Determination of α-Lactalbumin in Human Milk

ABSTRACT

α-Lactalbumin is a major protein in human milk and of considerable nutritional importance to the infant. However, there are discrepancies in the literature on content of this protein in human milk, which indicate a need for a method that would permit estimation from a large number of samples. The technique of immunodiffusion was adopted for human α-lactalbumin. Purification of α-lactalbumin for production of antigen is described as is the statistical evaluation of the method. The α-lactalbumin contents of pooled human milk samples as well as of the milk from one mother during 2 mo of lactation were analyzed. The α-lactalbumin content of human milk showed considerable variation. This variation and its relation to factors such as length of lactation, and nutritional status of the mother should be studied further.

INTRODUCTION

Knowledge of the protein composition of human milk and its variation is scanty. Generally, the opinion has been that the total protein content of human milk is relatively constant. However, due to lack of reliable methods for the determination of individual human milk proteins, little is known about variations in the content of different proteins in the milk.

Of special interest from the nutritional point of view is α-lactalbumin, which is a major component of human whey proteins (7, 11, 12). Studies by Forsum (3) have shown that bovine α-lactalbumin is a protein of high nutritive quality. Since human and bovine α-lactalbumin have similar amino acid composition (14), it is likely that α-lactalbumin in human milk contributes significantly to its high protein quality. Information about the α-lactalbumin content and its variation in human milk would be of value in the formulation of so-called “humanized breast milk substitutes” (3).

Nagasawa et al. (12) found 2.8 mg α-lactalbumin/ml of milk by electrophoresis in polyacrylamide gels. Ley and Jenness (7) reported higher values, namely 4.8 mg α-lactalbumin per ml of milk, using an enzymatic assay. As these methods are less suitable for routine analysis, immunological determination according to Mancini (9) was adopted for human α-lactalbumin.

MATERIALS AND METHODS

Human milk was obtained from Mjölkcentralen, Stockholm, Sweden, as were samples of pooled human milk. The pooled milk was sampled at weekly intervals from a milk bank consisting of milk from several mothers and intended for the feeding of infants in hospitals. Milk samples from one mother were collected as follows: 2 to 5 ml milk were collected by breast pump before and after nursing. Equal amounts of the two samples were mixed and kept frozen until analyzed.

α-Lactalbumin was purified by a modification of the procedure described by Phillips and Jenness (14). Five hundred milliliters of fresh human milk were defatted by centrifugation at 120 x g for 20 min. Casein was precipitated by adjusting the pH of the milk to 4.6 with 1 M HCl at 26 C. After the milk was at this temperature for 2 h, the casein was separated from the whey by centrifugation at 27,000 g for 15 min. The whey was divided into portions of 100 ml each and stored frozen until required. Each 100 ml-portion was transferred into .02 M phosphate buffer (pH 7.0, .13M NaCl) for subsequent ion-exchange chromatography, by gel filtration through Sephadex® G-25 course. The sample (130 ml) was then applied to an ion-exchange column (DEAE-Sephadex® A-25, 5 x 25 cm) equilibrated with the same buffer. The A-25 column was eluted with this buffer at a flow rate of 120 ml/h. No salt gradient was used to elute the
column. Fractions containing crude \( \alpha \)-lactalbumin were pooled, lyophilized, dissolved in 5 ml buffer, and equilibrated with buffer with an appropriate Sephadex\textsuperscript{R} G-25 column. The \( \alpha \)-lactalbumin thus obtained was purified further by gel filtration in a Sephadex\textsuperscript{R} G-75 column in the phosphate buffer. The purified \( \alpha \)-lactalbumin was desalted on Sephadex\textsuperscript{R} G-25 course and lyophilized.

Amino acid analyses were by an automatic amino acid analyzer (17) after hydrolysis of the sample with 6 M HCl for 24 and 72 h (5). Cysteine and methionine were determined after performic acid oxidation (10). Further details are given by Forsum (2). Tryptophan was determined after alkaline hydrolysis (1).

Electrophoresis in polyacrylamide (T\textsubscript{7.5} C\textsubscript{2.6}) was according to Ornstein and Davis (13). Experimental details for immunoelectrophoresis have been given by Forsum (2). Samples of human milk for immunoelectrophoresis were prepared by freeze-drying 10 ml human milk, defatted by centrifugation at 120 x g for 20 min, and dissolving the dried material in 1 ml of the appropriate buffer.

Analytical ultracentrifugation for determination of \( S_{20,w} \), was according to Schachman (15) in .01 M tris-HCl buffer, pH 8.0, .2 M NaCl.

Total nitrogen content of milk samples was estimated as described by Schuman (16).

Antisera against human \( \alpha \)-lactalbumin were produced in rabbits by Dako immunoglobulins A/S Denmark by their standard immunization procedure.

Preparation of standard solutions: Carbon, hydrogen, and nitrogen were determined quantitatively on purified \( \alpha \)-lactalbumin according to Kirsten (6). The nitrogen content of \( \alpha \)-lactalbumin was calculated from amino acid analysis, and thus standard solutions with an exact concentration of \( \alpha \)-lactalbumin could be prepared.

Quantitation by radial immunodiffusion was according to Mancini (9). All estimations were on milk defatted by centrifugation at 120 x g for 20 min.
TABLE 1. Amino acid composition of human α-lactalbumin.

