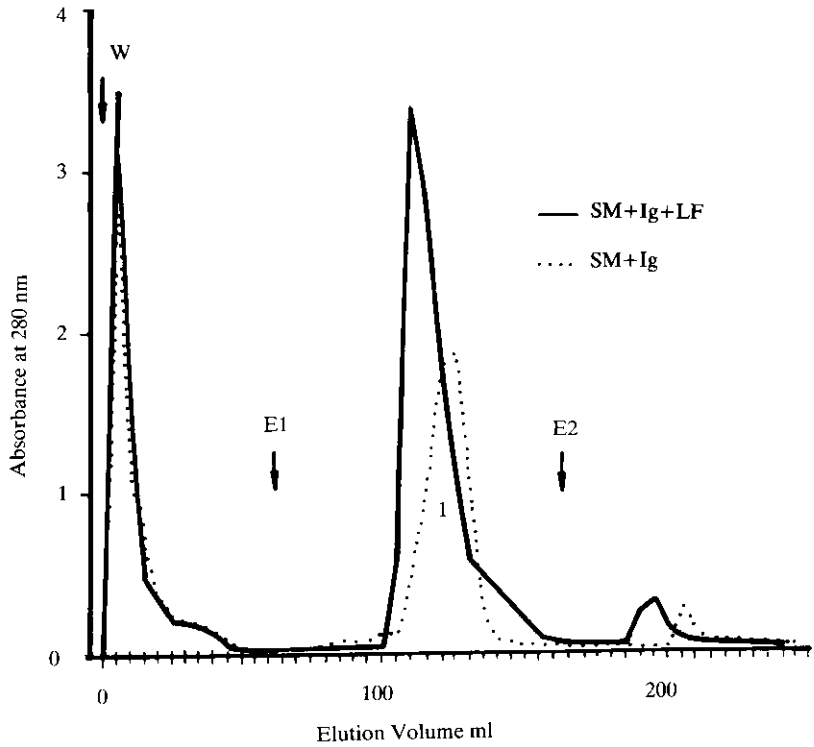

Fig. 3. Elution profiles of skim milk, Ig and Lf mixture on MCIC column. 60 ml of Ig and Lf were mixed with 1 ml skim milk and passed through MCIC column. W, washing with 0.02M phosphate buffer containing 0.5M NaCl, pH 7.0; El, elution with 0.01M imidazole; E2, elution with Trisacetate/ 0.5M NaCl, pH 3.0.

