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SHORT COMMUNICATIONS

Comparative study of the iron-binding strengths of equine, bovine and human lactoferrins

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Equine milk contains casein and various whey proteins such as β -lactoglobulin, α -lactalbumin, serum albumin, transferrin, lysozyme and lactoferrin (Bell *et al.* 1981). However, little is known about equine milk proteins compared with those of other species of mammal. The content of lactoferrin in equine milk is reported to be 0.2–2 mg/ml, while those in human and bovine milk are > 2 mg/ml and 0.02–0.2 mg/ml respectively (Masson & Heremans, 1971). Lactoferrin is a single polypeptide chain protein that can bind metal ions. It has been found not only in the milk of various mammals but also in other secretions and in polymorphonuclear cells. Many biological functions of lactoferrin have been characterized, such as its bacteriostatic effects, myelopoiesis, and other properties associated with defence systems (Spik & Montreuil, 1983). Some of the biological functions of lactoferrin have been considered to be due to its iron-binding properties. We have purified lactoferrin from equine milk and attempted to compare its iron-binding strength with that of human lactoferrin, bovine lactoferrin and bovine transferrin.

MATERIALS AND METHODS

Lactoferrin and other reagents

Equine (*Equus caballus*) lactoferrin was prepared from pooled milk of Mongolian mares according to the modified method reported by Bell *et al.* (1981). Firstly, casein was removed by ammonium sulphate precipitation (300 g ammonium sulphate/l skim milk). Further ammonium sulphate (200 g/l) was added to the supernatant to precipitate whey proteins. The whey protein component was desalted by dialysis against water and the solvent was changed to 0.05 M-Tris-HCl buffer, pH 8.0. The dialysed sample was applied to a column of DEAE-Sephadex A-25 equilibrated with the same buffer. The adsorbed fraction was eluted by the equilibrating buffer containing 0.5 M-NaCl, and this lactoferrin fraction was further purified by

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heparin-Sepharose CL6B affinity chromatography. The affinity column was equilibrated with 0.01 M-sodium phosphate buffer, pH 7.0. The coloured (pale red) fraction was eluted by a linear gradient of 0–1 M-NaCl in the adsorbing buffer and collected. This affinity chromatographic procedure was repeated. Lactoferrin obtained by this procedure was used for the measurements, except when another source is given.

Bovine lactoferrin was the same as that used in previously reported work (Shimazaki *et al.* 1992). Human lactoferrin (Lots 109F3910 and 61H3905) was obtained from Sigma Chemical Co. Ltd (St Louis, MO 63178, USA) and was saturated with iron by the method described previously (Shimazaki & Hosokawa, 1991).

Transferrin (holo type, Lot KPJ5494) from bovine plasma was purchased from Wako Pure Chemicals Ltd (Osaka, Japan) and another bovine transferrin, YU-transferrin type 2 (holo type, Lot FA0003) was a product of Yagai Co. Ltd (Yamagata, Japan). Heparin-Sepharose CL6B and DEAE-Sepharose A-25 were from Pharmacia LKB Biotechnology (S-751 82 Uppsala, Sweden).

Spectrophotometric titration and estimation of extent of iron saturation

The pH of a solution of lactoferrin or transferrin (concentration, 0.5–0.9 mg/ml) in 0.05 M-NaCl was measured using a Horiba pH meter type F-5 (Kyoto, Japan). Absorbance measurements were performed with a JASCO model UVIDEC 430 recording spectrophotometer (Japan Spectroscopic Co. Ltd, Tokyo) with a thermostated cell holder at 30 °C, using 10 mm quartz cells containing a small magnetic bar for stirring. HCl (0.5–3 M) was added to the lactoferrin solution in the quartz cuvette. The iron content ratio was calculated as $\alpha = (A - A_{apo}) / (A_{Fe} - A_{apo})$, where A is the absorbance at 280 nm of the sample solution at the given pH, A_{apo} the absorbance at acidic pH where ferric ions are dissociated completely, and A_{Fe} the absorbance of the solution of iron-saturated sample or the absorbance value before the addition of acid. Values of α were plotted against pH.

RESULTS AND DISCUSSION

The isolated equine lactoferrin had a molecular mass measured by SDS-PAGE of 80.3 ± 2.9 kDa. To confirm the identity of the protein isolated from Mongolian mares' milk, the N-terminal amino acid sequence was determined by protein sequencing. The sequence obtained was APRKSVRWXTISPAAE, which coincides with the N-terminus of equine lactoferrin as reported by Jollès *et al.* (1984).

