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Note

Fast protein liquid chromatography of antibacterial components in milk

Lactoperoxidase, lactoferrin and lysozyme

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In milk from various species there are several non-specific antibacterial factors. Among the most important of these are the three basic proteins: lactoperoxidase, lactoferrin and lysozyme (for a review, see ref. 1). The occurrence of these proteins differs between species, and also between colostrum and milk in the same species. During infections and other disturbances of the synthetic activity of the mammary gland, the increased antibacterial defence is reflected in the concentrations of these components. Therefore, the quantification and qualitative characterization of these proteins are of great interest as an indication of the general anti-infectious efficiency of milk. With the fast protein liquid chromatography (FPLC) method, the purified components can also be studied as to their physical properties, aggregation behaviour and stability.

The traditional preparation method for both lactoperoxidase and lactoferrin is founded on ion-exchange chromatography, and they accompany each other in these chromatographic techniques and both are mentioned in the first reports of the preparation method for the respective protein[2,3]. There has also been reported in the literature that during the preparation of lactoperoxidase a protein fraction occurred, which showed similarities with lactoperoxidase in physico-chemical properties, such as molecular weight and partial immunological cross-reactivity, but did not contain haem[4]. This was suggested to be a "non-haem lactoperoxidase". However, the haem group of lactoperoxidase is reported to be difficult to extract from the protein[5] and comparatively inaccessible[6]. By combining the FPLC chromatographic method with compositional analyses we investigated the nature of the peaks obtained by ion-exchange chromatography.

MATERIALS AND METHODS

Bovine milk lactoperoxidase and lyxozyme from human colostrum were purchased from Sigma (St. Louis, MO, U.S.A.) and lactoferrin from human colostrum

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from Calbiochem (La Jolla, CA, U.S.A.). Bovine milk lactoferrin was a gift of Dr. Ersson (Biomedical Center, Uppsala University, Uppsala, Sweden).

Skim milk was acidified and the resulting whey was dialysed against distilled water for 24 h. After the pH had been adjusted to 7.8, the whey sample was applied to a 400 × 26 mm I.D. column with CM-cellulose, equilibrated with 0.01 sodium veronal–hydrochloric acid buffer, pH 7.8. The proteins were eluted with a continuous salt gradient of 0–1 *M* sodium chloride.

FPLC chromatography was performed on a Mono S HR 5/5 column by means of the FPLC equipment (Pharmacia, Uppsala, Sweden). The buffer used for equilibration and elution was 0.01 *M* imidazole–hydrochloric acid (pH 7.0). The course of the gradient (0–1 *M* sodium chloride) was programmed on a gradient programmer. A sample volume of 0.2 ml was injected into the column. The eluent passed a UV monitor (UV-1, Pharmacia) which measured the absorbance at 280 nm.

Lactoperoxidase activity was measured with 2,2′-azino-di-[3-ethylbenzthiazoline sulfonate(6)] (ABTS) as chromogenic substrate[7]. Analyses of amino acid composition was performed at the Biomedical Center (Uppsala University, Uppsala).

RESULTS AND DISCUSSION

Fig. 1 shows the elution profile from a separation of whey proteins on CM-cellulose. The position of lactoperoxidase activity is consistent with earlier reports of the sodium chloride concentration needed for elution of lactoperoxidase[2]. The molecular weights of the fractions from the CM-cellulose chromatography were investigated by silver-stained sodium dodecyl sulphate polyacrylamide gel electrophoresis[8]. Fractions II and III turned out to have similar molecular weights.

The positions of the reference proteins on the FPLC chromatography are shown in Fig. 2. By use of the cationic Mono S ion-exchange column especially

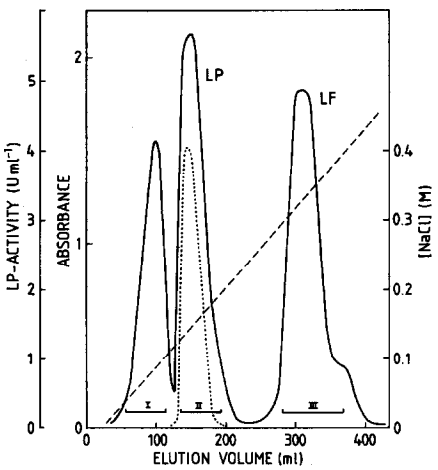

Fig. 1. Ion-exchange chromatography on CM-cellulose of whey proteins from bovine skim milk. Buffer: 0.01 *M* sodium veronal–hydrochloric acid buffer, pH 7.8. Gradient: 0–1 *M* sodium chloride. Column dimensions: 400 × 26 mm I.D. Flow-rate: 25 ml/h. (——) A_{280}; (· · · ·) lactoperoxidase activity; (----) sodium chloride concentration.

