ABSTRACT

The amino acid analysis, peptide mapping, and heat stability of bovine milk lysozyme are presented. The bovine milk lysozyme molecule contains approximately 154 amino acids and is strikingly different in amino acid content from human milk lysozyme and egg white lysozyme. Tryptic hydrolysis yielded 26 peptides, all of which are unique from tryptic peptides of human milk lysozyme and egg white lysozyme. In addition, bovine milk lysozyme was more heat stable than human milk lysozyme at pH 4.0 but more labile at pH 7.0 and 9.0. Possible explanations for the differences in heat stability are discussed.

INTRODUCTION

Lysozymes (N-acetylmuramide glycanohydrolase: EC 3.2.1.17) are enzymes widespread in most biological systems. The milk of several species of animals contains lysozyme. Bovine milk lysozyme (BML), human milk lysozyme (HML), and baboon milk lysozyme have been isolated in pure and homogeneous forms (5, 9, 10, 24). Isolated milk lysozymes are basic, small molecular weight proteins possessing the same biological activity as egg white lysozyme (EWL). However, they differ from each other and from EWL in specific activity, pH optimum, electrophoretic mobility, isoelectric point, and sedimentation coefficient (9, 24). The HML and EWL have molecular weights of approximately 15,000 (24) whereas BML is a slightly larger molecule with a molecular weight of approximately 18,000.

In 1967 Brew et al. (3) observed that the amino acid sequence of bovine α-lactalbumin compares closely to the sequence of EWL. Forty residues are in identical positions and 27 residues are in chemically similar positions. On this observation, it was proposed that genes for EWL and α-lactalbumin arose from a common genetic background.

Canfield et al. (8) reported that the primary sequence of human leukemia lysozyme (HLL) corresponds to the structure of HML shown by Jolles and Jolles (17). Comparison of the sequence of HLL to the sequence of bovine α-lactalbumin and EWL reveals that the three proteins have identical amino acids in 36 positions, further confirming the hypothesis that these proteins arose from a common ancestral gene (8). In addition, Findlay and Brew (11) determined the amino acid sequence of human α-lactalbumin which showed that 63 (51%) of the positions in the protein chain contained identical or closely related amino acids when compared to HLL. Canfield et al. (8) also concluded that various classes of lysozyme molecules have evolved along independent pathways since the NH2-terminal sequence of goose egg white lysozyme differs from that of EWL or the human lysozymes and all of the above differ from those of papaya (14) and T4 bacteriophage (15) lysozymes.

Our paper reports the amino acid composition of BML and compares the peptide mapping and heat stability of BML to HML and EWL to reveal the relationship of BML to other lysozymes and the α-lactalbumins.

MATERIALS AND METHODS

The BML and HML were isolated by methods reported in (10, 12), and EWL was purchased as a 3x crystallized, salt free preparation from Nutritional Biochemicals Corporation.
Amino Acid Analysis

The amino acid composition of BML was determined on the carboxymethylated derivative of the enzyme. The BMl was carboxymethylated by the method of Sela et al. (25) and then hydrolyzed for 24 and 72 h at 110 ± 2 °C in evacuated tubes as reported by Moore and Stein (22). Hydrolyzates were analyzed in a Beckman 120 C amino acid analyzer, and tryptophan was determined by the method of Spies and Chamber (26).

Peptide Mapping

The peptide mapping of the tryptic digests of carboxymethylated BML, HML, and EWL was by the procedure of Burns and Turner (4). The method of Bernard et al. (1) was used to remove trace chymotrypsin activity. The carboxymethylated lysozymes were dissolved in H₂O to a concentration of 5 mg/ml, and the pH was adjusted to 8.5 with 0.1 N NH₄OH. Three percent by weight of trypsin were added, and hydrolyses were run in duplicate for 24 h at 25 °C.

Standard 20 x 20 cm plates were coated with a 250 micron layer of cellulose (Whatman CC41 Microgranular Cellulose Powder) and washed by upward elution with 1% acetic acid. Fifteen microliters of the tryptic digests were applied to the plates in small aliquots, and the peptides were separated in the first dimension by high voltage electrophoresis in electrophoresis buffer-glacial acetic acid:90% formic acid:water (170:50:2800), pI 2.0, for 15 min at a constant voltage of 900.

The plates were air dried and then developed in the second dimension by thin layer chromatography with n-butanol:acetic acid:pyridine:water (15:3:12:12) as the solvent system. The plates were dried and rechromatographed to increase the resolution of the peptides. The second run increased greatly the resolution of the peptides, giving both increased separation and added sharpness to the spots. After drying, the peptides were visualized by spraying the plates with ninhydrin aerosol (Nutritional Biochemicals Corp.).