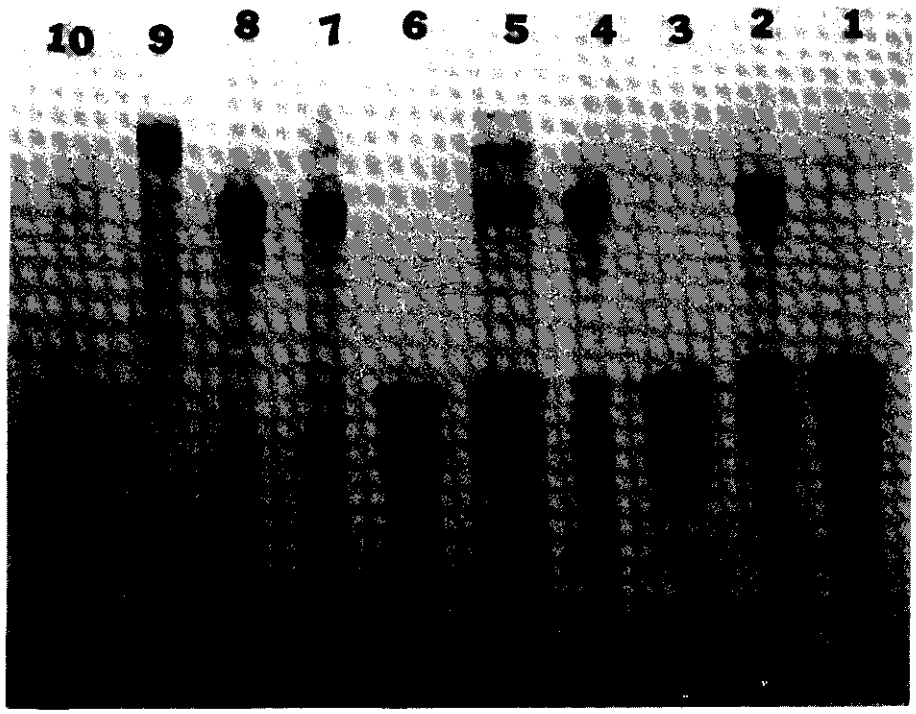

Fig. 4. SDS-PAGE of fractions obtained in Fig. 3. 1, skim milk; 2, skim milk and Ig mixture; 3, 4 and 5 are unbound, F1 and F2 of skim milk-Ig mixture application, respectively; 6, skim milk-Ig-Lf mixture; 7, 8 and 9 are unbound, F1 and F2 of skim milk-Ig-Lf application respectively; 10, 11 and 12 are standard Ig, Lf and alpha-casein respectively.

unbound proteins eluted with 0.01 M imidazole (F1) were immunoglobulins. Fraction (F2) eluted with Trisacetate/0.5 M NaCl, pH 3 was too small to detect by SDS-PAGE.

Fig. 3 shows the elution profile of a mixture of Ig and Lf in the presence of caseins in skim milk, under the same condition on MCIC column. Similar pattern to that of skim milk-Ig mixture was obtained; however, the amount of protein bound to the column was increased. SDS-PAGE profile (Fig. 4) indicated that the unbound fraction was basically casein fraction, while proteins eluted with 0.01 M imidazole were mixture of Ig and Lf. These bound proteins (Ig and Lf) could be separated by using gel filtration or 0 to .01 M imidazole gradient (unpublished data).

These results indicate that the type of ions in the buffer used for initial column equilibration and washing has great influence on proteins bound or unbound on the column of MCIC. Since caseins are classified as phosphoproteins [23]; it is believed that using phosphate buffer in the equilibration step of the column form a complex

with the copper ion (different color from that formed with Tris-acetic acid buffer) which may prevent the phosphoproteins from binding to the column the results also indicate the involvement of phosphoserine groups in the interaction with the copper ions which is dependent on the type of the negative radical of the buffer salt.

Mechanism of casein-metal interaction

The principle of separation of proteins by the MCIC column process lies in their different affinities for binding to immobilized metal ions, and is suggested to depend on availability of histidine, cystine and tryptophan residues of the proteins which form stable coordination with metal ions [24]. In general, absorption of protein to MCIC is performed at a slightly alkaline pH at high ionic strength to reduce non-specific electrostatic interactions. Elution is commonly accomplished by lowering the pH, which reverses protein coordination to metal-chelate and results in protein displacement. Alternatively, 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 or copper loaded column at pH 6.0, [3], elution profile of histidine-modified casein fractions was studied.

Alpha-casein

Alpha-casein represents more than 62.5% of total casein fraction in cow's milk and is composed of alphs-s and k-casein in ratio of 4:1.

Fig. 5 represents the elution profile of control alpha-casein and histidine-modified alpha-casein. After loading alpha-casein on Cu-chelate gel and washing with the starting alkaline pH buffer, only 10.7% of the total applied protein could be eluted under these conditions and the rest of protein (89.2%) (Table 2) was displaced by using a mild chelating agent i.e. 0.01 M imidazole. However, blocking 3.7 histidine residues by diethyl pyrocarbonate out of a total of 4 histidine per mole alpha-casein [25, pp 93-94] would increase the amount of unbound protein from 10.7% to 82.2% (Table 2) indicating the involvement of histidine groups in the interaction with copper ion. Less than 18% of protein bound was eluted with 0.01 M imidazole, which indicated the involvement of other amino acids (i.e. Trp, Cys, Tyr) in the interaction.

Alpha-s1 and beta-casein

Alpha-s1-casein is a subfraction of casein fraction which represents 50% of total casein proteins. Fig. 6 represents the elution profiles of alpha-s1-casein and histidine blocked alpha-s1-casein. Without modification of histidine residues of alpha-s1-casein, only a small amount (6.1%) of the protein applied was stripped off the column in the washing step and 93.9% of protein interacted with the copper under alkaline pH and was subsequently eluted with the eluting solution (Table 2). However, blocking 4.2 histidine residues by DEP out of a total of 5 histidine residues per mole alpha-s1-casein [23] would cripple the protein's ability to bind copper ion

