High Molecular Weight Mucin-like Glycoprotein in Bovine Milk

Yoshihiro Kanamaru, Takashi Toyoki, Satoshi Nagaoka, Yasuo Kuzuya, and Ryoya Niki *

Department of Food Science, Faculty of Agriculture, Gifu University, Gifu 501-11, Japan
* Laboratory of Food Biochemistry, Faculty of Agriculture, Hokkaido University, Sapporo 060, Japan

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A novel high-M, glycoprotein was found in bovine milk. It was eluted in the void volume fraction of the whey proteins by Sepharose 6B gel filtration and showed only restricted electrophoretic migration with SDS polyacrylamide gel electrophoresis (SDS-PAGE) in a composite gel of 1.5% acrylamide/1% agarose. By using the lectin staining technique after the electrophoresed gel had been transferred to nitrocellulose paper by Western blotting, the component was clearly visualized by eight out of the nine peroxidase-conjugated lectins used, including DBA and Con A. The similarity in SDS-PAGE pattern and lectin binding property to the high molecular weight mucin-like glycoproteins (HMGP) in human milk, 2,3 together with preliminary data of its amino acid composition, suggest that it is the bovine counterpart of HMGP. The occurrence of this type of glycoprotein has not previously been reported in bovine milk.

In 1982, Shimizu and Yamachi 3 originally reported the presence of one HMGP component (HMGP-C) in human milk, which was initially termed PAS-O because of its incapability to migrate into 10% acrylamide gel and its intense staining with periodic acid-Schiff (PAS) reagent (a carbohydrate content of 50% (w/w) was found). More recently, Shimizu et al. 4 have reported the occurrence of other HMGP components (HMGP-A and HMGP-B), revealing a much larger molecular size and containing more carbohydrate (80% (w/w)). These components were found both in human milk and on the human milk fat globule membrane (MFGM). Similar glycoproteins have never been discovered in bovine milk, 2,4,5 but have been found in moneky, 6 chimpanzee, 6 horse, 6 dog, 7 and rat 8 milk. Their exact biological significance still remains to be elucidated. Speculation on their role in protecting the surface of MFGM against attack by protease9 and in facilitating milk fat globule adherence to the mucus coating the gut, thus slowing globule movement and increasing digestive efficiency, 10 has been presented. If these important nutritive functions are indeed possessed by HMGP, elucidation of the presence or the absence of each component(s) in bovine milk seems to be critical, because bovine milk is widely used for the production of infant formula. In this study, we reexamined the possible occurrence of these types of glycoproteins in bovine milk.

Bovine whey was prepared after three-fold dilution of normal skimmed milk from Holstein cows at the University Farm by adjusting to pH 4.6 and removing the casein precipitate. After adjusting to pH 6.0, the whey was dialyzed against distilled water, and the whey proteins were lyophilized. Human milk was obtained from healthy mothers 2-7 days after parturition. The lyophilized whey proteins were obtained by the same methods as those used for preparing the bovine milk whey, except that the separation of acid whey from casein was done by centrifugation at 18,000 rpm for 3 h.

SDS-PAGE was conducted in a vertical slab unit according to the procedure employed for characterizing distinct species of human respiratory mucous glycoprotein. 6 The samples for electrophoresis were prepared by boiling the presence of 2% SDS without a reducing agent. The electrophoresed gel was transferred to nitrocellulose membranes (Schleicher & Schuell Inc., Keene, U.S.A.) according to the procedure of Towbin et al. 7 Blotting was conducted overnight with cooling by running tap water. The transferred nitrocellulose papers were stained by peroxidase-conjugated lectins as described by Moroi and Jung. 8 The peroxidase-conjugated lectins (Ulex europaeus, UEA-I; peanut agglutinin, PNA; Dolichos biflorus, DBA; soybean agglutinin, SBA; Ricinus communis, RCA-120; Concanavalin A, Con A; lentil lectin, LCA; wheat germ agglutinin, WGA; and Phaerulus vulgaris, PHA-E) 2 were purchased from Seikagaku Kogyo Co., Ltd., Tokyo, Japan.

To obtain the high-M, fraction, gel filtration on Sepharose 6B of the whey proteins was carried out in a 50 mm Tris-HCl buffer at pH 8.0 containing 0.15 M NaCl, 2 mM EDTA and 0.02% NaN 3 . The elution profiles monitored by absorption at 280 nm showed that the bovine whey proteins, as well as the human type, contained substantial amounts of components excluded by the Sepharose 6B gel matrix. The peak of the void volume (the 26th chromatographic fraction) was comparable between the bovine and human whey proteins. Bovine IgM (M, of 106 dalton) was found to elute at around the 40th fraction by the same gel chromatography, indicating that the glycoprotein under consideration would have been contained in the void volume fractions. These fractions were then pooled as the high-M, fraction (F1), concentrated, and rechromatographed in the same column. Each rechromatographed F1 from the bovine and human whey proteins was further analyzed in the following study.

