Proteinase Enzyme System of Lactic Streptococci
II. Role of Membrane Proteinase in Cellular Function

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The effect of several environmental conditions on the structure and activity of a membrane-associated proteinase from *Streptococcus lactis* was investigated. The activity of the enzyme varied with pH. Before storage at 3 C, maximal activity occurred at pH 6.0, but was minimal at this pH after storage. At all pH values tested, the enzyme was inactivated after storage. After storage at 3 C, the enzyme showed gross structural alterations with a concomitant loss of activity. Gel filtration and sedimentation velocity data indicated that inactivation of the enzyme was the result of aggregation to higher molecular weight forms. *p*-Hydroxymercuribenzoate prevented inactivation of the enzyme during storage by preventing aggregation. Activity was correlated with disaggregation of polymer forms of the enzyme to an active monomer. The storage-inactivated enzyme could be reactivated by treatment of the enzyme with cysteine, glutathione, or ferrous ion. Glutathione enabled stored cells to produce acid at their original rate when subcultured in milk. This was attributed to the effect of glutathione on the membrane proteinase. The data suggested that the biological activity of stored cells may be dependent upon the activity of the membrane proteinase.

Previous studies in our laboratory have shown that the lactic group of streptococci characteristically produces less acid after storage at refrigeration temperatures (1). Such stored cells also show a diminished residual proteinase activity. Subsequent studies of the proteinase system of *Streptococcus lactis* revealed the presence of two enzymes in cellular fractions (2a). The activity associated with the particulate fraction was observed to be very labile to storage, but that of the intracellular fraction was storage-stable. Moreover, the enzyme activity of the particulate fraction appeared to have properties similar to those observed in whole cells. The enzyme from the particulate fraction has been purified (Cowman, Ph.D. Thesis, North Carolina State University, Raleigh, 1966). The purified proteinase has been studied to determine how it is inactivated by storage, and to relate enzyme activity with cellular function.

**Materials and Methods**

**Enzyme.** The enzyme was purified from the particulate fraction obtained by sonic disruption of cells.

The particulate fraction was treated with lysozyme and then by differential centrifugation. During the steps of centrifugation, the activity remained with the membrane fraction of the cells. When the membrane fraction was treated with 2.0 M NaCl, the activity appeared in the supernatant liquid. After differential centrifugation, the enzyme was chromatographed on Sephadex G-50. The detailed procedure has been recorded elsewhere (Cowman, Ph.D. Thesis, North Carolina State University, Raleigh, 1966). In certain studies the enzyme was used as its *p*-hydroxymercuribenzoate (PHMB) derivative, obtained by treating the crude enzyme solution with excess PHMB prior to purification. Since the enzyme is labile to storage (at 3 C), purified enzyme solutions were maintained at 22 C as the experimental control. To standardize the exposure of these solutions to temperature, experiments were performed within 3 to 4 days after purification.

**Enzyme activity assay.** The activity of the enzyme was determined with casein as substrate according to the procedure described elsewhere (2a). Activity was expressed as the absorbance change at 650 mµ of the trichloroacetic acid filtrate when treated with the Folin-Ciocalteau phenol reagent. One unit of activity was defined as a 0.1 optical density change per 3 hr. All enzyme solution controls used in this study were standardized to give 300 units.

**Preparation of Sephadex G-100 column.** A 10-g amount of Sephadex G-100 was suspended in 0.05 M phosphate buffer, pH 6.0. After hydration of the gel grains, an analytical column (Sephadex K15/30) was...
packed in accordance with the procedures of Flodin (5). Final dimensions of the Sephadex column were 1.5 × 22 cm. The void volume of the column was 10 ml as determined by using Blue Dextran 2,000.

Before sample application, the column was equilibrated at room temperature with 0.05 M phosphate buffer, pH 6.0. During elution of a sample, effluent from the column was monitored continuously by ultraviolet absorption with the use of a 265-mu filter. Sedimentation velocity was determined with a Spinco model E analytical ultracentrifuge. In these studies, a synthetic-boundary single-sector cell was used. Dialysate served as solvent. Sedimentation was performed at 59,780 rev/min at either 3 or 20 C, and the schlieren patterns were photographed. The resultant patterns were analyzed by use of a microcomparator (Bausch & Lomb, Inc., Rochester, N.Y.) by the method of Trautman (12). Sedimentation coefficients were corrected to 20 C where necessary.

