ISOLATION OF LACTOFERRIN FROM HUMAN WHEY BY A SINGLE CHROMATOGRAPHIC STEP

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1. Introduction

Human milk, in contrast to bovine milk, is rich in the iron-binding protein lactoferrin. Lactoferrin is a single-chain glycoprotein with est. mol. wt 75 000–85 000. It has two iron-binding sites, each binding one atom of ferric iron [1–4]. In milk it is normally only partially saturated with iron [5]. Experiments in vitro indicate that lactoferrin is an important component of the bacteriostatic system present in human milk. Lactoferrin, by binding iron, prevents growth of iron-requiring bacteria in the milk. The bacteriostatic effect is augmented by specific antibacterial antibodies in the milk while it is abolished by denaturation of the protein or saturating it with iron [5,6]. To what extent this bacteriostatic effect is really operating in vivo, e.g., in the gastrointestinal tract of the breast fed infant, remains to be shown, although, this hypothesis is supported by data from in vivo experiments in guinea pigs [5]. Whether or not the high content of lactoferrin is part of an explanation why newborns absorb iron more efficiently from human milk than from bovine milk or formulas [7] is presently not known.

Further studies of the physiological role of lactoferrin will require large amounts of the isolated protein. Several methods for its isolation have been described but most of them are rather laborious [2,3,8,9]. This paper demonstrates that lactoferrin can be isolated from human whey in pure form in a high yield by a one step-chromatography on heparin–Sepharose.

2. Materials and methods

2.1. *Preparation of whey proteins*

Mature human milk, collected by a breast-pump from one mother over 24 h, was stored at 4°C until the end of the period, then pooled and frozen at −20°C until used. The milk was thawed at 4°C, centrifuged at 10 000 × *g* for 40 min and, after removal of the cream, the skim milk was adjusted to pH 4.7 with HCl and the caseins precipitated by heating the milk to 40°C for 30 min. After another centrifugation at 10 000 × *g* for 40 min the whey was decanted.

2.2. *Heparin–Sepharose chromatography*

Whey (50 ml) was dialyzed overnight against 0.05 M NaCl in 5 mM veronal–HCl (pH 7.4) then applied to a column of heparin–Sepharose [10] (detailed in legend to fig.1). When necessary the pooled fractions containing lactoferrin were dialyzed as above and then rechromatographed under identical conditions.

2.3. *Polyacrylamide gel electrophoresis and immunochemical analysis*

Sodium dodecylsulphate–polyacrylamide gel (SDS–gel) electrophoresis was performed according to [11] with the exception that the gels were 7% in polyacrylamide. The calibration proteins used for determination of molecular weight are indicated in fig.3. Staining and destaining were according to [12]. Staining with the periodic acid-Schiff reagent was according to [13] as in [14]. Commercial lactoferrin

was purchased from Sigma (St Louis, MO) and rabbit antiserum to human serum albumin from DACO-Immunoglobulins (Copenhagen). Rabbit antisera to human whey and to isolated lactoferrin were prepared by use of Freund's complete and incomplete adjuvants in the first and subsequent booster injections, respectively. 0.2 mg of protein was used in the initial and 0.1 mg in the booster injection. The immunoglobulin fractions were then prepared [15] and immunodiffusions were performed as in [16].

2.4. *Amino acid analysis and NH_2-amino acid sequence determination*

Samples were hydrolyzed using constant boiling HCl, in vacuo, at 110°C for 24 h or 48 h. A modified Beckman 120 C amino acid analyzer with a single-column system (DC 6 A, Durrum) and an iso-pH buffer system [17] was used for analysis. Methionine and cysteine were also determined as methionine sulphone and cysteic acid after performic acid oxidation and tryptophane determined spectrophotometrically [18]. The NH_2-terminal amino acid sequence was determined according to [19].

2.5. *Carbohydrate and iron analysis*

Hexosamines were determined, using the amino acid analyzer, after hydrolysis of the samples in 2 M HCl at 110°C, in vacuo, for 3 or 6 h [20]. Hexoses were determined by use of the anthrone reagent [21]. Iron content was determined as in [22].

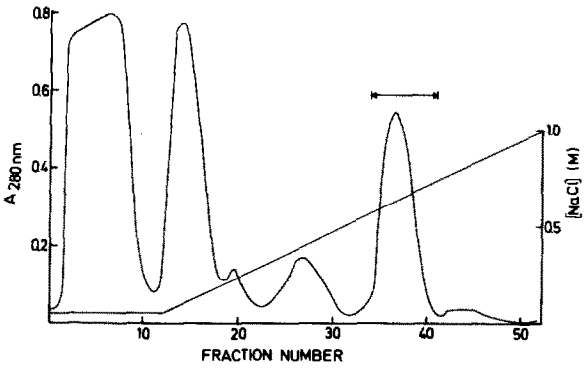

Fig.1. Heparin–Sepharose chromatography of human whey. Whey (50 ml) dialyzed against 0.05 M NaCl in 5 mM veronal–HCl, (pH 7.4) was applied to the column (10 ml settled gel). The column was washed with 50 ml of the same buffer and then eluted with a linear gradient of NaCl (400 ml) as indicated. The flow rate was 52 ml/h. Fractions 34–42 (total vol. 78 ml) were combined and used for further analysis.

