ISOLATION OF LACTOFERRIN FROM HUMAN MILK BY METAL-CHELATE AFFINITY CHROMATOGRAPHY

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1. Introduction

Lactoferrin, the iron-binding protein of breast milk, is considered to be of great importance for the breast-fed infant. It has been shown [1,2] that lactoferrin can bind iron in vitro and in vivo, thereby preventing the growth of iron-requiring microorganisms. In addition the iron of breast milk is much more readily absorbed by the infant than iron in commercial formulas, where it is present in the form of added iron-salts [3].

Lactoferrin was first isolated almost simultaneously by Johansson [4], Montreuil and Mullet [5] and Blanc and Isliker [6]. It has been called 'red protein' and 'lactotransferrin' [6,7] although the commonly accepted name is lactoferrin. It has a mol. wt 80 000 and is capable of binding 2 atoms of iron molecule. The concentration of lactoferrin in human milk varies from 3–4 mg/ml in early colostrum to 1–2 mg/ml in mature milk [8,9].

In order to further investigate the physiological role of lactoferrin, it is necessary to have a pure preparation of this protein. The only commercially available source contains about 20% impurities and the purification procedures described earlier are laborious. We have recently developed a new isolation procedure, metal-chelate affinity chromatography [10] which is based on the property of certain proteins to bind transition metals. A chelate-forming ligand is covalently bound to a gel-forming matrix. The chelate is then established by saturating the gel-bound ligand with the desired metal-ion.

The present paper describes the first application of the method. Since lactoferrin is capable of binding copper [11] as well as iron and since the copper-chelate–gel complex is much more stable than the iron complex, the copper-chelate–gel complex was chosen for the isolation of lactoferrin.

2. Materials and methods

2.1. *Human milk*

Mature human milk was obtained from healthy well-nourished Swedish mothers 2–3 months after parturition. The fat was removed by centrifugation at 5000 × *g* for 20 min. Casein was precipitated by adjusting the skim milk with acetic acid to pH 4.6 allowing to stand 1 h and centrifuging for 30 min at 15 000 × *g*. The resulting whey (100 ml) was then dialyzed against the starting buffer of the affinity chromatography.

2.2. *Commercial lactoferrin*

Lyophilized human lactoferrin was obtained from Wakodo Co. (Tokyo, Japan). The purity was designated 81% electrophoretically. Ten mg of the preparation was dissolved in 10 ml of the starting buffer.

2.3. *Metal chelate affinity chromatography*

The gel, bis-carboxymethyl amino agarose (Sepharose 4B) was prepared as described by Porath et al. [10]. The gel was packed in a column (3.2 × 5.5 cm) with total vol. 44 ml. The upper two-thirds of the column were saturated with copper-ions by frontal development with $CuSO_4$ (1 mg/ml). The bed thus prepared was washed with 0.05 M Tris–acetic acid buffer, pH 8.2 in 0.5 M NaCl. The copper-ion-free part of the column serves to prevent any copper-ion emerging in the effluent from the column. The sample was followed by one column vol. of starting buffer and then eluted with a linear-gradient consisting of 500 ml starting buffer and 500 ml Tris–acetate acid, pH 2.8 in 0.5 M NaCl or stepwise by Tris–acetic acid buffer, pH 4.0, in 0.5 M NaCl. An 18 ml/h flow-rate and 6 ml fraction vol. were used.

2.4. *Molecular sieve chromatography*

Separation of the material eluted from the Cu–gel was carried out on Sephadex G-150 (Pharmacia Fine Chemicals, Uppsala, Sweden) (2.0 × 104.0 cm column) equilibrated with the starting buffer 14 ml/h flow-rate and 4 ml fraction vol.

2.5. *Immunoelectrophoresis*

Quantitation of lactoferrin, secretory IgA and serum albumin was achieved by immunoelectrophoresis according to Laurell [12]. The 'fused rocket' technique described by Svendsen [13] was used to localize the specific proteins in the chromatograms. The agarose was alkali-treated to minimize electro-endosmosis [14]. Immunoelectrophoresis as described by Scheidegger [15] was used for the analysis of the eluted materials. Anti-human whey proteins were obtained from Dakopatts (Copenhagen, Denmark) and Boehringerwerke (Mannheim, FRG).