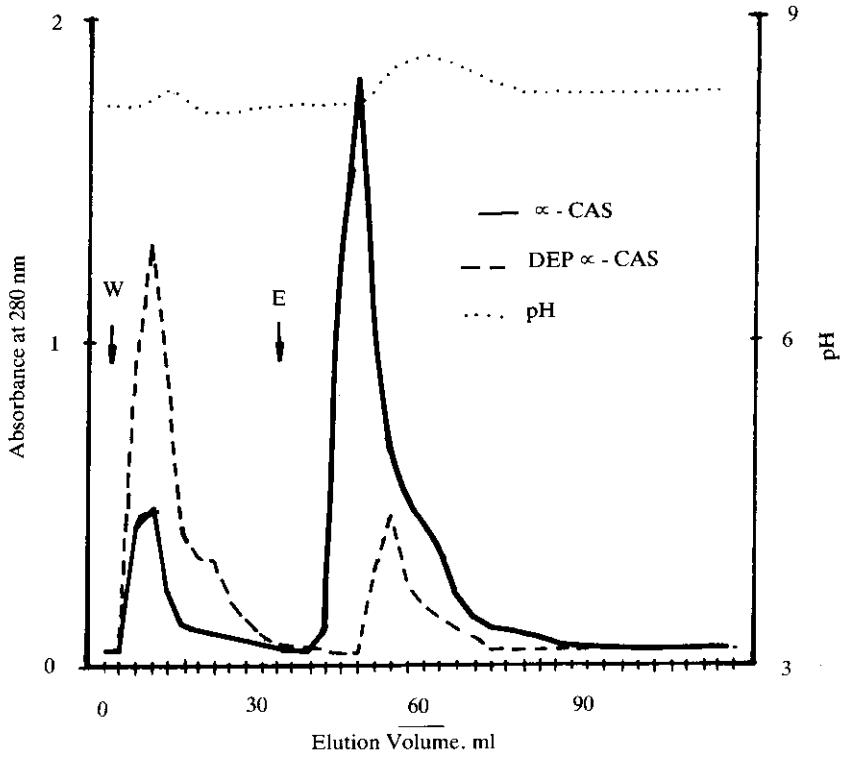

Fig. 5. Metal chelate interaction chromatography of alpha-casein. 3 ml of protein with and without diethylpyrocabonate modification were equilibrated with 0.05M Tris-acetate/0.5M NaCl, pH 8.2 and applied to Cu-chelate Sepharose 6B. W, washing with the same equilibrating buffer; E, elution with 0.01M imidazole. Flow rate was 30 ml/hr.

immobilized on agarose. Based on total absorbance unit calculation, 94.3% of applied protein was recovered in the washing step, while only small amount of applied sample (5.7%) was bound and subsequently eluted with 0.01 M imidazole.

The second major protein of bovine casein is beta-casein which represents 30% of total casein fraction. Fig. 7 shows the elution profiles of beta-casein before and after histidine modification with DEP. Unbound fraction of control beta-casein represented only 13.6% while the unbound fraction of DEP treated beta-casein accounted for 54.7% of protein applied (Table 2). This indicated that even though 4.4 histidine residues were modified out of a total of 5 histidine residues per mole beta-casein [23], 45.3% of protein applied was bound to the copper ion and subsequently eluted with 0.01 M imidazole solution. This might also indicate that some other amino acids and its availability were involved in the interaction with copper ions.

Table 2. 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 k-casein	87.9	12.1
DEP-Polymer-k-casein	94.3	5.7
SSS-k-casein	38.5	61.5
DEP-SSS-k-casein	98.9	1.1

[a] Calculated based on total absorbance units
[b] See Figures 52, 53, 54 and 55 for abbreviation identity.

K-casein

The k-casein represents 12.5% of total casein in cow's milk, and exists in the form of a mixture of polymers of k-casein held together by intermolecular disulfide bonds [23]. K-casein prepared by the method of Zittle and Custer [14] was considered to be assuming an aggregate structure. Fig. 8A shows the elution behaviour of this preparation, with and without histidine groups modification, on Cu-chelate agarose. The amount of unbound k-casein for control and histidine modified k-casein were 87.9% and 94.3%, respectively (Table 2), indicating that the amount of adsorbed k-casein on Cu-chelate and recovered by 0.01 M imidazole was quite small for control k-casein. This behaviour of polymer k-casein on MCIC column might be due to its high molecular weight and its aggregated structure which probably restrict the interaction with metal ions. To demonstrate whether the aggregated structure of k-casein would effect copper-k-case interaction, MCIC of monomer k-casein was performed. Fig. 8B shows the elution profiles of k-casein monomers formed by reducing disulfide bonds and protecting them with sodium tetrathionate. The amount of bound protein in reduced k-casein was more than 61% of the total sample applied, compared to 12.1% of polymer k-casein adsorbed onto the same column (Fig. 8A). However, blocking 2.7 histidine residues out of 3 histidine residues per mole of monomer k-casein [23] reduced the amount of boundmodified k-casein to 1.1%, which demonstrates the involvement of histidineresidues in the interaction of k-casein with copper ions. This might also indicate that disulfide bonds of polymer k-casein had no role in the interaction with metal ions.

