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Note

Isolation of human lactoferrin by affinity chromatography using insolubilized bovine β-lactoglobulin

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Lactoferrin is an iron-binding protein found in many external secretions. In mammals, lactoferrin is present in milk in concentrations that vary depending on the stage of lactation [1] and the species. Human milk contains between 1 and 2 mg/ml [2], whereas bovine milk contains only ca. 0.1 mg/ml [1].

The biological role of lactoferrin is still obscure. The ability of this protein to sequester iron has suggested that lactoferrin can act as a bacteriostatic agent making the metal unavailable to bacteria [3]. A bactericidal activity for lactoferrin has also been reported resulting from blockage of the biosynthesis of the bacterial cell wall, or alteration of the membrane permeability [4]. On the other hand, human lactoferrin may be involved in growth mechanisms, acting as a growth factor for some intestinal cells [5].

Human lactoferrin interacts with acidic proteins such as casein, α-lactalbumin and albumin [6]. These interactions have been ascribed to the high isoelectric point of lactoferrin [7]. However, interaction of lactoferrin with some basic proteins, such as secretory immunoglobulin A (IgA) [8] and lysozyme [9], suggests the involvement of non-electrostatic forces. These interactions may be important in intestinal maturation by promoting the binding of lactoferrin to the intestinal mucosa, where it can exert its function as microbial inhibitor or growth stimulator.

In a recent paper we have demonstrated that isolated bovine lactoferrin is bound by insolubilized β-lactoglobulin, in a way that is strongly dependent on

the ionic strength of the buffer [10]. This interaction is mainly due to electrostatic forces, but shows some degree of specifity. In this paper we describe a new method for the isolation of human lactoferrin using this interaction between lactoferrin and bovine β-lactoglobulin.

EXPERIMENTAL

Materials

Human milk was obtained from healthy donors. Whey was prepared from skimmed milk by rennin coagulation followed by centrifugation. Antiserum against human lactoferrin was kindly provided by Dr. J. Brock (Glasgow, U.K.). Bovine β-lactoglobulin (Sigma, Poole, U.K.) was insolubilized on Sepharose 4B (Pharmacia, Uppsala, Sweden) previously activated with cyanogen bromide as described by Cuatrecasas [11].

Lactoferrin purification

Human whey (5 ml) was dialysed overnight against 0.1 M sodium chloride–0.01 M potassium phosphate buffer (pH 7.2) and applied at a flow-rate of 7 ml/h to a column containing 8 ml of β-lactoglobulin-Sepharose (2.9 mg of protein per ml of settled gel) equilibrated in the same buffer. The column was washed and bound protein eluted with 0.3 M sodium chloride (40 ml). Absorption at 280 nm was monitored, and the lactoferrin concentration measured by radial immunodiffusion [12]. Fractions containing lactoferrin were pooled and concentrated by ultrafiltration in an Amicon cell using a PM10 membrane.

The purity of the lactoferrin obtained was tested by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) using gels of 15% acrylamide and 0.2% bisacrylamide, by electrophoresis in polyacrylamide-agarose and by gel filtration in Sephacryl S-200 (Pharmacia). For this chromatography, the concentrate (0.5 ml) was loaded on a column (70 cm×1.1 cm I.D.) equilibrated with 0.5 M sodium chloride–0.01 M potassium phosphate buffer (pH 7.2). The column was eluted with this buffer at a flow-rate of 8 ml/h.

Interaction of human lactoferrin in whey with insolubilized β-lactoglobulin was also analysed by frontal analysis chromatography using a column containing 1 ml of the gel. The apparent affinity constant was calculated using the half-saturation point of the lactoferrin saturation curve [13].