3. Results and discussion

A typical profile of human whey proteins, eluted with a linear gradient of NaCl from a heparin–Sepharose column, is shown in fig.1. The large peak that elutes with about 0.6 M NaCl corresponds to lactoferrin, while the peak that elutes with about 0.4 M NaCl contains albumin and the bile salt-stimulated lipase (L. B., O. H., in preparation). Thus, the lactoferrin binds comparatively strongly to heparin-

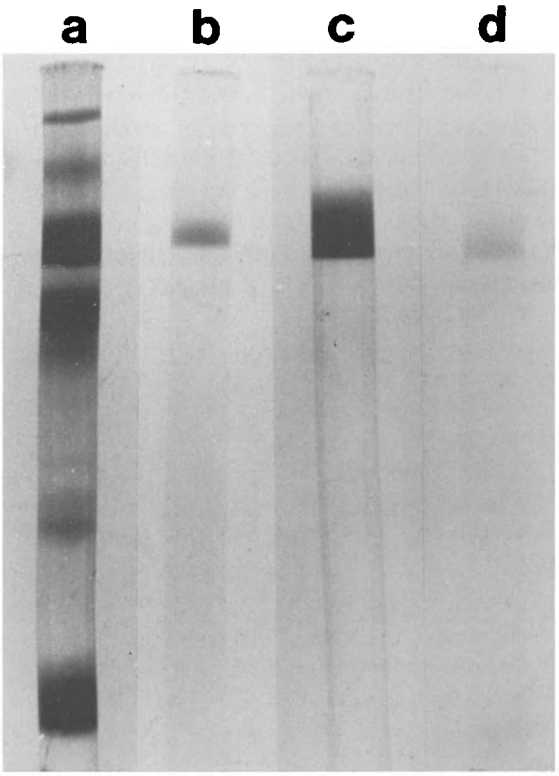

Fig.2. SDS–gel electrophoresis. Samples applied to the gels were: whey protein, 120 μg (a); isolated lactoferrin, 8 μg (b); 60 μg (c,d). Gels a–c were stained for protein with Coomassie blue and gel (d) with the periodic acid-Schiff reagent for carbohydrate. Samples were treated with 2-mercaptoethanol prior to application. The gels were 60 × 7 mm and the current 8 mA/tube. Migration was towards the anodic end (the lower part of the photograph).

Sepharose and separation from the other whey proteins can be obtained by a single chromatographic step. In some preparations however, the protein peak, combined as indicated by the arrow, contained small impurities as revealed by SDS–gel electrophoresis. These were easily removed simply by a rechromatography under identical conditions. SDS–gels of an aliquot from the combined peak fractions in the described preparation revealed no impurities, not even when the gel was somewhat over-loaded with protein (fig.2). The isolated protein also stained with the periodic acid-Schiff reagent (fig.2) indicating that it contained carbohydrate. The molecular weight was estimated to 77 100 by SDS–gel electrophoresis (fig.3). This value is in good agreement with [2,23].

By immunodiffusion technique the isolated lactoferrin gave only one precipitate against the rabbit antiserum to human whey while a commercial lactoferrin revealed several precipitates (fig.4a). Conversely, the antiserum prepared to isolated lactoferrin revealed only one precipitate to whey proteins which in turn had identical immunological determinants to the isolated lactoferrin and to one component of the commercial lactoferrin preparation (fig.4b). Since albumin, according to the elution profile from the heparin–Sepharose chromatography, was a potential impurity in our preparation, human whey, commercial lactoferrin and isolated lactoferrin were set up against an antiserum to human serum albumin. This revealed as expected a precipitate against human whey, but also against commercial lactoferrin (fig.4c). The isolated lactoferrin however, gave no precipitate. By

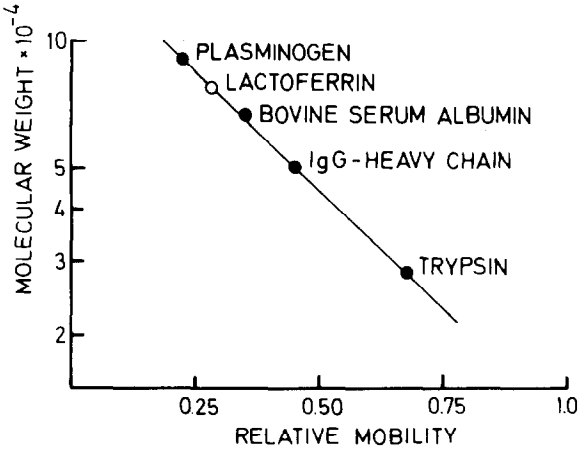

Fig.3. Determination of molecular weight. Lactoferrin (20 μg) and the respective calibration proteins (20 μg) were applied to SDS–gels and electrophoresis was performed as in legend to fig.2.

