Identification of a Calcium-Binding Protein in Human Milk as α-Lactalbumin

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ABSTRACT
Calcium-binding activity of proteins in human milk was surveyed by Chelex-100 assay. Two calcium-binding proteins besides caseins were found by gel filtration on Sephadex G-100 and chromatography on diethylaminoethyl Sephadex. Results of the amino acid analysis and immuno-double diffusion confirmed that these two components were the same protein and identical with α-lactalbumin.

Studies about the effect of ethyldiaminetetraacetate on electrophoretic (Disc-polyacrylamide-gel electrophoresis) mobility and on thermal stability suggested that human α-lactalbumin is a calcium metalloprotein. One mole of human α-lactalbumin appeared to bind one mole of calcium ions, and the formation constant for the calcium-human α-lactalbumin complex was 1.0 x 10^5 M^{-1}.

INTRODUCTION
Calcium in human milk is essential for growth of infants. The concentration of calcium in the milk is about 8 mM (16). The concentration of caseins, which can form colloidal micelles by binding calcium, is much less in human milk than in other mammalian milks, and, therefore, calcium in human milk is present mainly as a soluble (14) rather than as a colloidal form. However, calcium-binding components in addition to caseins may be in human milk.

Zittle et al. (25) reported that β-lactoglobulin in bovine milk bound calcium, but this component is absent from human milk (3).

Recently, Hiraoka et al. (12) demonstrated that bovine α-lactalbumin was a calcium metalloprotein. Tamura et al. (20) found two calcium-binding proteins in bovine milk and suggested that these components might be identical with α-lactalbumin.

In this paper, we surveyed the distribution of calcium-binding proteins in human milk and found a calcium-binding protein in addition to caseins. This protein was identified as α-lactalbumin.

MATERIALS AND METHODS

Human Skim Milk
Human milk samples were obtained from a healthy mother after 1 mo of parturition. Each sample immediately was defatted by centrifugation and stored at -20°C. Samples were defrosted and pooled prior to experiments.

Survey and Preparation of Calcium-Binding Protein in Human Milk
Twenty milliliters of human skim milk was applied to the column of Sephadex G-100 (2 x 120 cm) equilibrated with 50 mM imidazole-HCl buffer, pH 6.3, according to Armstrong et al. (1). Flow rate was 10 ml/h, and eluent was collected in 5-ml portions at 4°C. The effluent was monitored by measurement of absorbance at 280 nm, and calcium-binding activity of each portion was measured by Chelex-100 assay using 45Ca (see later). The portions having calcium-binding activity were collected, concentrated, and then applied to the ion exchange chromatography of diethylaminoethyl (DEAE)-Sephadex A-50 (1.6 x 10 cm) equilibrated with the same buffer. Elution was with a linear gradient of NaCl from 0 to .3 M at room temperature, and the effluent was monitored at 280 nm. Fractions having high calcium-binding activity were collected and dialyzed against de-
ionized water and then lyophilized. We called these fractions human milk calcium-binding proteins (Hm-CaBP) and used them for further experiments.

Assay for Calcium-Binding Activity

Calcium-binding activity was measured by Chelex-100 resin according to the method of Wasserman et al. (23). One milliliter of each test sample was pipetted into a small test tube, and .2 ml of Chelex resin suspension, containing .05 to .1 ml of packed resin equilibrated with 50 mM imidazole-HCl buffer, pH 6.3, was added. One-tenth milliliter of $^{45}$Ca (.5 µCi) was added to the suspension and stirred for 15 s vigorously. After centrifuged at 1500 rpm for 10 min, .2 ml of the supernatant was pipetted into a liquid scintillation vial containing 10 ml of liquid scintillator (Bray’s solution (4)). Assays were at room temperature. Radioactivity of $^{45}$Ca was measured with Aloka liquid scintillation spectrometer LSC-670. Calcium-binding activity was expressed as the percentage of $^{45}$Ca remaining in the supernatant.

Human α-Lactalbumin and Antihuman α-Lactalbumin Serum (Rabbit)

Human α-lactalbumin (Hα-La) prepared by the method of Armstrong et al. (1, 2) and antihuman α-lactalbumin serum (rabbit) kindly were provided by Jun-ichi Kurisaki in our laboratory.

Disc Polyacrylamide Gel Electrophoresis (Disc-PAGE)

Disc-PAGE was according to the method of Davis (9) with 10% acrylamide-gel in the presence and absence of .2 mM CaCl$_2$ or .2 mM EDTA.