Heat Stability

The BML, at a concentration of 100 μg/ml, was adjusted to pI 4.0, 7.0, or 9.0, with either dilute HCl or NaOH. Control assays were run on unheated enzyme solutions. One-milliliter quantities of the enzyme solutions were transferred to capped test tubes and placed in a 100 °C water bath for varying times. At periodic intervals tubes were removed, cooled to room temperature, and assayed for enzymatic activity according to the method of Parry et al. (23).

Circular Dichroism

Circular dichroism measurements of BML and HML were with a Cary 60 recording spectropolarimeter (CO Model) as reported previously (12).

RESULTS AND DISCUSSION

Amino Acid Analysis of Bovine Milk Lysozyme

Results of several replicate analyses of 24 and 72 h hydrolyzates of carboxymethylated BML are in Table 1. The number of residues for each amino acid was calculated with a molecular weight of 18,000 determined from the sedimentation data reported earlier (9) and diffusion studies (unpublished data). Inasmuch as the number of tryptophan and methionine residues, 1.0 and 2.05, respectively, were exceedingly close to the integral numbers, the amino acid (molecular ratio) or composition strongly supports the accuracy of the molecular weight. Moreover, the minimum molecular weights, independent of centrifugal or physical measurements, based on tryptophan and methionine moieties were 18,000 and 9000, respectively.

The amino acid analysis revealed BML's composition was considerably different from HML and EWL. The most striking differences appeared to be in the histidine and tryptophan contents. The BML contained seven residues of histidine whereas HML and EWL contain only one histidine residue (7, 24). Also, only six half-cystines were in the BML molecule compared to eight half-cystines in HML and EWL.

On the other hand, BML contained only one tryptophan residue while HML contained five (24) and EWL contained six tryptophan residues (7). The low tryptophan content of BML is a notable difference from EWL as three tryptophan residues are involved directly in the substrate binding of EWL. Nevertheless, other lysozymes with low tryptophan contents have been isolated from microorganisms, bacteriophages,
TABLE 1. Amino acid composition of bovine milk lysozyme.

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Number of residues&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Number of residues corrected for destruction&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>24 hr hydrolyzate</td>
<td>72 hr hydrolyzate</td>
</tr>
<tr>
<td>Lysine</td>
<td>10.0</td>
<td>10.1</td>
</tr>
<tr>
<td>Histidine</td>
<td>6.6</td>
<td>6.7</td>
</tr>
<tr>
<td>Ammonia</td>
<td>18.4</td>
<td>21.4</td>
</tr>
<tr>
<td>Arginine</td>
<td>15.0</td>
<td>14.5</td>
</tr>
<tr>
<td>CM cysteine</td>
<td>6.3</td>
<td>6.2</td>
</tr>
<tr>
<td>Aspartic acid</td>
<td>22.3</td>
<td>23.1</td>
</tr>
<tr>
<td>Threonine</td>
<td>7.8</td>
<td>7.5</td>
</tr>
<tr>
<td>Serine</td>
<td>7.4</td>
<td>6.9</td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>13.7</td>
<td>14.2</td>
</tr>
<tr>
<td>Proline</td>
<td>9.4</td>
<td>9.3</td>
</tr>
<tr>
<td>Glycine</td>
<td>10.6</td>
<td>10.9</td>
</tr>
<tr>
<td>Alanine</td>
<td>4.8</td>
<td>5.3</td>
</tr>
<tr>
<td>Valine</td>
<td>4.5</td>
<td>5.9</td>
</tr>
<tr>
<td>Methionine</td>
<td>2.05</td>
<td>2.05</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>8.5</td>
<td>9.9</td>
</tr>
<tr>
<td>Leucine</td>
<td>5.6</td>
<td>5.4</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>6.1</td>
<td>4.5</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>7.4</td>
<td>7.1</td>
</tr>
<tr>
<td>Tryptophan&lt;sup&gt;c&lt;/sup&gt;</td>
<td>1.0</td>
<td>1.0</td>
</tr>
</tbody>
</table>

<sup>a</sup>Calculated by assuming a molecular weight of 18,000 which was previously determined by sedimentation and diffusion studies.

<sup>b</sup>Values for destruction of threonine, cysteine, tyrosine, and serine extrapolated to zero time. Ammonia was similarly extrapolated. Hydrolyses for valine and isoleucine were assumed complete after 72 hr.

