Sequential purification of lactoferrin, lysozyme and secretory immunoglobulin A from human milk

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1. INTRODUCTION

Human milk protects the immunologically immature infant from gastro-intestinal invasion of pathogenic microbes by providing significant quantities of 3 proteins: sIgA, LF, and Lys [1–4]. sIgA and LF constitute ~ 5% each of the total milk protein, while Lys represents < 0.5% [1]. LF, a 75 000–80 000 M_r single polypeptide chain [5], binds 2 atoms of ferric iron with a K_1 260 × larger than that of serum transferrin [6]. By sequestering iron so strongly, LF acts to deprive the gut flora of the free iron required for proliferation and is strongly bacteriostatic in vitro for many common gut-associated pathogens [7,8]. Lys (EC 3.2.1.17; *N*-acetylmuramide glycanohydrolase) is an enzyme which hydrolyzes the glycosidic bonds between *N*-acetylglucosamine and *N*-acetylmuramic acid, components of the bacterial cell wall peptidoglycan. Breast milk [Lys] are 3–10 mg% compared with serum levels of 0.6–1 mg%, and the human milk enzyme [1] has been reported to have a specific activity 3.5 × that of egg white lysozyme [4]. The mechanism of its role in the defense of the infant gut from pathogens, yet to be fully explained, most probably is intricately interrelated with antibody and possibly complement components for full effectiveness [9]. Our long-range purpose is to systematically study such concertive or synergistic relationships involving host defense factors in human milk. Both LF and Lys represent very effective, however non-specific, antimicrobial activities. However, milk sIgA confers upon the infant specific antibacterial, antiviral, antitoxic antibodies [10] reflective of the spectrum of the mother's antigenic experience. sIgA in milk is ~ 150 mg% [1]. The intake of specific antimicrobial antibodies is quite high throughout the nursing period thus providing passive immunity during the time in which the infant immune system is maturing. By mechanisms which are not yet clear, sIgA potentiates the antibacterial activity of LF [7].

We describe here a protocol for sequential isolation of all 3 of these proteins from the same sample of pooled human milk. The very high isoelectric pH-values of Lys and LF enable them to be separated from the remainder of milk (whey) proteins and from each other in a single chromatographic separation on heparin–Sepharose, a technique described in [11] for the isolation of pure LF. From the remaining proteins, pure sIgA has been isolated. It is of great interest to begin to examine the factors in human milk which, both singly and in concert, protect against a battery of potential pathogens.

Abbreviations: sIgA, secretory immunoglobulin A; LF, lactoferrin; Lys, lysozyme; HS, heparin–Sepharose.

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2. MATERIALS AND METHODS

Human milk was obtained from the Neonatal Intensive Care Unit (University of Missouri Health Sciences Center, Columbia MO). Aliquots of individual samples were taken and pools were made of milk from at least 6 mothers. The milk was stored frozen at −20°C in 500 ml aliquots. Heparin–Sepharose and Sephadex filtration gels were obtained from Pharmacia (Uppsala). Monospecific

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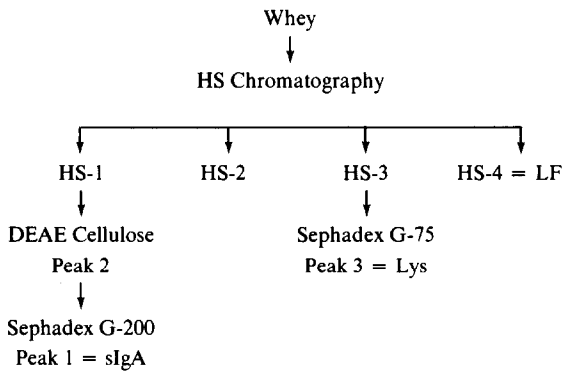
antisera to human LF (rabbit) and sIgA (goat) were obtained from Behring Diagnostics and Cappel Laboratories, respectively. Rabbit anti-serum against whole milk proteins was obtained from Nordic Immunological Laboratories. Dried *Micrococcus lysodeikticus* was obtained from Sigma.

Protein concentrations were determined as in [12] using crystalline bovine serum albumin as a standard. SDS–polyacrylamide gel electrophoresis was done as in [13]. Lys activity was quantitated spectrophotometrically [14] using heat-killed *M. lysodeikticus*. Large numbers of column fractions were screened for Lys activity using the lysoplate method [15] at pH 6.3 using egg white lysozyme as standard. Dialysis of whey prior to HS chromatography was done in Spectrapor dialysis tubing (Spectrum Medical Industries) with an ~3500 M_r cut off to prevent loss of Lys. Pressure concentrations of Lys fractions was done on Amicon UM2 membranes and all other fractions on Amicon PM30.