Immuno-Double Diffusion

This was by the Ouchterlony method (17) on 1% agarose gel in Veronal buffer, pH 8.6. Samples were dissolved in phosphate buffered saline, pH 6.3, to made .1% solution. Eight microliters of the sample solution was pipetted into a well, and the equal volume of the antiserum was pipetted into another well. The gel was left to stand overnight at room temperature, and then formation of immunoprecipitine lines was observed.

Amino Acid Analysis

After hydrolysis of Hm-CaBP for 24 h in 6 N HCl at 110°C, the amino acid composition was determined on a Hitachi Model 835 amino acid analyzer.

Formation Constant

Formation constant (Kf) for the calcium-Hm-CaBP complex was determined by the ion exchange method of Schubert et al. (19). Varying concentrations of Hm-CaBP (.5 to 4.0 µM) were prepared in 1 mM imidazole-HCl buffer, pH 7.2, containing .16 M NaCl. One milliliter of Dowex 50 W-X4 (100 to 200 mesh) resin suspension (containing 5 mg of the resin equilibrated with the same buffer) and .5 ml of $^{45}$Ca (.5 µCi) were added to the solution. The final volume was adjusted to 5 ml with the buffer. Equilibrium was achieved by mixing on a shaker at room temperature for 16 h. After centrifuging at 1500 rpm for 10 min, .2 ml of the supernatant was taken for measurement of $^{45}$Ca-radioactivity. The Kf was calculated by the following expression,

\[ K_f = 1/K_c = [(K_d^0/K_d) - 1]/(A^b)^n \]

where Kd = distribution coefficient, Kd$^0$ = distribution coefficient in the absence of Hm-CaBP, and A$^b$ = concentration of Hm-CaBP of charge b.

Differential Scanning Calorimetric Analysis

The Hm-CaBP was dissolved in 20 mM Tris-HCl buffer, pH 7.1, in the presence and absence of 1 mM CaCl$_2$ or 1 mM EDTA. The concentration of the protein was 2.1%. Sixty microliters of the sample was pipetted into a silver cell and was analyzed by a differential scanning calorimeter (Daini-Seikosha SSC 60). Conditions were: sensitivity, 100 µV; heating rate, .6°C/ min. Reference was 4.5 mg of Sephadex G-200 in 50 µl of distilled water.

Reagents

All chemicals were of analytical grade.

RESULTS

A typical gel filtration pattern of human skim milk is in Figure 1. Calcium-binding activ-
ity of each fraction is indicated. Human skim milk was separated into two large fractions by gel filtration on Sephadex G-100. The first protein peak that eluated at void volume was considered to contain mainly caseins as precipitation occurred when the pH of this fraction was brought to 4.6. Calcium-binding activity of this fraction was not as high as the second pro-
tein peak. We designated the second protein peak, which had high calcium-binding activity, as a human milk calcium-binding protein (Hm-CaBP) fraction and further purified it on DEAE-Sephadex A-50.

On DEAE-Sephadex chromatography (Figure 2), the Hm-CaBP fraction obtained in Figure 1 was separated into two fractions (P-I and P-II). Such a phenomenon was observed by Tamura et al. (20) for bovine milk CaBP. These two protein peaks possessed calcium-binding activity (Figure 2), and the activity measured by Chelex-100 assay was higher for P-II than for P-I.

The effect of EDTA on Hm-CaBP was studied on Disc-PAGE in the presence and absence of calcium or EDTA (Figure 3). The two proteins (P-I and P-II) showed the same mobility in each case. Moreover, the mobility was the same as that of α-lactalbumin. In the presence of EDTA, the mobility of Hm-CaBP was higher than in the presence of calcium as for calmodulin (13). These P-I and P-II might be the same protein and identical with α-lactalbumin. We tried further investigation to identify these two proteins.

Figure 4 shows the immuno-double diffusion patterns of Hm-CaBP (P-I and P-II) and human α-lactalbumin (Hα-La) with antihuman α-lactalbumin serum. Both P-I and P-II formed a fuse with Hα-La. Thus, both proteins were immunologically identical with Hα-La. From the results of amino acid analyses, both proteins are α-lactalbumin.

Furthermore, a peak of α-lactalbumin on DEAE-chromatography in the presence of 1 mM CaCl₂ and 1 mM EDTA appeared at different positions (Figure 5). The elution volumes of peak A and peak B were identical with those of P-I and P-II (Figure 2), respectively.

From these results, we concluded that the Hm-CaBP obtained here is α-lactalbumin. Calcium bound at the different amounts to this protein caused the chromatographic heterogeneity in Figure 2.