Chromatographic studies. The influence of storage at 3 C on the enzyme was analyzed by use of the Sephadex G-100 analytical column. The eluting buffer was 0.05 M phosphate buffer (pH 6.0) unless otherwise specified. In some instances, solutions from sedimentation velocity experiments were immediately analyzed on the column. The effects of PHMB, metal ions, and reducing agents on the elution of the enzyme from Sephadex G-100 were analyzed after treatment. Stock enzyme solution (300 units) was treated with 0.2 ml of 10-3 M PHMB or 0.2 ml of 10-2 M ferrous sulfate. Sedimentation velocity experiments with reducing agents, 0.2 ml of 10-3 M reduced glutathione (GSH) or 0.2 ml of 10-2 M cysteine was added to stock enzyme. Treated enzyme solutions were allowed to equilibrate 30 min at 22 C; then 200 to 400 ml of buffer was applied to the column and the elution profile was recorded.

Influence of glutathione on acid production by whole cells. Glutathione was studied for its effect on the ability of stored cells to produce acid. Cells from 2 liters of casein medium (2a) were obtained by centrifugation, and were then resuspended in 20 ml of 10% nonfat milk solids (NFM). Before and after storage at 3 C, a 1% inoculation of the cell-milk mixture was made into 100 ml of 10% NFM supplemented with various concentrations of glutathione. Milk alone served as the control. At selected intervals during incubation at 22 C, a sample was removed and then the pH was recorded.

RESULTS

The activity of the purified enzyme as a function of pH was determined by incubating enzyme at 37 C with casein adjusted to pH 5.0, 6.0, 7.0, 8.0, and 9.0. Prior to storage at 3 C, maximal activity was observed at pH 6.0 (Fig. 1); minimal activity was at pH 8.0. After storage at 3 C for 3 days, the enzymatic activity was reduced at all pH values, but the most significant decrease was at pH 6.0.

In previous studies the enzyme as its PHMB derivative was observed to be fully active even at 3 C, but the native enzyme was labile to this storage temperature. This difference in storage behavior between the native enzyme and its PHMB derivative was investigated further by use of ultracentrifugal procedures and column chromatography. The PHMB derivative of the enzyme, when maintained at 22 C, sedimented at a rate corresponding to 1.43S (Table 1). Storage of the derivative at 3 C for 3 days did not alter the sedimentation rate. Conversely, when the native enzyme was maintained at 22 C it had a sedimentation coefficient of 4.89S, but storage at 3 C for 24 hr resulted in an increase of sedimen-

![Figure 1](http://jb.asm.org/)

**FIG. 1. Effect of pH on activity of membrane proteinase from Streptococcus lactis 3; solvent, 0.2 M phosphate.**

**TABLE 1. Influence of storage on sedimentation velocity of Streptococcus lactis 3 membrane proteinase in 0.05 M phosphate buffer, pH 6.0**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Sedimentation coefficient</th>
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<tr>
<td>PHMB derivative maintained at 22 C</td>
<td>1.42&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>PHMB derivative stored for 3 days at 3 C</td>
<td>1.43&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Native enzyme maintained at 22 C</td>
<td>4.89&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Native enzyme stored for 3 days at 3 C</td>
<td>7.43&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
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<sup>a</sup> Rotor velocity at 59,780 rev/min at 22 or 3 C.
<sup>b</sup> All coefficients corrected for temperature at 20 C.
tation constant to 7.43S. Measurement of enzyme activity of the solutions showed that, when the native enzyme or its PHMB derivative was maintained at 22°C, each contained 300 units of activity. The activity of the PHMB derivative after storage for three days at 3°C remained unchanged, but under the same conditions the native enzyme lost 86% of its activity.