3. RESULTS AND DISCUSSION

Pooled human milk was thawed at room temperature and the cream removed by centrifuging at 10 000 × *g* for 30 min. All procedures were carried out at 4°C except where noted. The resultant skim milk was adjusted to pH 4.7 with 4 N HCl and incubated at 40°C for 30 min to precipitate predominantly the caseins. After centrifugation as above, the whey was decanted and the precipitate redissolved in 0.1 M Tris–HCl buffer (pH 7.5) containing 0.1 M NaCl. The latter was frozen at – 20°C and was used to estimate the amount of sIgA, Lys and LF lost at this step. A simplified flow diagram of the subsequent whey fractionation is shown below:



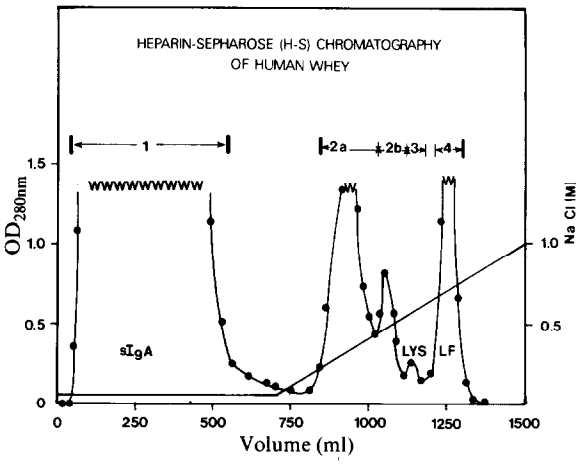


Fig.1. Heparin–Sepharose chromatography of 310 ml human whey in 0.005 M Veronal–HCl (pH 7.4), 0.05 M NaCl followed by a linear 0.05–1.0 M NaCl gradient. Flow rate was 20 ml/h. Of the total of 2864 mg which was loaded on the column, 1768, 260, 19 and 350 mg were recovered after concentration from pools 1, 2, 3 and 4, respectively.

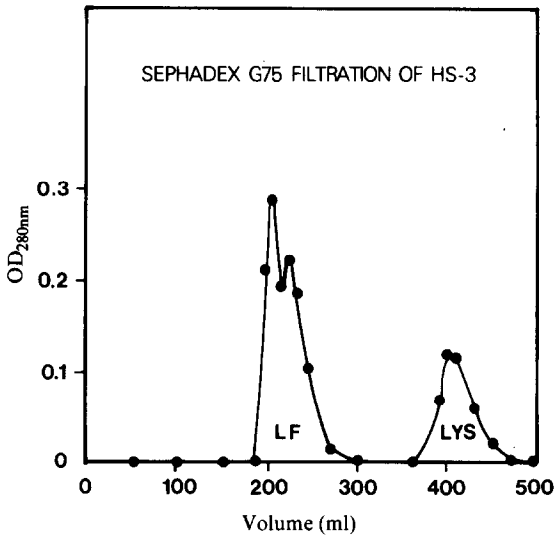


Fig.2. Sephadex G-75 gel filtration of HS-3 in 0.1 M sodium acetate (pH 6.5). Flow rate was 20 ml/h. The protein load was 37 mg protein representing ~600 ml whey from which 8 mg pure lysozyme was recovered after concentration. This separation was very reproducible with the size of the first peak varying with the degree of contamination with LF. Every attempt was made to pool fractions containing high enzyme activity into HS-3 rather than following the UV elution pattern.

Whey was dialyzed against 3 changes of a 10 × vol. 0.05 M NaCl in 0.005 M Veronal HCl (pH 7.4). HS chromatography essentially followed the method in [11]. Fig.1 shows a representative fractionation of 310 ml whey on a 2 cm × 30 cm column of HS. HS-1 contains all the whey proteins not basic enough to have affinity for the strongly negatively charged heparin moiety. This peak usually contains all of the sIgA; however, with some milk pools, a slight amount may be retained and elute off with relatively low molarity NaCl in HS-2. The first peak contains no LF and only 5–7% of the total Lys activity which is probably loosely complexed with acidic proteins. After washing the column with starting buffer, a linear 800 ml gradient from 0.05–1.0 M NaCl was initiated at a relatively slow flow rate of 20 ml/h. HS-2, HS-3 and HS-4 eluted with ~ 0.2 M, 0.4 M, and 0.6 M NaCl, respectively. HS-3 contained most of the Lys activity applied to the column and HS-4 contained all of the LF. At this point, LF was essentially pure. However it could be recycled on HS to eliminate any trace contaminants such as lysozyme. About 350 mg pure LF was obtained from 310 ml whey.