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Data obtained in this study</th>
<th>Data according to Findlay et al. (4)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lys</td>
<td>12</td>
<td>13</td>
</tr>
<tr>
<td>His</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>Arg</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Asx</td>
<td>16</td>
<td>16</td>
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<td>Thr</td>
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<tr>
<td>Ser</td>
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<td>8</td>
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<tr>
<td>Val</td>
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<td>2</td>
</tr>
<tr>
<td>Met</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>Ile</td>
<td>11</td>
<td>12</td>
</tr>
<tr>
<td>Leu</td>
<td>13</td>
<td>14</td>
</tr>
<tr>
<td>Tyr</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>Phe</td>
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<td>4</td>
</tr>
<tr>
<td>Trp</td>
<td>3</td>
<td>3</td>
</tr>
</tbody>
</table>

*Calculations based upon the assumption of one arginine residue per mole.

RESULTS

Purification of α-Lactalbumin

The elution profile obtained after ion-exchange chromatography of human whey on DEAE-Sephadex A-25 is in Fig. 1. The α-lactalbumin containing fractions were pooled as indicated in the figure.

Criteria of Purity

Polyacrylamide electrophoresis of purified α-lactalbumin (Fig. 2) showed one single band. Analytical ultracentrifugation of 1% α-lactalbumin solution showed one single symmetrical boundary with a sedimentation constant, $S_{20,w}$, of $1.63 \times 10^{-13}$ which agreed with results of other authors (14).

Results of amino acid analysis are in Table 1 and also are compared to those of Findlay et al. (4). The number of amino acid residues, based on the assumption of one arginine residue, is given. This amino acid composition corresponds to a molecular weight of 13,860 as compared to 14,100 based on the values of Findlay and Brew (4). (In this calculation, asparagine and glutamine were treated as aspartic and glutamic acid, respectively.) The values agree with the results of Findlay and Brew (4).

Antisera

The quality of the rabbit antisera produced against purified α-lactalbumin was tested by immunoelectrophoresis. Antibodies to milk proteins other than human α-lactalbumin were not detectable in the antisera (Fig. 3).

Quantitation of Human α-Lactalbumin

The mean value of 54 determinations of α-lactalbumin in one sample of breast milk was 2.02 mg/ml. The standard deviation was 0.06, or 2.7% of the mean value, and the range was 1.94 to 2.10 mg α-lactalbumin per ml milk.

The content of α-lactalbumin in pooled human milk samples is in Table 2. Crude protein content ($N \times 6.25$), as well as the percentage of α-lactalbumin of crude protein also are in the table. The α-lactalbumin content of these samples varied from 2.78 to 4.37 mg/ml although the crude protein content of these milk samples varied little.

Figure 4 shows the content of α-lactalbumin in the breast milk of one single mother during the first 2 mo of lactation. The α-lactalbumin

TABLE 2. Contents of α-lactalbumin and crude protein in pooled human milk.

<table>
<thead>
<tr>
<th>Sample</th>
<th>α-Lactalbumin</th>
<th>Crude protein content, $N \times 6.25$</th>
<th>α-Lactalbumin in percent of crude protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pooled human milk I</td>
<td>2.78</td>
<td>11.8</td>
<td>23.6</td>
</tr>
<tr>
<td>Pooled human milk II</td>
<td>4.37</td>
<td>10.2</td>
<td>42.9</td>
</tr>
<tr>
<td>Pooled human milk III</td>
<td>3.13</td>
<td>11.4</td>
<td>27.5</td>
</tr>
</tbody>
</table>

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The simplicity, accuracy, and reproducibility of immunological methods for determination of proteins in biological fluids are appreciated widely. However, these methods require that the antigen be available in a high degree of purity. The method for purification of human α-lactalbumin used here is a further simplification of that described by Phillips and Jenness (14) since no salt gradient is used for elution of the ion-exchange column. By this method, at least 30 to 70 mg purified α-lactalbumin can be prepared in a single operation.

Statistical evaluation of the immunodiffusion test showed that standard deviation in percent of the mean value was 2.7%, which is fairly close to the 2% given by Mancini in his description of the quantitative immunodiffusion method, an acceptable accuracy of the estimation.

Analyses of pooled human milk as well as of milk samples from one mother indicated considerable variation in α-lactalbumin content. This finding, which was further confirmed (8), explains the great discrepancy in α-lactalbumin content of breast milk reported in the literature (7, 11, 12). Thus, a thorough investigation of the variation of α-lactalbumin in breast milk from different mothers with respect to stage of lactation, diurnal variation, etc., is needed. Furthermore, since α-lactalbumin probably contributes significantly to the high nutritional quality of breast milk protein, it would be of considerable interest to study the α-lactalbumin content of the milk in relation to the nutritional status of the mother and child. Such studies should also include determination of other proteins of breast milk. The influence of different factors on the content of α-lactalbumin as well as of other proteins in human milk is presently under investigation.

Content was relatively high in colostrum. A general decrease in the α-lactalbumin content occurred in transitional and mature milk. This decrease was more pronounced during the first period of lactation. However, different milk samples varied greatly in their α-lactalbumin content. Values from 2.98 to 4.84 mg/ml were in the milk of this mother.

**DISCUSSION**

The simplicity, accuracy, and reproducibility of immunological methods for determination of proteins in biological fluids are appreciated widely. However, these methods require that the antigen be available in a high degree of purity. The method for purification of human α-lactalbumin used here is a further simplification of that described by Phillips and Jenness (14) since no salt gradient is used for elution of the ion-exchange column. By this method, at least 30 to 70 mg purified α-lactalbumin can be prepared in a single operation.

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ACKNOWLEDGMENTS

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REFERENCES