The high-M, glycoprotein components in F1 were clearly detected when lectin-staining was carried out after electrophoretic transfer to the

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Table Staining of the High-M, Glycoproteins in Whey with Different Lectins

<table>
<thead>
<tr>
<th>Lectin</th>
<th>Specificity</th>
<th>Human HMGP</th>
<th>Bovine</th>
</tr>
</thead>
<tbody>
<tr>
<td>UEA-I</td>
<td>a-L-Fucose</td>
<td>±</td>
<td>±</td>
</tr>
<tr>
<td>PNA</td>
<td>Galactose-β(1→3)-N-acyetylgalactosamine</td>
<td>+</td>
<td>±</td>
</tr>
<tr>
<td>SBA</td>
<td>α- and β-D-N-</td>
<td>+</td>
<td>±</td>
</tr>
<tr>
<td></td>
<td>Acetylgalactosamine</td>
<td></td>
<td>+</td>
</tr>
<tr>
<td>RCA-120</td>
<td>β-D-Galactose</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>DBA</td>
<td>α- N-Acetylgalactosamine</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Con A</td>
<td>α-D-Mannose, α-D-glucose</td>
<td>-</td>
<td>±</td>
</tr>
<tr>
<td>LCA</td>
<td>α-D-Mannose, α-D-glucose</td>
<td>-</td>
<td>±</td>
</tr>
<tr>
<td>WGA</td>
<td>β-N-Acetylgalactosamine</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>PHA-E</td>
<td>Galactose-β(1→4)-N-</td>
<td></td>
<td>++</td>
</tr>
<tr>
<td></td>
<td>acetylgalactosamine-β(1→2)-mannose</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

++ = dark staining; + = intermediate staining; ± = slight staining; - = no staining.
nitrocellulose membrane from the electrophoresed gel. The lectin-staining profiles of both bovine and human F1 are presented in Fig. These data, as well as others, are summarized in Table. As can be seen in the figure, human F1 contains two electrophoretically distinct high- $M_r$ glycoprotein species (designated as HMGP-A and -C). They were distinguished not only by their different electrophoretic mobility, but by their different reactivity to various lectins, as shown in Table. These differences between the two components are compatible with the properties of HMGP-A and -C reported by Shimizu and Yamauchi$^{39}$ and by Shimizu et al.$^{2,3}$

As shown in Fig., several bands were detected in bovine F1 by this technique; one with very low electrophoretic mobility and recognized by all lectins, except UEA-I (designated as A), one visualized as a broad band at the intermediate migrating position, and others with high electrophoretic mobility. All of them were recognized by WGA and, although not so obviously, by Con A. The specificity of PNA and DBA was strikingly restricted to component A. The reaction specificity of the latter is especially worthy of note, because only bovine component A was recognized by this lectin among the various human and bovine components. SBA, PHA, RCA-120, and LCA also recognized component A (see Table). These results clearly show that an HMGP-like component does exist in bovine milk.

Whether or not component A also occurs on bovine MFGM seemed to be one of the most important aspects to be elucidated. Human HMGP components have been reported to exist in the glycoalyx (the outer surface) of the milk fat globule$^{11}$ and contain a considerable amount of carbohydrate. So we prepared the soluble apoprotein fraction from bovine MFGM according to Kanno et al.$^{11}$, which was found to be rich in carbohydrate. When the apoprotein preparation was analyzed by SDS-PAGE and lectin-staining, the typical band of component A with low mobility was clearly detected.

Bovine component A was further purified from F1. Because several other components in F1 were separated from component A in the presence of SDS (see Fig.), gel filtration with the buffer system containing SDS$^{10}$ was conducted on Sepharose CL-2B. When the component in the void volume fraction was analyzed by SDS-PAGE and lectin-staining, only a single band corresponding to component A was detected. The amino acid composition of this component was then investigated by a Hitachi 835 automatic amino acid analyzer. Different from human HMGP-A or -C, the bovine component contained less threonine and serine (one third and two thirds of the value in HMGP-A),$^{2}$ respectively. On the other hand, the amount of glycine was more than double that reported for human HMGP-A. The profiles for the composition of the other amino acids were very similar to those of human HMGP-A. Less threonine and serine implies that less glycosylation might occur in bovine component A. This would explain the weak staining in the electrophoresed gels of component A in bovine milk by the PAS reagent, compared with the intense staining of HMGP components in human milk, and may further explain why component A has never previously been discovered in bovine milk. Only the PAS staining technique was employed for detecting the component in the studies reported so far.$^{2,3,4}$

From the results presented here, it can be concluded that component A found in bovine F1 could be the counterpart of human HMGP in bovine milk. The occurrence of a glycoprotein of this kind does not seem to be restricted to the milk of a particular species, as has previously been thought; rather, it seems likely that the milk of mammals contains HMGP-equivalent component(s), whose role in milk would therefore be common among mammals, as has been postulated in the literature.$^{2,3}$

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References