After ultracentrifugation and activity measurements, the solutions were chromatographed on Sephadex G-100 and eluted with 0.05 M phosphate buffer, pH 6.0. When the native enzyme that had been maintained for 48 hr at 22°C was chromatographed, three components were eluted. A high molecular-weight component (I) was eluted immediately after the void volume, and this was presumed to be a higher polymer (Fig. 2A). This component was enzymatically inactive. Two additional components which eluted more slowly were possibly a dimer (component II) and a monomer (component III). These two components were not separated sufficiently to permit activity measurement of each component. Consequently, tubes comprising these components were pooled and were found to contain 300 units of activity. Storage of the native enzyme for 24 hr at 3°C resulted in a 75% loss in activity. Chromatography of the stored enzyme indicated that considerable association of the enzyme had occurred (Fig. 2B). Concomitant with the association was the complete disappearance of component II (dimer) and considerable reduction in component III (monomer). The amount of protein applied to the column corresponding to Fig. 2B was identical to that for Fig. 2A, indicating that the observed decrease in ultraviolet absorption (at 265 μm) was not due to a loss of protein. All of the protein was accounted for from peak areas of a sedimentation velocity diagram obtained from the same solution used for the column chromatography experiments.

In contrast, the PHMB derivative of the enzyme, which had been maintained at 22°C, was eluted mostly as a monomer (component III). Smaller peaks representing polymer (I) and dimer also were observed (Fig. 3A). Tubes of eluate comprising components I and III were assayed for enzyme activity. Before chromatography, the activity was 300 units, of which 290 units appeared as component III; component I was inactive. Storage of the PHMB derivative for three days at 3°C resulted in no change in the elution profile (Fig. 3B). The stored solution had an activity of 300 units, which was recovered as component III after chromatography.

Ferrous ion has been observed to increase the activity of the membrane enzyme. To study the effect of this ion on enzyme structure and activity, native enzyme was treated with ferrous ion (Fe²⁺), then allowed to stand at room temperature (22°C) for 30 min before chromatography on Sephadex G-100. Untreated enzyme (300

![Fig. 2. Elution profile of membrane proteinase from Sephadex G-100 as affected by temperature of storage.](image)

![Fig. 3. Elution of the p-hydroxymercuribenzoate derivative of the membrane proteinase from Sephadex G-100; eluting buffer, 0.05 M phosphate, pH 6.0.](image)
units) maintained at 22°C was eluted as shown previously (Fig. 2A). After treatment with Fe++, the enzyme was eluted mainly as a monomer (III), with a smaller peak representing polymer (I), as shown in Fig. 4A. Moreover, the ultraviolet absorbance (at 265 μm) of both components, especially III, was increased significantly, and this could not be considered simply a redistribution of component II. Measurement of enzyme activity of the separated components showed the polymer to be inactive, but the activity of component III (468 units) was 56% higher than the untreated control (300 units). Storage of the enzyme for 24 hr at 3°C, followed by treatment with Fe++, resulted in 85% reactivation of the initial activity (468 units). After chromatography, the monomer (III) contained all of the activity (Fig. 4B). The stored enzyme in the presence of Fe++ also showed a considerable increase in absorbance (at 265 μm).

The increased absorbance (at 265 μm) of the enzyme was not considered to be caused by an interaction between Fe++ and sulfhydryl group(s) in the protein. This was examined further, with glutathione as a model peptide. Solutions used were (i) GSH control, 0.5 ml of 0.2% GSH plus 1.5 ml of double-distilled (glass) water; (ii) PHMB control, 0.5 ml of 0.2% PHMB plus 1.5 ml of water; (iii) GSH and PHMB, 0.5 ml each of solutions i and ii plus 1.0 ml of water; (iv) GSH and Fe++, 0.5 ml of solution i, 0.2 ml of 10⁻² m ferrous sulfate, and 1.0 ml water; and (v) 0.5 ml each of solutions i and ii, 0.2 ml of 10⁻² m ferrous sulfate, and 0.8 ml of water. The absorbance of the solutions was measured at 250, 255, and 257 μm. GSH alone showed low absorbance at these wavelengths, whereas absorbance by the PHMB control was high. When GSH was treated with PHMB, the absorbance at 255 and 257 μm was increased. When GSH was treated with Fe++, absorbance was not increased. Also, treatment of GSH with both PHMB and Fe++ did not result in increased absorbance, except for that due to the GSH-PHMB reaction.