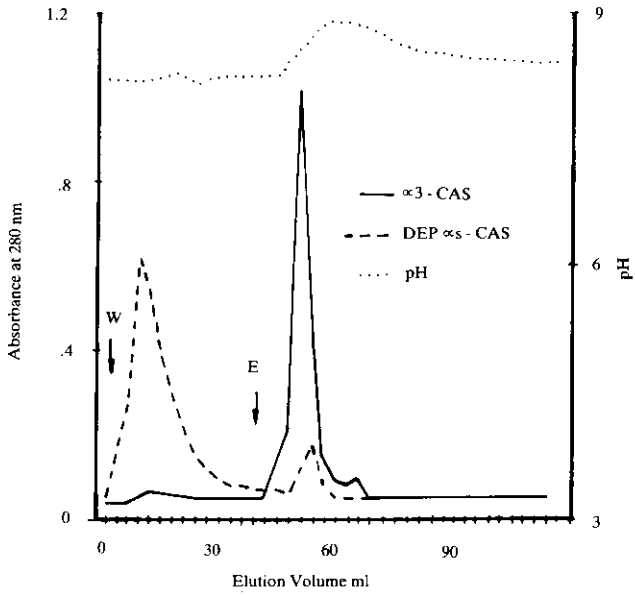

Fig. 6. Metal chelate interaction chromatography of alpha-sl-casein. See Fig. 5 for conditions of separation.

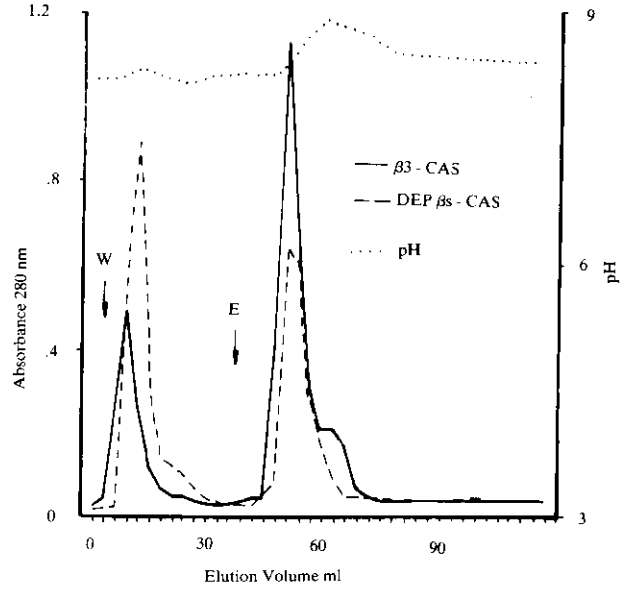

Fig. 7. Metal chelate interaction chromatography of beta-casein. See Fig. 5 for conditions of separation.