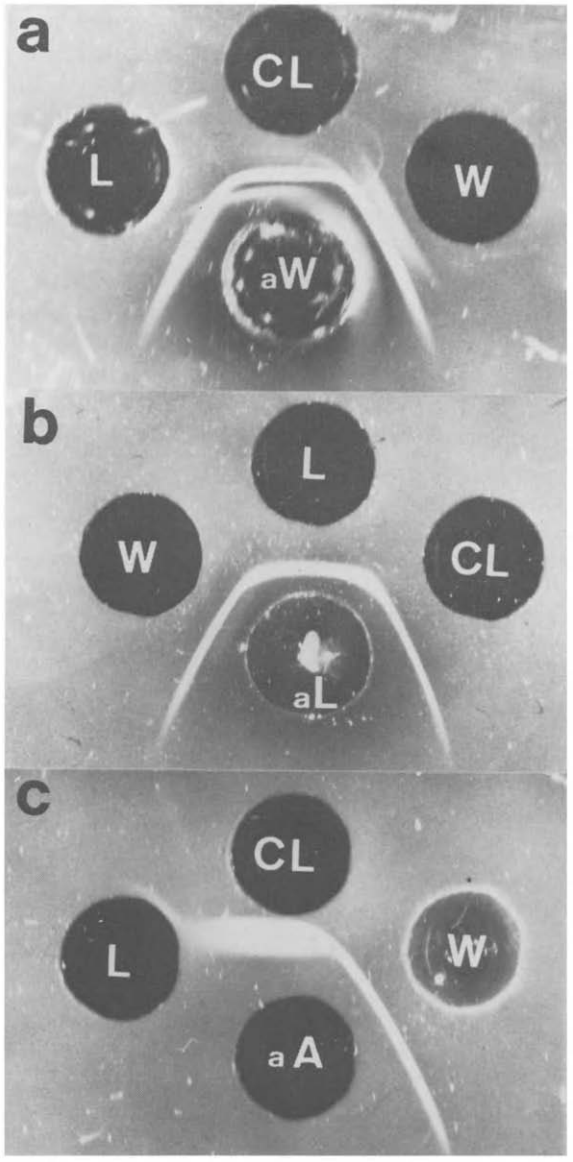

Fig.4. Immunodiffusion studies of lactoferrin. The figure shows immunodiffusion plates (double diffusion in one dimension). Antibodies used were against human whey (top), isolated lactoferrin (middle) and human serum albumin (bottom). Their respective wells are indicated by aW, aL and aA. Antigens used were whey proteins (W), isolated lactoferrin (L), and commercial lactoferrin (CL).

Table 1
Amino acid composition of lactoferrin isolated from human whey

Amino acid	Residues/mol[a]
Aspartic acid/asparagine	71
Threonine	29
Serine	45
Glutamic acid/glutamine	78
Proline	28
Glycine	59
Alanine	62
Cysteine	23[b]
Valine	37
Methionine	5[b]
Isoleucine	13
Leucine	55
Tyrosine	20
Phenylalanine	32
Lysine	39
Histidine	7
Arginine	46
Tryptophane	10[c]
Hexose	14[d]
Glucosamine	4[e]

NH_2-terminal amino acid sequence: H_2N–Gly–Arg–

[a] Calculated from mol. wt 77 100
[b] Values obtained after performic acid oxidation
[c] Determined spectrophotometrically
[d] Determined by the anthrone method
[e] Determined by use of amino acid analyzer

these criteria the isolated protein was lactoferrin and the preparation contained no impurities.

The amino acid composition of the isolated lactoferrin (table 1) was in good agreement with [2,8,24], perhaps with the exception that the glutamic acid/glutamine content was higher than in [2,8] but, on the other hand, it was lower than in [24]. The NH_2-terminal amino acid sequence was H_2N–Gly–Arg–. Glycine as NH_2-terminal is in agreement with [25] while no NH_2-terminal amino acid was detected in [8]. There are no previous data on the second residue in the sequence. The iron content was 0.3 mol/mol protein and the contents of hexoses and hexosamines was around 4% of which hexoses comprised 3% and hexosamine 1%, respectively (table 1). The value for glucosamine is underestimated by the amino acid analyzer and if corrections are made our valu' are comparable to those in [23].

In conclusion this paper describes for the first time a method to isolate human whey lactoferrin in pure form by a single chromatographic step. The capacity for heparin–Sepharose to bind lactoferrin is high and it should be easy to adjust the method for purification in larger scale.

Acknowledgements

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