The effect of Fe++ on the enzyme might be attributable to its role as a reducing agent. To test whether Fe++ functioned only as a reducing agent, native enzyme was treated with both cysteine and ferrous ion. The effect of these agents (in combination) on the activity and structure of the enzyme was investigated. The necessary control elution profiles were the same as those shown in Fig. 2A and 2B and Fig. 4A and 4B. When the enzyme was treated with cysteine only (Fig. 5A), the enzyme was eluted primarily as the monomer (III). Before chromatography, the treated enzyme contained 468 units of activity, an increase over that of the control (300 units). After chromatography, 95% of the observed activity was as monomer (III). The elution profile remained unchanged in the presence of both cysteine and Fe++, except for the increased absorbance (at 265 μm) due to Fe++. The activity of the enzyme treated with both agents increased to 555 units, the majority being associated with the monomer. After storage at 3°C for 24 hr, the enzyme was reactivated 45% (of 468 units) by cysteine alone. This appeared to be correlated with dissociation of polymer forms to active monomer (Fig. 5B). In the presence of both cysteine and Fe++, 60% reactivation (of 555 units) resulted, due apparently to an increase in monomer.

Polymer forms of the enzyme also were effectively dissociated to monomer by treatment of the enzyme with GSH. When enzyme maintained at 22°C for 48 hr was treated with GSH, mostly monomer was eluted (Fig. 6A). The activity of the enzyme was increased by 30% over that of the control (300 units). After 24 hr at 3°C, the stored enzyme showed 65% reactivation upon addition of GSH, with monomer being the major component eluted (Fig. 6B).

Since glutathione and cysteine both were effec-
tive in reactivating the storage-inactivated proteinase, glutathione was selected for additional studies with whole cells. Before and after storage at 3°C, cells were subcultured in milk and in milk containing glutathione. During incubation at 22°C, samples were removed and the acid produced by the cells was measured. Unstored cells in milk reached pH 4.75 after 14 hr of incubation at 22°C. Addition of glutathione to the milk did not result in increased acid production by unstored cells (Table 2). After storage at 3°C for 6 days, stored cells produced less acid in milk alone, with a pH of only 5.0 being attained after 14 hr of incubation. However, the stored cells reached pH 4.75 after 14 hr when subcultured in milk with 0.1% glutathione added. Higher concentrations of glutathione did not enhance the acid produced by stored cells.

**Discussion**

The lactic group of streptococci appears to be particularly sensitive to the environmental stress of low-temperature storage (1, 2). The decreased biological activity of stored cells is manifested by their diminished proteolytic activity and by their reduced ability to produce acid in milk. Subsequently, it was shown that the cell contained an intracellular proteinase that was stable during storage (3) and a membrane proteinase that was labile. The similar behavior of the purified proteinase and whole cells toward storage at 3°C, PHMB, reducing agents, and ferrous ions suggests the possibility that the activity of cells in milk was regulated by this enzyme.

**Table 2. Effect of glutathione on acid production by Streptococcus lactis 3 when cultured in milk at 22°C**

<table>
<thead>
<tr>
<th>Storage</th>
<th>Incubation period (hr)</th>
<th>Percentage of glutathione added to milk</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>0.0</td>
</tr>
<tr>
<td>None</td>
<td></td>
<td></td>
</tr>
<tr>
<td>116 days at 3°C</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>0.015</td>
<td>0.025</td>
</tr>
<tr>
<td>4</td>
<td>0.035</td>
<td>0.030</td>
</tr>
<tr>
<td>6</td>
<td>0.050</td>
<td>0.048</td>
</tr>
<tr>
<td>8</td>
<td>0.073</td>
<td>0.073</td>
</tr>
<tr>
<td>10</td>
<td>0.093</td>
<td>0.103</td>
</tr>
<tr>
<td>12</td>
<td>0.123</td>
<td>0.136</td>
</tr>
<tr>
<td>14</td>
<td>0.135</td>
<td>0.137</td>
</tr>
</tbody>
</table>

* Change in pH per hour. Average of duplicate trials.
The activity of the membrane proteinase was affected by variation in pH. This would be expected since pH can change the hydrogen-ion equilibrium at the active center, or alter the active structure (4, 6, 8, 13). Regardless of pH, the activity of the enzyme was decreased after storage. The severity of inactivation at pH 6.0 may indicate a pH dependence.

