

THE ISOLATION AND CHARACTERIZATION OF LACTOFERRIN FROM SOW MILK AND BOAR SEMINAL PLASMA

T. K. ROBERTS* AND J. C. BOURSNEILL

A.R.C. Unit of Reproductive Physiology and Biochemistry, Cambridge†

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Summary. Lactoferrin isolated from sow milk (about 0.6 mg/ml) was shown to be chromatographically homogeneous, an observation supported by electrophoresis and by reaction against monospecific anti-lactoferrin antiserum. Isoelectric focusing showed multiple forms of the protein (i.e.p., 9.3 to 10.0) converted by neuraminidase to one form (i.e.p., 9.65). Boar seminal plasma contains immunologically identical lactoferrin (0.1 to 0.5 mg/ml) which binds strongly to boar spermatozoa.

Lactoferrins are a group of iron-binding proteins (Groves, 1960; Johansson, 1960) found widely in mammalian external secretions (Masson, Heremans & Dive, 1966). The concentrations observed show marked variations between species (Masson, 1970). The protein usually occurs in an almost iron-free form.

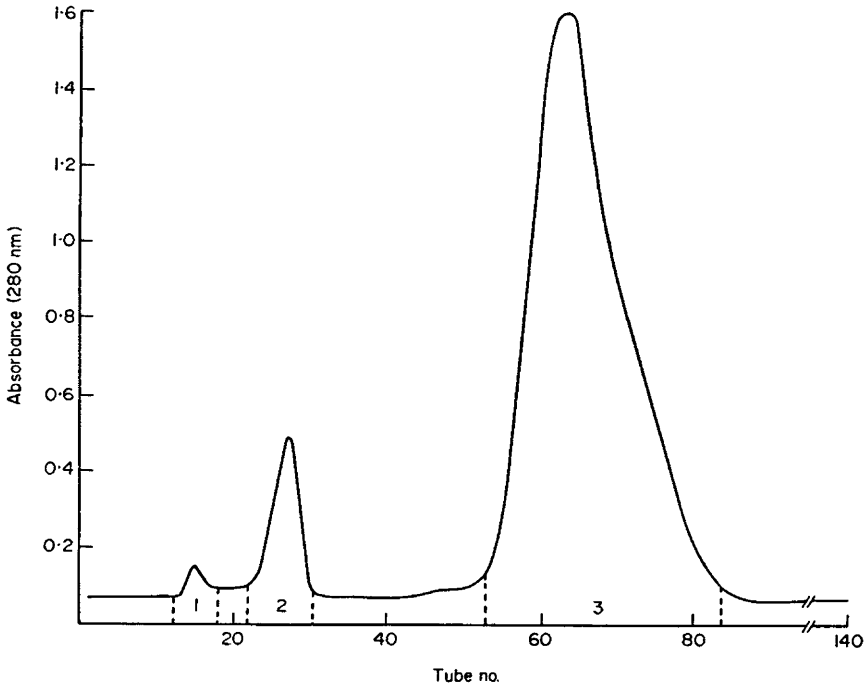
There have been earlier reports of the distribution of lactoferrin in the reproductive tract secretions of a number of animals (Hekman & Rümke, 1968; Roberts & Boettcher, 1971). This report concerns the isolation, by a modification of the method used for human lactoferrin by Querinjean, Masson & Heremans (1971), and characterization of lactoferrin from sows' milk. The study also concerns the occurrence of lactoferrin in boar seminal plasma.

Whole milk, collected from at least ten lactating sows, was pooled and frozen until use. The milk (700 ml) was defatted by centrifugation (10,000 *g* for 20 min), filtered through a coarse gauze and adjusted to pH 7 with 400 mM- Na_2HPO_4 . Ammonium ferrous sulphate (10 mg) was added to the product (650 ml) to saturate the lactoferrin with iron. The mixture was stirred for 2 hr, diluted with an equal volume of buffer, 20 mM- Na_2HPO_4 , 150 mM- NaCl , pH 7, and the conductivity was adjusted to that of the diluting buffer with 4 M- NaCl . After adding 3 g CM-Sephadex (Pharmacia, G.B.), the mixture was stirred for a further 4 hr, allowed to stand and the supernatant fluid was then decanted. The CM-Sephadex containing the red iron-lactoferrin complex (Johansson, 1960) was washed twice, each time with 1.1 ml buffer, and was then used to form a column (2.5 × 25 cm). This was eluted with the 150 mM- NaCl -phosphate buffer until protein was no longer detected in the washings at 280 nm (Uvicord,

* Present address: Department of Biological Sciences, The University of Newcastle, New South Wales 2308, Australia.

† Postal address: Animal Research Station, 307 Huntingdon Road, Cambridge CB3 0JQ.

L.K.B. Instruments, Croydon). The lactoferrin was eluted with 500 mM-NaCl-20 mM-phosphate, pH 7 buffer. The fractions containing the red protein were concentrated by ultrafiltration and dialysed against the 150 mM-NaCl buffer. This crude lactoferrin was applied to another equilibrated CM-Sephadex column and, after passage of the 150 mM-NaCl buffer, a linear NaCl (150 to 750 mM) gradient in 20 mM-phosphate buffer, pH 7 was applied. The lactoferrin eluted as a single peak at about 400 mM-NaCl (Text-fig. 1). The lactoferrin fractions, obtained from a number of runs, were concentrated, dialysed against water and freeze dried. The total weight (450 mg) represented 0.6 mg/ml whole milk.