RESULTS AND DISCUSSION

Fig. 1 shows the profile of human whey chromatographed on the bovine β-lactoglobulin-Sepharose column. At 0.1 M sodium chloride most of lactoferrin was retained by the column. The total binding capacity was 7 mg of human lactoferrin for a column containing 23 mg of β-lactoglobulin. Lactoferrin eluted at high ionic strength from the affinity column seemed to be slightly contam-
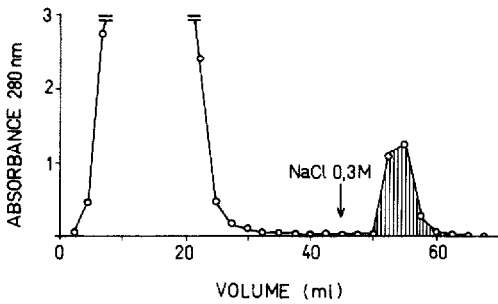
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Fig. 1. Elution profile of human whey on β-lactoglobulin-Sepharose affinity derivate. The shaded area corresponds to the retained lactoferrin eluted with 0.3 M sodium chloride.
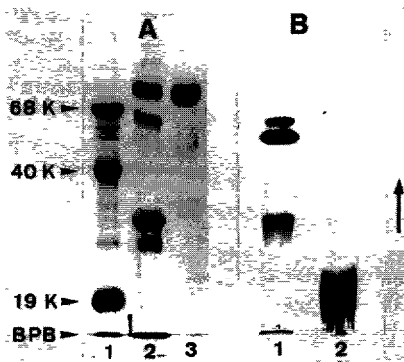

Fig. 2. (A) SDS-PAGE of purified human lactoferrin Lane 1, molecular mass markers; lane 2, human whey; lane 3, proteins eluted from the column of β-lactoglobulin-Sepharose. The anodal front was tracked with bromophenol blue (BPB). (B) Polyacrylamide-agarose gel electrophoresis of human lactoferrin. Lane 1, human whey; lane 2, human lactoferrin eluted from the column of β-lactoglobulin-Sepharose. The arrow indicates the direction of the electrophoretic run.

inated, by SDS-PAGE (Fig. 2A), with some other proteins with apparent molecular mass of ca. 50 000 and 30 000. Nevertheless, a single band was observed by polyacrylamide-agarose electrophoresis (Fig. 2B) using non-denaturing conditions. Densitometric analysis of electrophoretic runs in SDS showed that intact lactoferrin represents more than 92% of the proteins eluted with 0.3 M sodium chloride.

However, the contaminant proteins could not be separated from lactoferrin by gel chromatography using a buffer containing 0.5 M sodium chloride, which is sufficient to break all known interactions between lactoferrin and other proteins [10]. It is possible that these contaminant proteins were fragments derived from the proteolysis of lactoferrin, which may remain associated in non-denaturing conditions [14]. In bovine colostrum, the presence of a lactoferrin fragment with a molecular mass of ca. 45 000 has been demonstrated [15].
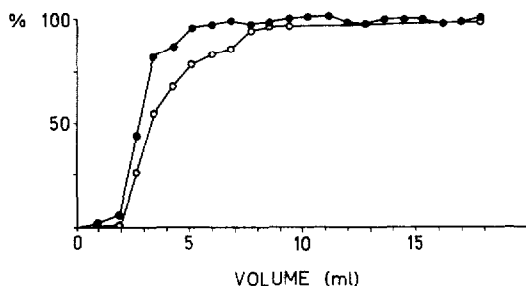
Fig. 3. Frontal analysis chromatography of human whey on β-lactoglobulin-Sepharose derivate. The total protein saturation curve was measured as the absorbance at 280 nm (●). The lactoferrin saturation curve was determined by radial immunodiffusion (○).

The interaction of human lactoferrin with insolubilized bovine β-lactoglobulin was examined by frontal analysis chromatography. Fig. 3 shows the lactoferrin saturation curve obtained by frontal analysis in a β-lactoglobulin-Sepharose column. The apparent affinity constant was calculated using the half-saturation point of the curve as elution volume. At low ionic strength (0.1 M sodium chloride) the K_a is $6.5 \cdot 10^3$ M^{-1}.

Lactoferrin was first isolated by ion-exchange chromatography [16], but owing to the interest in this protein several other methods have been developped to improve its isolation, such as affinity chromatography with Cibacron Blue-Sepharose [17], heparin-Sepharose [18], copper chelate affinity chromatography [2] and immunoadsorption with monoclonal antibodies [19]. Some of the methods described above involve denaturing conditions in the isolation that may affect the behaviour of the protein.

The main advantage of the method described here is that it allows the isolation of highly purified lactoferrin from a small volume of milk in just one chromatographic step with a good yield. Furthermore, the conditions of the isolation do not affect the iron-binding capacity of lactoferrin, so that studies of its physiological functions can be carried out.

The method is only suitable for isolation of lactoferrin from milks that do not contain β-lactoglobulin, such as human milk. If the milk contains β-lactoglobulin, as in ruminants, the competition of free β-lactoglobulin impedes the binding of lactoferrin to the insolubilized ligand.

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