In Figure 6, ((Kd°/Kd) - 1) versus Aᵇ plot on log-log paper is shown at concentrations of .5, 1.0, 2.0, and 4.0 μM of Hm-CaBP (Hα-La) to obtain the formation constant (Kf). Slope, n, molar ratio of calcium to Hα-La in the calcium-Hα-La complex was .80, i.e., 1 mole of calcium associated with 1 mole of Hα-La. The Kf was calculated from the equation under the assumption that n = 1 to obtain 1.0 × 10⁵ M⁻¹.

Figure 7 shows the differential thermogram of Hα-La. Intact Hα-La showed a broad endothermic peak at 35 to 40°C and a second one at about 65°C. The EDTA caused increase of the former peak and decrease of the latter. Calcium, however, caused decrease of the former and increase of the latter. This indicates that the thermal stability of this protein is decreased by addition of EDTA and increased by addition of calcium. Similar phenomena were observed by Hiraoka et al. (12) on bovine α-lactalbumin by CD measurement.

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Figure 5. Chromatogram of human α-lactalbumin on DEAE-Sephadex A-50 in the presence of 1 mM CaCl₂ (---), and 1 mM EDTA (----). ⋮⋯ shows NaCl concentration. A and B indicate the peaks of the elution volumes of chromatographed solutes.

DISCUSSION

Human skim milk was separated into two large fractions by gel filtration on Sephadex G-100. The first fraction (casein fraction) did not show high calcium-binding activity by Chelex-100 assay. Calcium in casein micelles may be not so easily exchangeable with soluble calcium as Hm-CaBP for bovine milk (24). It is also likely that the binding constant for the calcium-casein complex is much lower than that for the calcium-Chelex resin complex, and, therefore, this causes the low calcium-binding activity of the casein fraction.

**TABLE 1.** Amino acid composition of human milk CaBP (P-I and P-II) obtained by DEAE A-50 chromatography. Preparation of these fractions and amino acid analyses were as described in Materials and Methods.

<table>
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<th>Amino acid</th>
<th>P-I Molar ratio</th>
<th>Nearest integer</th>
<th>P-II Molar ratio</th>
<th>Nearest integer</th>
<th>Human α-lactalbumin (μM)</th>
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¹ND, not determined.
Lysozyme in egg white is one of calcium-binding proteins (15). This component is also in human milk in much higher concentration than in bovine milk (8). The primary structure of this protein is highly homologous to α-lactalbumin (5), but we could not find the calcium-binding activity corresponding to lysozyme by this survey.

By surveying calcium-binding proteins in human milk, two components were observed on DEAE-Sephadex chromatography. The results of amino acid analysis (Table 1) and immmuno-double diffusion (Figure 4) show that these proteins are α-lactalbumin. The Ha-La shows two protein peaks on DEAE chromatography (18). When the chromatography was in the presence of 1 mM CaCl₂ or 1 mM EDTA, the chromatogram showed a single peak in each case, and their elution volumes (peak A, in the presence of CaCl₂ and peak B, in the presence of EDTA in Figure 5) corresponded to those of P-I and P-II (Figure 2). These two proteins were considered to be the same protein that binds calcium ions at different rates.

Bovine α-lactalbumin is a calcium-binding metalloprotein (12). The band corresponding to Hm-CaBP on Disc-PAGE ran faster in the presence of EDTA than in the presence of calcium. The effect was similar for calmodulin (13) on Disc-PAGE where EGTA was used instead of EDTA. Measurement of the formation constant of Ha-La for calcium shows that 1 mole of calcium bound to 1 mole of this protein as well as bovine α-lactalbumin (12). The curve of differential scanning calorimetry suggests that calcium stabilizes Ha-La against heat (Figure 6). The endothermic heat effect near 65°C was caused by denaturation of the protein which contained calcium. In contrast, the endothermic peak, between 30 and 40°C, was explained by an unfolding of α-lactalbumin that contained no calcium. The primary structures of both human (11) and bovine (5, 6, 21) α-lactalbumins have been established. Although the difference is found in about one-third of the sequences, both structures are similar, so the mode of metal-protein complex formation may be similar. By model building studies of bovine α-lactalbumin on three-dimensional structure (7, 22), a cleft of the region also appears in the region corresponding to the metal-binding site of lysozyme. A similar cleft may be in Ha-La.

α-Lactalbumin is a modifier protein in lactose biosynthesis (10). Whether α-lactalbumin requires calcium ions for biological activity is an interesting subject for further study.

ACKNOWLEDGMENTS

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REFERENCES