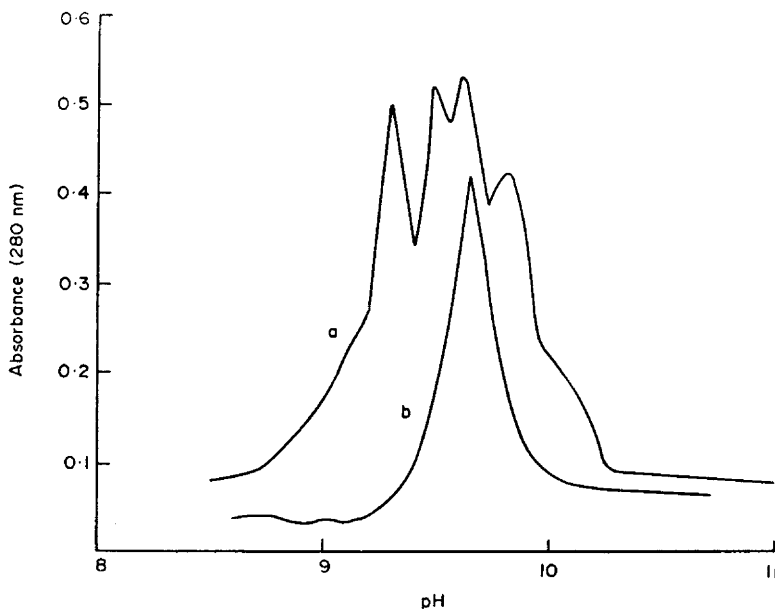


TEXT-FIG. 1. Protein absorbance at 280 nm of the eluate from a CM-Sephadex column (2.5 × 30 cm) of a lactoferrin preparation obtained from sows' milk. The column and sample were equilibrated with 20 mM-phosphate buffer pH 7.0, containing 150 mM-NaCl. A linear gradient, 150 mM to 750 mM, in the phosphate buffer was used for elution. The fractions were grouped as indicated. Group 3 contained the lactoferrin.

The red preparation showed no detectable impurities either by chromatography on Sephadex G-100 and CM-cellulose (each of which gave a single protein peak), or by electrophoresis on agarose gel at pH 8.6 (Johansson, 1972) which revealed only one band. Confirmation that the protein preparation was lactoferrin was shown by immunoelectrophoresis against rabbit antisera to (1) pig lactoferrin and (2) sows' milk. In both cases, only one precipitin arc was observed.

The isoelectric point of 50 mg of this protein was determined by isoelectric focusing on a 110-ml column (8100-10, L.K.B. Instruments, Croydon) in a sucrose density gradient containing carrier ampholine pH 7 to 10 (L.K.B. Instruments, Croydon). At least four peaks were visible in the pH range 9.4 to

10 (Text-fig. 2). All the peaks were red and all gave a positive reaction with anti-lactoferrin antiserum. Five such experiments showed similar isoelectric points between 9.3 and 10.



TEXT-FIG. 2. Isoelectric focusing of a sample of sows' milk lactoferrin in the iron-saturated form (a) before and (b) after treatment with neuraminidase.

Another sample (200 mg) of the lactoferrin was incubated at room temperature with neuraminidase (Behringwerke AG) at pH 5.5, following the method of Roberts, Masson & Heremans (1972) to remove sialyl groups, and was again subjected to isoelectric focusing. This enzyme-treated lactoferrin showed only one peak at pH 9.65 (Text-fig. 2).

The distribution of lactoferrin in the male reproductive tract was examined by using monospecific anti-lactoferrin antiserum. Lactoferrin was shown to be present in seminal plasma, seminal vesicle secretion and to a slight extent in epididymal fluid. Isolation of lactoferrin on a quantitative basis from pooled seminal plasma (nine samples from six boars), using the method described for milk, yielded about 0.3 mg/ml seminal plasma. Inhibition studies, using anti-lactoferrin antiserum, revealed that ejaculated spermatozoa contained absorbed lactoferrin which could not be washed off at physiological pH with buffers of ionic strength equivalent to 500 mM-NaCl, but that it could be removed by 4 M-NaCl.

In this study, the isoelectric point found for the sows' milk lactoferrin (9.3 to 10) is in accordance with the basic nature of this protein (Masson, 1970); this range is, however, apparently somewhat higher than that found for human (8.2 to 9.2; Roberts *et al.*, 1973) or for bovine lactoferrin (7.8 to 8.0; Groves, 1960).

The reduction in the number of peaks seen on isoelectric focusing by the removal of sialyl residues with neuraminidase is in accordance with the observations on human lactoferrin by Roberts *et al.* (1972). This suggests

that in porcine lactoferrin, heterogeneity is also due to differing numbers of sialyl groups in the protein molecule.

Other investigations in the course of the present study suggest that the primary origin of the immunologically identical seminal lactoferrin in the boar is the vesicular secretion. The evidence presented points to a strong binding of the seminal lactoferrin by the ejaculated spermatozoa. This binding also accords with the evidence for human seminal lactoferrin given by Hekman & Rümke (1968).

The present paper adds the pig to the list of animals containing iron-binding proteins in the reproductive tract (Roberts & Boettcher, 1971). The rôle of these proteins is unclear although there have been many studies reporting correlations between reproductive performance and transferrin types (Khattab, Watson & Axford, 1964; Kristjansson, 1964; Ashton, 1965). Studies of lactoferrin types and fertility have been initiated but difficulties have been encountered in typing lactoferrin (Groves, Peterson & Kiddy, 1965). The present paper offers a simple isolation procedure for obtaining lactoferrin for use in such studies.

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