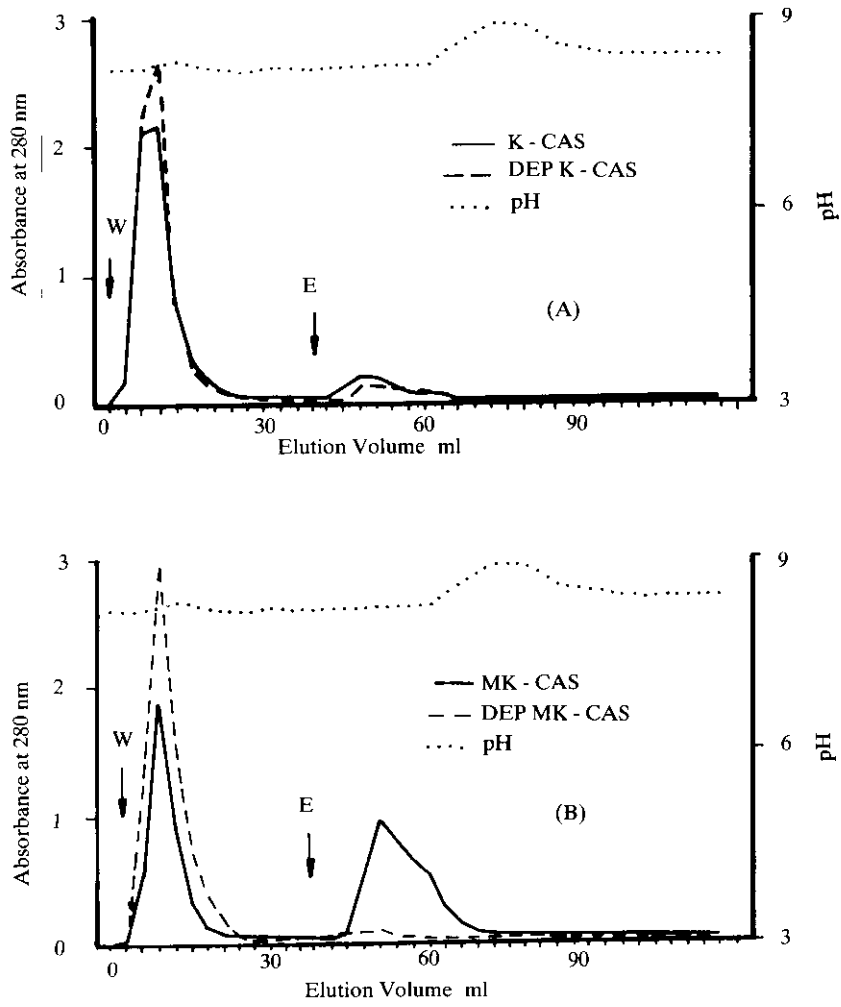

Fig. 8. Metal chelate interaction chromatography of (A) polymer k-casein and (B) monomer k-casein. See Fig. 5 for conditions of separation.

Conclusions

In conclusion, the use of the skim milk directly as a starting material for Ig and Lf separation is feasible. Using Trisacetate buffer as an equilibrating buffer provokes competition between Ig and caseins to bind copper ions and then decreases the

capacity of MCIC column to bind and retain immunoglobulins. However, the use of phosphate buffer as an equilibrating buffer prevents phosphoproteins from binding to the chelating gel and allows these phosphoproteins to be collected as unbound fraction. Whereas Ig and Lf retained on the column can subsequently be eluted separately. Under these conditions Ig and Lf may be collected directly from skim milk and used for nutritional or pharmaceutical purposes. Under Trisacetate buffer conditions, chemical modification studies indicate the involvement of histidyl residues of casein fractions in the interaction with copper ions.

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فصل بروتينات المناعة واللاكتوفيرين من الحليب الفرز مباشرة باستخدام كروماتوجرافي الألفة المعدنية

شعلان علوان المشايخي و شوريو نقاي

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ملخص البحث. استعمل في هذه الدراسة كروماتوجرافي المعادن المرتبطة لفصل بروتينات المناعة واللاكتوفيرين مباشرة من الحليب الفرز. حيث استعمل محلول منظم ترس الخلات عند أسس هيدروجين مقداره (٨) ووجد أن مكونات الكازين قد ارتبطت بقوة على أيونات النحاس الموجودة على عمود كروماتوجرافي المعادن المرتبطة. هذا الارتباط قلل من قابلية بروتينات المناعة واللاكتوفيرين للارتباط مع أيونات النحاس. وعند استعمال التنافذ المناعي الشعاعي وجد أن بروتينات المناعة واللاكتوفيرين كانت في الجزء غير المرتبط من الحليب الفرز أما عند استعمال محلول منظم الفوسفات فقد لوحظ إزاحة الكازين من العمود أولاً وبقاء بروتينات المناعة واللاكتوفيرين مرتبطة كما هو موضح في الفصل بالهجرة الكهربائية على هلام الأكريلامايد الحاوي على مواد مفككة. هذه البروتينات المرتبطة بالعمود يمكن إزاحتها إما باستعمال محلول منظم الخلات عند أس هيدروجين مقداره (٤) أو باستعمال محلول الأميدازول بالإضافة إلى ذلك فقد تم دراسة دور المستيدين والمجاميع الكبريتية في التداخلات الحاصلة في العمود أعلاه.