Storage of the enzyme produced clearly identifiable structural changes, resulting in decreased absorbance (at 265 μm) of the enzyme and in loss of activity. Correlation of sedimentation velocity data and elution profiles indicated that storage promoted aggregation of the enzyme. Concomitant with the structural alterations was a decrease in absorbance (265 μm). The decreased absorbance was not due to a difference in protein concentration, but could result from changes in the exposure of chromophoric groups to solvent. Loss of biological activity appeared to be a further manifestation of gross changes in structure. Several investigators have suggested that storage inactivation of enzymes may be caused by induced conformational or structural changes (7, 10, 11). Scrutton and Utter (10) and Havir et al. (7) observed that inactivation of various enzymes by low-temperature storage was due to dissociation of the molecules into subunits. The inactivated enzymes could be reactivated by warming to room temperature. In the present study, storage inactivation of the proteinase was attributed to aggregation. The inactivated enzyme could not be reactivated by placing it at room temperature. The aggregation of the enzyme at 3°C was suggested by the increase in rate of sedimentation and was confirmed by gel chromatography. The change in molecular weight correlated with enzyme inactivation.

The decidedly lower sedimentation coefficient of the PHMB derivative of the enzyme can be attributed to the ability of PHMB to prevent enzyme, suggesting that sulfhydryl groups may have a key role in enzyme structure. The 1.42S molecule observed with the PHMB derivative may be the smallest active form of the enzyme. This was suggested when Fe++, cysteine, or glutathione dissociated the aggregated enzyme into a molecule of similar size.

Ferrous ion was observed to promote consistently an increase in absorbance by the protein (at 265 μm). This increased absorbance must be attributed to an interaction of ferrous ion. The absorbance increase cannot be accounted for simply as the result of a metal-sulfhydryl complex. When the PHMB derivative of the enzyme was treated with Fe++, absorbance (at 265 μm) was still increased (data not shown). Furthermore, the absorbance of glutathione was not increased by Fe++. These data would suggest that the Fe++ interaction does not involve a sulfhydryl group. Studies on the Fe++ interaction with the enzyme are in progress.

The ability of Fe++ to reactivate the stored proteinase may be due to its role as a reducing agent. Sadana and Rittenberg (9) found that the hydrogenase of Desulfovibrio desulfuricans, inactivated by storage at 0°C, could be reactivated by Fe++. They postulated that Fe++ reduced disulfide bonds formed during storage. In the present study, cysteine and glutathione also appeared to function in a manner similar to Fe++. However, these reducing agents failed to promote the absorbance increase observed with Fe++. This would suggest that Fe++ may have a secondary effect on the enzyme.

S. lactis requires an organic source of nitrogen for growth. When cultured in milk, it is dependent upon its proteinase enzyme system to furnish needed nitrogenous compounds. Any deleterious effect on this enzyme system would be expected to retard growth of the cells with a concomitant reduction in the rate of acid production by the cell. However, a relief of the imposed stress would be expected to result in the cells having characteristics of normally growing cells. Glutathione enabled stored cells to produce acid at their original rate when subcultured in milk. The membrane proteinase was reactivated by glutathione, and possibly in stored cells the effect of glutathione was at the site of this enzyme. It has been shown previously (1) that stored cells subcultured in milk containing pancreas extract produced acid at a rate comparable to that of unstored cells. In this case, an essential step in obtaining needed nitrogenous compounds was bypassed by the cells. In the present study, glutathione, not acting as a metabolite, apparently relieved the stress at the essential step. These data would suggest that the membrane proteinase performs a key cellular function. Studies are now underway to clarify further the role of the enzyme in cellular function.

Acknowledgments

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