Purification of Membrane-bound Lactoferrin from the Human Milk Fat Globule Membrane

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Although lactoferrin is known as a basic soluble glycoprotein, the presence of the membrane-bound form of this protein has also been demonstrated in human milk. Membrane-bound lactoferrin was extracted from the human milk fat globule membrane with a detergent mixture of 1% Tween-20, 0.5% C12E8, and 0.5 mM KCl in 20 mM Tris-HCl (pH 7.4). Lactoferrin in the detergent-soluble fraction was purified by affinity chromatography with Concanavalin A and by hydrophobic chromatography with phenyl-Superose. The purified protein gave a single band of 80 kDa by SDS-PAGE. Its N-terminal amino acid sequence was consistent with that of human lactoferrin.

Key words: lactoferrin; human milk; milk fat globule membrane; purification

Lactoferrin (LF) is a multifunctional iron-binding glycoprotein with potent antimicrobial properties. A large amount of LF is secreted from the lactating mammary gland into milk, and especially in human colostrum. This protein has also been found in some forms of epithelial tumour and shown to be down-regulated in them. A human casein fraction prepared by both acid and ultracentrifugal precipitation contained a substantial amount of LF. LF in the membrane-bound form has also been demonstrated in epithelial cells of the mammary gland and identified in the membranes of human milk fat globules. To date, the isolation of membrane-bound LF has not been demonstrated and, therefore, the function of LF in the membrane remains unknown.

The membrane surrounding the fat globule in milk, which is referred to as the milk fat globule membrane (MFGM), is derived from the apical plasma membrane of mammary secretory cells during the secretion of milk lipid. Since it is not easy to obtain lactating human mammary gland epithelial cells, the membrane fraction from fat globules of human milk is often used as a substitute for the membrane of the mammary epithelial cell. We describe in this study a method for purifying membrane-bound LF from human MFGM (hMFGM).

Mature human milk was obtained from volunteers introduced by Tochigi National Hospital (Utsunomiya, Japan). The human milk was centrifuged for 90 min at 100,000 x g and 4°C on a swing rotor (Hitachi RPS 27-2) to separate into four fractions: cream, whey, skim milk membrane, and casein pellet. The floating cream layer was churned by hand-shaking after its dispersion with 2.5 volumes of chilled distilled water. In this process, MFGM is released from the fat globules and drained as buttermilk. The resulting buttermilk was collected as the membrane fraction. This membrane fraction (80 mg as protein) was washed with a 0.1 M sodium-citrate buffer (pH 7.0) to remove the casein and whey protein contaminants, and then centrifuged at 100,000 x g and 4°C for 1 h to yield a pellet of hMFGM. The resulting hMFGM (43 mg) was rendered soluble with a mixture of 1% Tween 20, 0.5% nonionic detergent C12E8, and 0.5 mM KCl in 20 mM Tris-HCl (pH 7.4) containing 2 mM PMSF, 1 mM EDTA and 1% aprotinin. After stirring for 30 min at 4°C, the mixture was centrifuged at 100,000 x g for 1 h, and the resulting supernatant fraction was used for the next purification steps. Protein was determined by the method of Peterson. SDS-PAGE was performed by using 10% acrylamide gel, the protein bands being visualized by Coomassie brilliant blue R-250.

Step 1. Affinity chromatography with Con A
The supernatant fraction (15.1 mg of protein/50 ml) was directly fractionated through a Con A-Sepharose 4B column (10 x 0.5 cm, Sigma) that had been equilibrated with 20 mM Tris-HCl (pH 7.4).

Abbreviations: LF, lactoferrin; TF, transferrin; MFGM, milk fat globule membrane; C12E8, n-dodecylpolyethylene glycol monoether; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; Con A, Concanavalin A; HPLC, high-performance liquid chromatography; PMSF, phenylmethylisulfonyl fluoride
containing 1% Tween 20, 0.5% C12E8 and 2 mM PMSF (buffer A). The unadsorbed protein was washed with several column volumes of buffer A, and the adsorbed protein was eluted with 0.25 M of \(\alpha\)-d-methylmannospyranoside in buffer A. The eluted protein fraction was concentrated to 5 ml by ultrafiltration with a PM 10 membrane (Amicon), and to the resulting concentrate was added solid (NH4)2SO4 to make a final concentration of 2 M.