The iron content of equine lactoferrin was estimated from the ratio of absorbance at 280 and 460 nm, and was calculated to be 107%. Two other equine lactoferrin preparations from Percheron mares' milk were also saturated with iron. However, the possibility that lactoferrin became saturated with iron contained in the ammonium sulphate during the purification process cannot be ruled out.

The pH-dependent absorbance changes were measured at 280 and 460 nm. The magnitude of the difference in absorbance of apolactoferrin and hololactoferrin at 280 nm was about four times larger than that at 460 nm. Moreover, the tendencies of the pH-dependent absorbance changes were the same at 280 and 460 nm, so the values at 280 nm were plotted as shown in Fig. 1. The pH values at the position of $\alpha = 0.5$, i.e. the pH at which the protein was 50% saturated with iron, were 2.2, 2.1, 2.7 and 3.3 for equine lactoferrin, human lactoferrin, bovine lactoferrin and bovine transferrin respectively. Transferrin (a product of Wako Pure Chemicals) contained

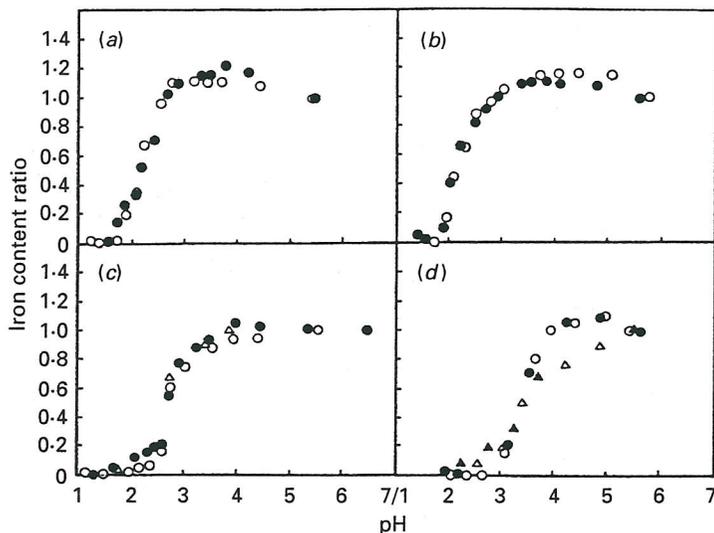


Fig. 1. pH titration curves of lactoferrins and transferrin measured by absorption at 280 nm: (a) equine lactoferrin, (b) human lactoferrin, (c) bovine lactoferrin, (d) bovine transferrin. Different symbols on the same plot denote different series of measurements. In (d) transferrin (○, ●) from Wako and YU-transferrin (△, ▲) from Yagai were used. The iron content ratio is given by $\alpha = (A - A_{apo}) / (A_{Fe} - A_{apo})$, where A is absorbance of the sample at a given pH, A_{apo} absorbance of the sample at acidic pH and A_{Fe} absorbance of the iron-saturated sample.

haemopexin as an impurity, because the characteristic absorption peak at 412 nm was observed (Roberts *et al.* 1966). The contaminant did not influence the titration experiments because the titration curves for the two transferrins were essentially the same (Fig. 1d).

It is known that whereas the affinity of lactoferrin for iron is largely retained at pH values of < 4.0, transferrin completely releases ionic iron under such conditions (Montreuil *et al.* 1960; Masson & Heremans, 1968). However, transferrin retained iron at pH 4 under our experimental conditions. This may be explained by experimental differences, since the pH at which lactoferrin or transferrin releases iron is considerably influenced by ionic strength and buffer composition and concentration, as reported by Mazurier & Spik (1980). The difference in the metal-binding strengths of human lactoferrin and human transferrin has also been studied by equilibrium dialysis (Aisen & Leibman, 1972). A difference in the pH-dependence of iron release for bovine lactoferrin and transferrin was confirmed in our experiments. A further study of iron binding and release by a sequestering agent has been reported and the difference in iron binding of lactoferrin and transferrin explained by differences in the stability of the 'closed' form of the hololactoferrin conformation and that of transferrin (Chung & Raymond, 1993). Moreover, in our experiments it was found that the iron-binding behaviour of equine lactoferrin is similar to that of human lactoferrin but not bovine lactoferrin. The 'physiological' meaning of these differences in milk lactoferrins remains to be resolved.

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