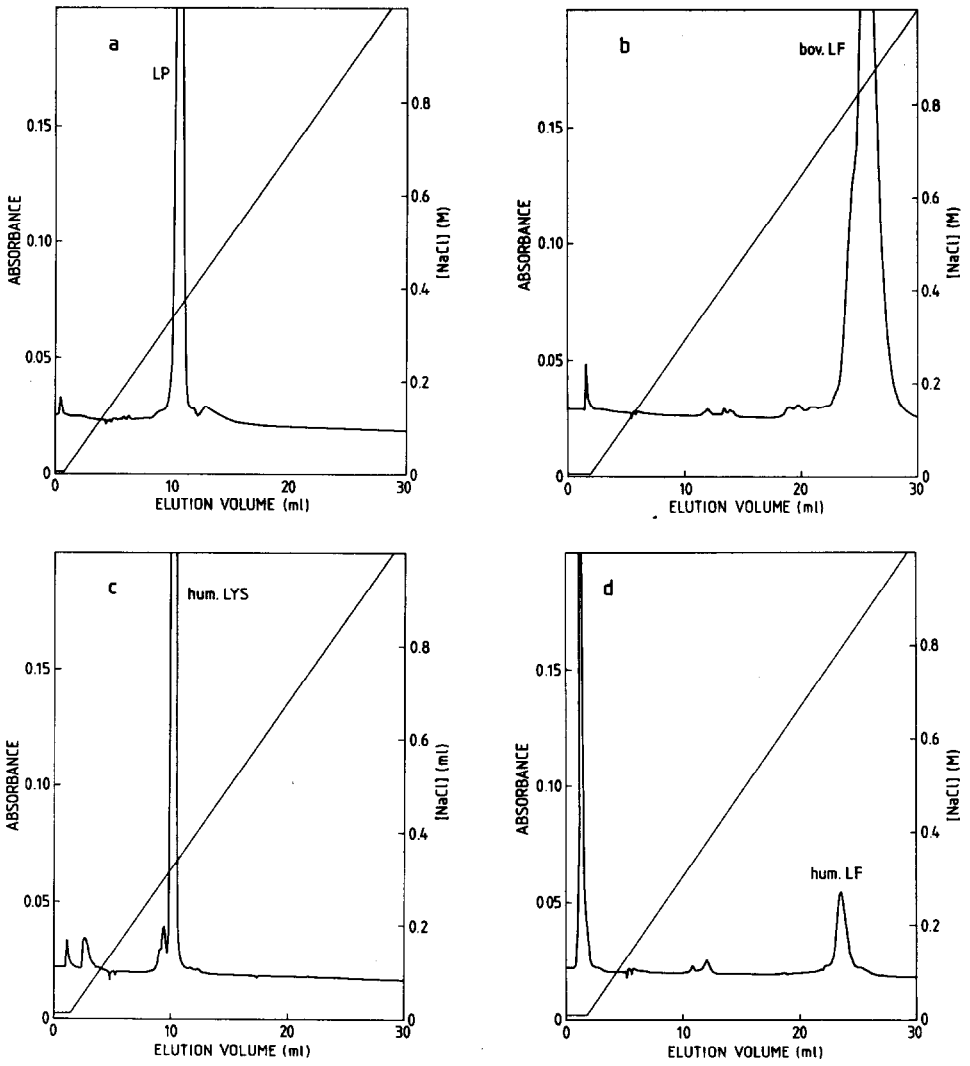

Fig. 2. FPLC ion-exchange chromatography of antibacterial proteins in bovine and human milk whey. Column: Mono S. Column dimensions: 50 × 5 mm I.D. Flow-rate: 60 ml/h. (a) Bovine lactoperoxidase (LP); (b) bovine lactoferrin (LF); (c) human lysozyme (LYS); (d) human lactoferrin (LF).

developed for FPLC, and the microprocessor programming unit, it was possible to design a reliable and sensitive separation method for the antibacterial proteins in milk with a duration of each run of only about 10–20 min as compared to conventional chromatography, which requires several hours. Less than a few hundred microlitres of sample are required. The salt concentrations at which the proteins were eluted showed great reproducibility between runs.