<sup>c</sup>Tryptophan was determined by the method of Spies and Chambers (24).

The absolute tryptophan content ranged from 9.2 to 11.0 μg with an average of 10.07 μg per mg of BML protein, amounting to 1.007% tryptophan in the lysozyme.

and rabbit spleen (15, 19, 21). The role of tryptophan in the substrate binding by BML and HML is discussed in another publication (13). The total number of residues in BML was approximately 154 compared to either 125 or 129 (15, 17, 20) in HML and 129 in EWL (7).

Peptide Mapping

Peptide maps from trypsin hydrolysis of carboxymethylated BML, HML, and EWL are in Fig. 1. The peptide maps of BML indicated 26 peptides which agreed with the amino acid analysis values of 10 lysines and 15 arginines. However, 21 and 23 peptides were obtained for HML and EWL, respectively. This is three peptides in excess of the expected theoretical value for HML and five in excess for EWL.

Appearance of peptides in excess of the theoretical value for EWL was not unexpected, as Canfield (6) obtained more tryptic peptides than calculated from the amino acid analysis. He isolated some peptides containing more than one lysine and arginine and concluded that overlapping of peptides was due to trypsin susceptible bonds in carboxymethylated lysozyme that were cleaved relatively slowly during hydrolysis. Concerning the excess peptide in the HML preparation, Jolles and Jolles previously obtained 21 peaks during ion-exchange separation of the tryptic hydrolyzate (17).

Comparison of the mobility of the peptides indicated that there were no identical peptides in BML as compared to HML and EWL tryptic peptides.

Heat Stability of BML

Most lysozymes are relatively heat stable at acid pH and more labile at alkaline pH. As shown in Fig. 2, BML was stable at pH 4.0,
losing only 43% of its activity after 20 min at 100°C. Under the same condition, HML has been reported to lose 85% of its activity after 20 min (24). However, at pH 7.0 and 9.0, BML was more labile than HML.

Beychok and Warner (2) concluded that the extreme stability of lysozyme at acid pH's does not depend upon the hydrogen-bound structure but on other factors such as disulfide crosslinking. The BML appears to possess three disulfide bonds as shown by the present amino acid analysis and the determination of free SH groups in the native and reduced BML molecule (12). There is some controversy, however, as to the disulfide bonds of HML. Jolles and Jolles (17) observed that reduced HML contains eight half-cystine residues, as does EWL, but only three disulfide bonds as in BML. This is in opposition to an earlier report of Jolles (20) that HML contained only six half-cystine residues. Likewise, studies in our laboratory by Parry et al. (24) showed that HML contained only six half-cystine residues. Friend et al. (12) noted that titration of native HML with p-mercuri-
benzoate revealed no free SH groups while titration of completely reduced HML revealed six. These inconsistencies have been partially resolved by the determination of the complete amino acid sequence of HML by Jolles and Jolles (18) which confirmed eight half-cystines. It appears that HML contains at least three disulfide bonds and possibly may contain some free cysteine residues. Jolles et al. (16) reported that as the number of disulfides decreases, the heat stability of the various lysozymes also decreases. A priori, one would expect that both BML and HML possess heat stabilities of similar degrees. Apparently heat stability is dependent upon additional factors.

**Circular Dichroism**

Another possibility is that BML has a lower \( \alpha \)-helical content than HML and EWL which could result in a lowering of its heat stability at the alkaline pH values. Figure 3 presents the circular dichrographs of BML and HML. The circular dichroism data are expressed in terms of \((\Sigma L - \Sigma r) \times 10^3\) which at 208 and 222 nm reflect the amount of \( \alpha \)-helix in the sample. The data indicate that BML, in fact, possesses a greater \( \alpha \)-helical content than does HML.

This study has shown that BML differs from HML and EWL in amino acid composition and, based upon peptide mapping, contains no similar segments of primary structure. The many differences in BML's amino acid composition when compared to the amino acid compositions of HML and EWL suggest that BML is not related genetically to either bovine or human \( \alpha \)-lactalbumin as apparently is the case for HML and EWL. However, no definite conclusion can be made on this point until the primary sequence of BML is known. Our current investigations also have shown that the lower heat stability of BML at higher pH values cannot be explained by differences in disulfide bonds as has been reported previously for other lysozymes (16). It also was shown that the lower heat stability of BML at alkaline pH is not related to secondary structure of the lysozymes.

**ACKNOWLEDGMENT**

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17. Jolles, J., and P. Jolles. 1969. La structure chim-