**Step 2. Phenyl-Superose HPLC**
The LF-enriched fraction in 2 M (NH4)2SO4 was centrifuged at 15,000 \(\times\) g for 10 min, and an aliquot of the resulting supernatant (3.2 mg of protein/5 ml) was subjected to hydrophobic HPLC in a phenyl-Superose HR 5/5 column (Pharmacia Biotech) which had been equilibrated with a 50 mM Tris-HCl buffer (pH 7.4) containing 0.2% Tween 20, 0.2% C12E8 and 2 M (NH4)2SO4. The unadsorbed fraction was washed out with the equilibration buffer until no protein could be detected. The adsorbed portion was eluted with a decreasing linear gradient of (NH4)2SO4 from 2 to 0 M in 30 min. The eluted LF fraction (0.7 mg) was pooled and used for SDS-PAGE and an N-terminal amino acid sequence analysis.

About 54% of membrane protein was recovered from the buttermilk after washing with a citrate buffer (pH 7.0). LF in hMFGM was efficiently extracted with the detergent mixture. The extract was then applied to Con A affinity chromatography (Fig. 1-A). LF adsorbed to the Con A column was eluted by 0.25 M \(\alpha\)-d-methylmannospyranoside. Subsequent hydrophobic HPLC gave a single protein peak of LF that was eluted at 1 M (NH4)2SO4 (Fig. 1-B). The recovery of this preparation was about 1.6% of hMFGM protein by weight.

The SDS-PAGE patterns during the purification process are shown in Fig. 2. hMFGM was composed of major band of 155, 70, 58, 52, 45, and 39 kDa (lane 2). Its pattern was very close to that reported for hMFGM prepared from washed cream\(^{10,11}\) and showed that bands resulting from whey protein and casein were not present. The purity of the preparation from hydrophobic chromatography was confirmed in lane 5 by a single band with an apparent molecular weight of 80,000. Its N-terminal amino acid sequence was analyzed with a gas-phase protein sequencer (Applied Biosystems model 476 A) after electroblotting onto a polyvinylidene difluoride membrane.\(^{12}\) The sequence was determined up to 23rd residue as G-R-R-R-S-V-Q-W-X-T-V-S-Q-P-E-A-T-K-X-F-Q-W, which is similar to the published sequence for human LF,\(^{13}\) except for the 11th residue (T in our preparation instead of A). In addition, the amino acid composition was almost...
identical to that published for human LF\textsuperscript{13} (data not shown). The possibility of this protein being transferrin (TF) can be excluded, because glycine and the following four arginine residues at the N-terminus are characteristic of LF, although TF is known as a homologous protein to LF and as a major cytosolic protein in mammary epithelial cells.\textsuperscript{10} PAS-4 glycoprotein in MFGM, which is homologous to CD36 and has a molecular mass (76 kDa)\textsuperscript{10} close to that of LF, was successfully separated from LF by affinity to phenyl-Superose. Thus, membrane-bound LF could be successfully purified from hMFGM by taking advantage of the difference in affinity to Concanavalin A and phenyl groups.

We have shown in this study that a substantial amount of LF was associated with MFGM in human milk, because LF could not be released from hMFGM without 1% Tween 20 or 0.5 m KCl. Non-ionic detergents such as Tween 20 and C\textsubscript{12}E\textsubscript{8} were necessary to render soluble and separate LF from hMFGM by column chromatography. The interaction between LF and membrane proteins is thought to be through hydrophobic bonding. The basic property of LF resulting from its high pI value\textsuperscript{17} may initiate their interaction. The expression of LF receptors has been demonstrated in many kinds of cell, including epithelial cell lines from a non-malignant human breast.\textsuperscript{17,18} although the interaction between LF and MFGM is not likely to be through the receptor since LF did not dissociate from MFGM under low pH or high salt conditions (data not shown). LF could also be extracted from hMFGM with 1 m MgCl\textsubscript{2}, Triton X-100 or by sonification.\textsuperscript{5}

We propose the following explanation for the occurrence of membrane-bound LF. Just after the secretion of milk fat globules, the membrane surrounding the fat globules undergoes structural rearrangement in the alveolar lumen.\textsuperscript{7} LF molecules might be involved in this process to form one of the constituents of MFGM through hydrophobic interaction. It is under investigation which protein in MFGM is responsible for the interaction with LF.

**References**