All HS fractions prior to pooling were tested immunochemically for the presence of sIgA, LF and, using the lysoplate method, Lys activity. Pools were then assayed spectrophotometrically for Lys activity: 72% of the Lys activity in the starting whey was contained in HS-3. After concentration, HS-3 was gel filtered on a 2.5 cm × 90 cm Sephadex G-75 column since the predominant contaminants were of much higher M_r-value. A symmetrical peak, eluted in the small size region (see fig.2), contained all the Lys activity. The yield of lysozyme protein was 1–1.5 mg lysozyme protein/100 ml whey.

sIgA was purified from HS-1 in a 2-step procedure following the method in [16]. The HS-1 pool was

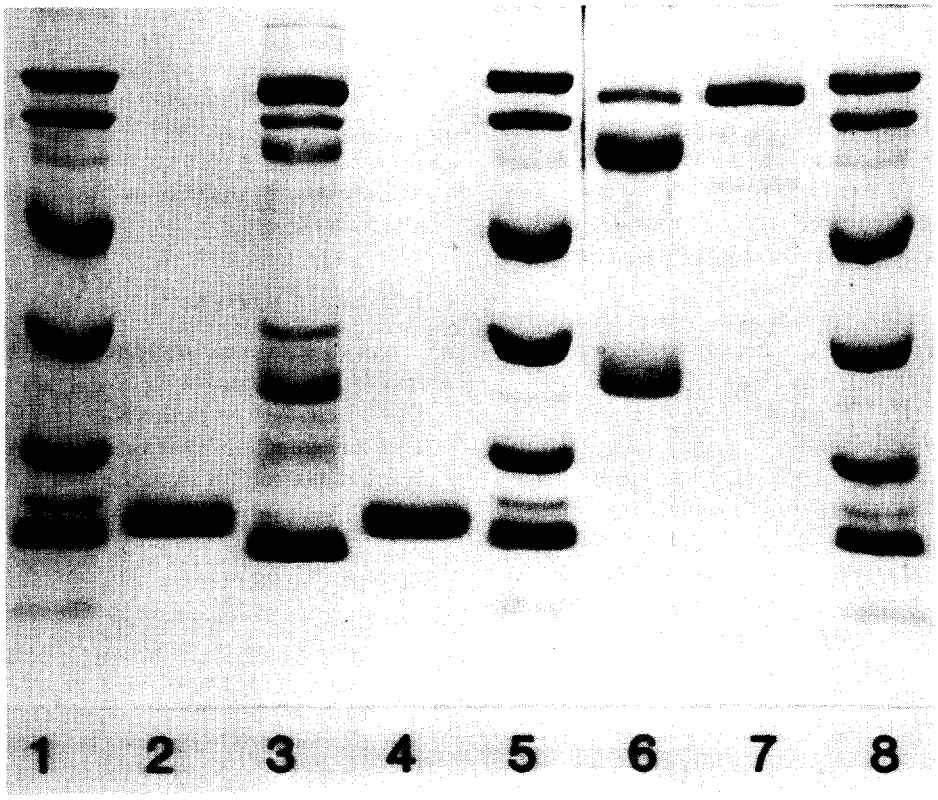


Fig.3. 12.5% SDS–polyacrylamide gel electrophoresis under reducing conditions of: whey 10 μl (3), sIgA 30 μg (6), LF 25 μg (7), Lys 20 μg (2,4), and Bio-Rad low-M_r standards (1,5,8).

concentrated 2-fold to 250 ml, dialyzed against 0.01 M sodium phosphate (pH 7.0) and applied to a 150 ml bed vol. column of DEAE–cellulose (DE 52) equilibrated with the same buffer. The column was pumped at ~50 ml/h until the first peak was completely eluted and baseline was maintained for at least 2 column vol. The bulk of the sIgA eluted with 0.1 M NaCl in the same buffer. Of the 1.7 g protein applied to this column, the peak eluting with starting buffer contained only ~1% of the protein. The second peak contained 300–350 mg total; however, only those fractions containing sIgA were pooled and concentrated for subsequent gel filtration. A similar separation was done using a 440 ml bed vol. column of DE52 with twice the length. The first peak was again only ~1% of the protein while second peak region, even though bulk eluted, clearly resolved into 2 peaks, the first of which contained all the sIgA (~130 mg) while the second contained predominantly and unidentified protein which was similar in size to, but was not, lysozyme. Regardless of which column size was used, sIgA was obtained pure from the subsequent gel filtration. After concentration, the sIgA-containing pool, still < 50% pure, was gel filtered at 20 ml/h on a 2.5 cm × 90 cm Sephadex G-200 column in 0.05 M Tris–HCl (pH 7.5) containing 0.2 M NaCl, 0.001 M EDTA and 0.003 M sodium azide. The resultant first peak contained pure sIgA with no detectable lysozyme activity. About 0.4 mg/ml whey were recovered.