2.6. *Gel electrophoresis*

Polyacrylamide gel electrophoresis was performed by using a gel containing 8% acrylamide which in turn contained 2% bis-acrylamide (T_8C_2). The gels were run in 0.9 M acetic acid for 3 h with current 2 mA/gel. The staining solution was 0.1% Amido Black B in ethanol (20%) and acetic acid (7%). Excess stain was removed by washing with 7% acetic acid.

2.7. *Biological activity*

The bacteriostatic effect of lactoferrin was assayed by the inhibition of the growth of *Escherichia coli* with the method described by Reiter et al. [16].

3. Results and discussion

The metal-chelate affinity chromatography of the commercial lactoferrin preparation is shown in fig.1. The column was eluted stepwise with the pH 4.0 buffer. Immunoelectrophoresis by the method of Scheidegger [15] revealed that the material which passed through the metal-chelate column contained several proteins but no detectable amounts of lactoferrin. The eluted material contained the same amount of lactoferrin applied to the column and no other proteins.

The elution profile was somewhat different when human whey proteins were separated on the column (fig.2a). The change in pH of the influent was linear but the pH of the effluent was higher at the beginning of the gradient due to the buffering capacity of the participating ions. All of the lactoferrin was adsorbed by the gel and all was desorbed by gradient-elution. However, secretory IgA and serum albumin also were adsorbed by the gel and desorbed by gradient-elution (fig.2b). Serum albumin was separated from lactoferrin by adjusting the gradient of the eluting buffer but secretory IgA was not completely separated from lactoferrin. The separation of secretory IgA (mol. wt 370 000) and lactoferrin (mol. wt 80 000) was however easily achieved by gel-filtration on Sephadex

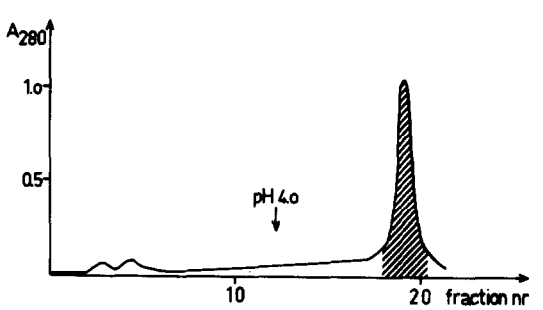

Fig.1. Metal-chelate affinity chromatography of a commercial lactoferrin preparation. The column (3.2 × 5.5 cm) was equilibrated in 0.05 M Tris–acetic acid buffer, pH 8.2, in 0.5 M NaCl. Elution: same buffer as above, at pH 4.0.

Fig.2a

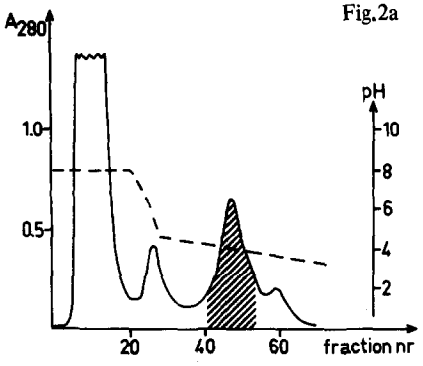

Fig.2b

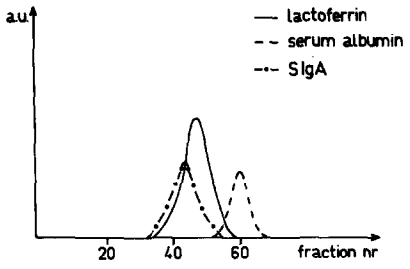

Fig.2(a) Metal-chelate affinity chromatography of human milk proteins. The column (3.2 × 5.5 cm) was equilibrated in 0.05 M Tris Tris–acetic acid buffer, pH 8.2, in 0.5 M NaCl. Elution: 500 ml Tris–acetic acid buffer, pH 8.2 – 500 ml Tris–acetic acid buffer pH 2.8 (both in 0.5 M NaCl). (b) 'Fused rocket' immunoelectrophoresis of the fractions from fig.2a against anti-IgA, anti-lactoferrin and anti-serum albumin.