Fractions I–III obtained by ion-exchange chromatography on CM-cellulose (indicated in Fig. 1) were analysed with the FPLC technique (Fig. 3). When compared

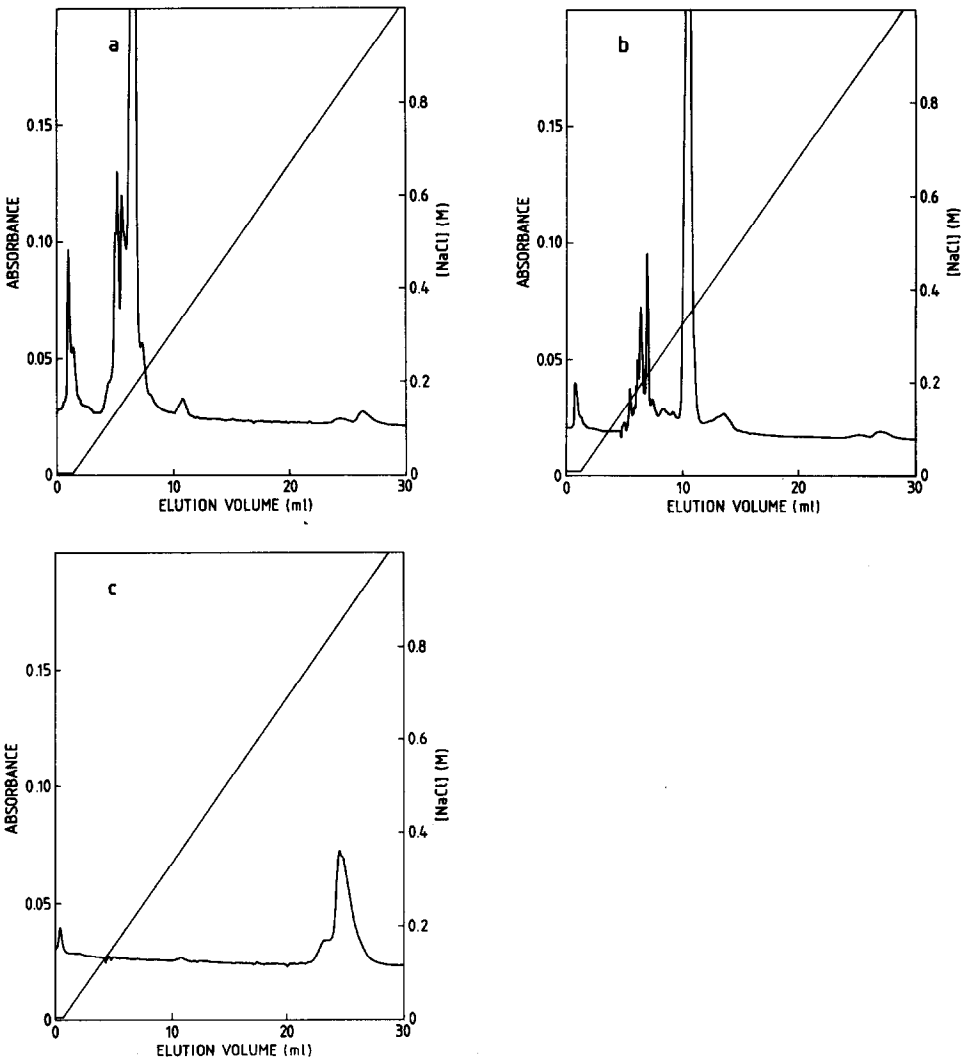

Fig. 3. FPLC ion-exchange chromatography of fractions from large-scale separation on CM-cellulose (Fig. 1). In (a)–(c) are shown the three main fractions I–III as indicated in Fig. 1. Conditions as in Fig. 2.

to the position of the reference substance (Fig. 2) it is obvious that fractions II and III were substantially lactoperoxidase and lactoferrin, respectively. As to the composition of fraction I, it did partially coincide with human milk lysozyme, but activity measurements indicated that the eventual content of lysozyme is small, as is expected from the reported lysozyme activity in bovine milk under normal conditions[9]. In ruminants, the milk lysozyme activity is due mainly to the blood-associated lysozyme *g*, and should be different from lysozyme *c*, found in their gastrointestinal tract and in human milk[10].

When the fraction corresponding to the "non-haem lactoperoxidase" prepared

according to Dumontet and Rousset[4] was analysed with FPLC, it coincided with the lactoferrin peak. The amino acid composition of this fraction was compared to the amino acid composition of lactoperoxidase[11] and bovine lactoferrin[12], and the so-called Metzger indices[13] were: 11.7 for the comparison between lactoperoxidase and the "non-haem lactoperoxidase" and 4.4 for the comparison between lactoferrin and the "non-haem lactoperoxidase". Considering experimental errors, this and other evidence suggests that the fraction corresponding to the "non-haem lactoperoxidase" is identical with lactoferrin.

CONCLUSIONS

The development of a fast and reliable method for quantitative and qualitative analysis of the antibacterial proteins in milk will make it possible to study the variation in these components during different stages of milk secretion and in the case of f. ex. mastitis. It is also possible to closely analyse the interrelationship between the stability of protein structure and enzyme activity during practical applications in composite samples.

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