LF, Lys and sIgA, prepared as described, were shown to be pure by electrophoresis in 12% SDS–polyacrylamide gels (fig.3). LF was a single protein band of ~78 000 M_r. Lys also showed a single band approximately the size of the egg white lysozyme standard (~15 000 M_r). sIgA under these reducing conditions separated into its component chains – secretory component, heavy and light chains, and J chain. In double gel diffusion using heterospecific antiserum to milk proteins both LF and sIgA showed single precipitins and, using specific anti-LF and anti-sIgA, no cross-contamination was demonstrable in the purified preparations of LF, Lys and sIgA (fig.4). The specific (i.e., units/A_{280}) enzyme activity was identical in all the fractions contained in the Sephadex G-75 lysozyme peak, again demonstrating purity of the preparation. Antisera are now being prepared to all 3 proteins for use in their quantitation during our ensuing studies of these as host-defense factors in human milk.

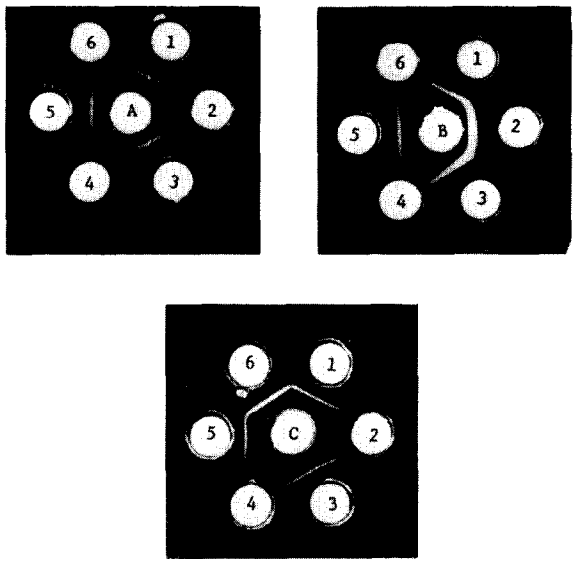


Fig.4. Ouchterlony double gel diffusion of whey (1,3,5), LF (2), Lys (4), and sIgA (6) against anti-whole milk (A), anti-LF (B) and anti-sIgA (C). The additional precipitin line detected by anti-sIgA in whey represents a contaminating antibody in the commercial antiserum used. This line is never seen with our purified sIgA preparations.

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REFERENCES

[1] McClelland, D.B.L., McGrath, J. and Samson, R.R. (1978) Acta Paediatr. Scand. Suppl. 271, 2–20.
[2] Hanson, L.A. (1961) Int. Arch. Allergy Appl. Immunol. 18, 241–267.
[3] Johansson, B. (1960) Acta Chem. Scand. 14, 510–512.
[4] Jolles, J. and Jolles, P. (1967) Biochemistry 6, 411–417.
[5] Querinjean, P., Masson, P.L. and Heremans, J.F. (1971) Eur. J. Biochem. 20, 420–425.

[6] Aisen, P. and Leibman, A. (1972) Biochim. Biophys. Acta 257, 314–323.
[7] Bullen, J.J., Rogers, H.J. and Leigh, L. (1972) Brit. Med. J. 1, 69–75.
[8] Reiter, B., Brock, J.H. and Steel, E.D. (1975) Immunology 28, 83–95.
[9] Adinolphi, M., Glynn, A.A., Lindsay, M. and Milne, C.M. (1966) Immunology 10, 517–526.
[10] Ogra, P.L. and Dayton, D. (1979) in: Immunology of Human Breast Milk, pp. 145–157, Raven, New York.
[11] Blackberg, L. and Hernell, O. (1980) FEBS Lett. 109, 180–184.
[12] Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265–275.
[13] Laemmli, U.K. (1970) Nature 227, 680–685.
[14] Zucker, S., Hanes, D.J., Vogler, W.R. and Eanes, R.Z. (1970) J. Lab. Clin. Med. 75, 83–92.
[15] Osserman, E.F. and Lawlor, D.P. (1966) J. Exp. Med. 124, 921–952.
[16] Pincus, C.S., Lamm, M.E. and Nussenzweig, V. (1971) J. Exp. Med. 133, 987–1003.