G-150 (fig.3). Only one component was detected by gel electrophoresis (fig.4) following the gel-filtration. Immunoelectrophoresis [15] also indicated that the purified lactoferrin did not contain detectable amounts of contaminating proteins. Atomic absorption spectroscopy of the purified lactoferrin showed that no copper leaked from the column to the unsaturated lactoferrin and that no substitution of the lactoferrin-bound iron occurred. These findings coincide with the very strong binding of the copper to the gel and the weaker binding of iron.

The recovery of lactoferrin was quantitative and the capacity of the column appeared to be very high. In fact, only a few millimeters of the column, as detected by the red colour of lactoferrin, was occupied by the lactoferrin (200 mg) in the chromatographic run of fig.2a,

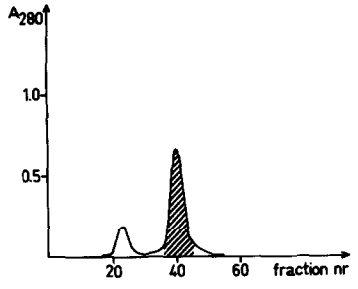

Fig.3 Gel-filtration of pooled material from fig.2a on Sephadex G-150 (2.0 × 104.0 cm) in 0.05 M Tris–acetic acid buffer, pH 8.2, with 0.5 M NaCl.

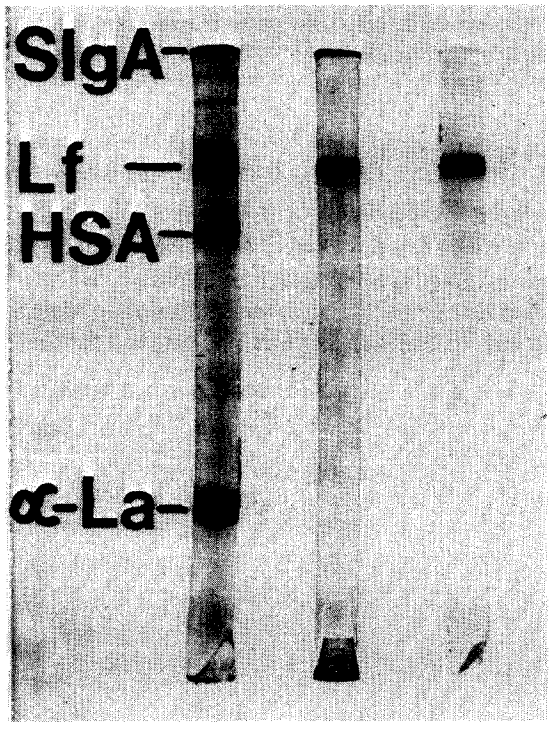

Fig.4. Polyacrylamide gel electrophoresis. Current 2 mA/gel, running time 3 h. Lf lactoferrin, HSA human serum albumin, SIgA secretory IgA, α-La α-lactalbumin.

suggesting that the binding capacity was about 70 mg lactoferrin/ml gel. The copper content of the gel was 50 μmol/ml gel. It should be pointed out that no protein adsorption occurred in a control experiment in which no copper had been complexed to the gel. As lactoferrin has been shown to be saturated to about 10–40% of its total iron-binding capacity [2] and all of the lactoferrin was adsorbed it is probable that surface-exposed histidine (and/or cysteine) residues of lactoferrin interact with Cu^{2+}-ions with excess binding capacity. The bacteriostatic activity of the lactoferrin as shown by the inhibition of growth of *E. coli* was intact after the purification procedure, indicating that the purification method does not cause any detectable damage to the protein.

The present study indicates that metal-chelate affinity chromatography together with a gel-filtration provides a simple method for the isolation of human lactoferrin.

In addition the method has the advantages of:

(1) High capacity
(2) Quantitative recovery
(3) No detectable damage to the protein
(4) Easy regeneration.

Acknowledgements

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