Mechanism of Excretion of a Bacterial Proteinase: Demonstration of Two Proteolytic Enzymes Produced by a Sarcina Strain (Coccus P)

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A Sarcina strain (Coccus P) produces two proteolytic enzymes. One is found only extracellularly, is far more prevalent, and is actively excreted during exponential growth. It is the enzyme responsible for the known strong proteolytic activity of the cultures of this strain. A second protease is, however, produced which remains associated with the intact cells but is released by the protoplasts. The two enzymes appear unrelated in their derivation. Calcium ions play an essential role in preventing autodigestion of the excreted enzyme.

Bacterial proteins are found outside the cell boundary as a consequence either of passive processes such as leakage or lysis or of active excretion. Under conditions in which leakage and lysis do not occur, as during exponential growth, the cell boundary is a barrier causing a complete separation of the bulk of the intracellular proteins from the one or very few extracellular proteins, with no trace of either type being detectable on the wrong side of the boundary. Since in bacteria there is no evidence of protein being produced other than internally, the separation into intra- and extracellular proteins should occur after peptide chain formation. The question arises as to whether the structure of the cell boundary or that of the excreted proteins themselves determines this separation.

Coccus P, a Sarcina closely related to Micrococcus lysodeikticus (3), produces an extracellular proteinase during the exponential phase of growth so that the process appears to be active excretion. The organism grows exponentially in a defined synthetic medium (12) to relatively high cell density (10⁸ cells/ml); therefore the mechanism of excretion can be studied over an extended period of time without the difficulties of changing growth rates. Coagulation of reconstituted skim milk provides a simple and sensitive assay for enzyme activity (11). The extracellular proteinase has also been purified and partially characterized (6–8). It has been shown that extracellular proteolytic activity can be found only when Ca²⁺ is present in the medium (4). However, there is always a low level of proteolytic activity associated with the cells irrespective of the presence of Ca²⁺. This paper is concerned with the relationship between these two extra- and intracellular proteolytic activities. It is found that they are due to two different proteins, only one of which is actively excreted.

MATERIALS AND METHODS

Bacterial strains. Wild-type Coccus P (3) was maintained routinely on tryptone-agar slants (Field’s medium, Difco). Nutritional requirements are: guanine (or guanosine), biotin, and catechol (or any other orthophenol; reference 12).

Proteinaseless mutants were induced with N-methyl-N’-nitro-N-nitrosoguanidine by a slightly modified procedure used for Escherichia coli (1). Wild-type Coccus P was grown in 10 ml of Field’s medium to 6 x 10⁶ cells/ml. After centrifugation, the cells were resuspended in 0.5 ml of 0.2 M acetate buffer (pH 5), and 0.12 ml of a standard solution (4 mg/ml) of nitrosoguanidine was added. This suspension was incubated at 37 C for 3.5 hr. After centrifugation and washing, a sample of cells was inoculated into Field’s medium. After a long lag (2 days), the survivors (~10⁻⁴) grew.

Dilutions were plated on Field’s medium and on agar-milk plates. Nonproteolytic colonies occurred at a frequency of 0.25 x 10⁻² of the survivors. Twenty-three of these colonies were analyzed further; one strain, CP10, used in these experiments has the same generation time as the wild type in minimal medium. It has no extracellular proteolytic activity regardless of Ca²⁺ concentration, whereas it retains its cell-associated activity.

Minimal medium. The basal medium was the minimal medium (MMCP) described by Gorini and Lord.

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(12) with the following composition (grams per liter): sodium glutamate, 3; sodium acetate, 3; glycine, 1; (NH₄)₂SO₄, 0.04; MgCl₂, 0.2; KH₂PO₄, 0.137; guan

inosine, 0.3; biotin, 0.0023; trishydroxymethylamino-
methane (Tris), 12.1. The pH was adjusted to 7. After

autoclaving, the following were added: catechol (steri-

lized by filtration), 0.0029 g/liter, and sterile glucose, 2

g/liter. The calcium content of this medium was de-
termined by atomic absorption (kindly performed by K.

Fuwa) and was consistently of the order of 10⁻⁴ M, presumably attributable to calcium contamination in the chemicals, water, and glassware used. Such distinctly cal-

cium-free conditions were not obtainable, all experiments and cultures to which no calcium was intentionally added are designated as "low-calcium" and assumed to con-
tain a concentration of Ca²⁺ ions of the order of 10⁻⁴ M.

To obtain the "high-calcium" cultures, 0.111 g/liter of CaCl₂ was added to MMCP, which brought the concentra-
tion of Ca²⁺ to 10⁻³ M.

Selective solid media. Enriched solid medium was 1.5% Field's medium (Difco) and 2% agar. Minimal solid medium was MMCP with 2% agar.

Selective solid media. To prepare milk plates (17), 10 ml of a 10% solution of skim milk (Difco) in 0.1 M Tris-

hydrochloride buffer (pH 7) was added to 100 ml of enriched or minimal "high-calcium" agar medium. To prepare lactose plates, 10 ml of a 10% solution of casein

(Hammersten) in 0.1 M Tris-hydrochloride buffer (pH 8) was added to 100 ml of minimal "high-calcium" agar medium. After 24 hr at 37 C, the proteolytic colonies were detected on the milk plates by a clear halo around the colony (attributed to rapid digestion of the milk proteins) and on the casein plates by a halo of turbidity, presumably because, under the action of the proteinase-
casein complexes before being digested. Mutant CP10 showed neither a clear halo on milk plates nor turbidity on casein plates within incubation time identical to that of the wild type.

Growth conditions. Coccus P is a strict aerobe. In MMCP the doubling time is 2 hr and 15 min at 37 C, irrespective of the calcium concentration. Lags of un-

predictable length before initiation of growth were

avoided by using a rather large inoculum (1% volume of the inoculated medium) taken from a culture in low-Ca

MMCP at mid-exponential phase of growth (≈ 4 × 10⁹

cells/ml). The culture thus started from a density of ≈4

× 10⁹ cells/ml, and growth was usually stopped at a
density of 6 × 10⁹ cells/ml (or at the density indicated in the text). Separation procedures were performed with the aid of a 10% solution of casein (or skim milk) added to each fraction. The Ca²⁺ concentra-
tion in the supernatant fluid was then immediately raised to 10⁻² M when necessary. The pellet was washed three times by suspension in distilled water (not saline) followed by centrifugation. Since enzyme B tends to leak out from the cells, reproducible results were obtained only when the above manipulations were quickly done in a standard manner.

Preparation of supernatant fluid concentrates. For the analysis of all supernatant proteins, concentration by evaporation at 35 C in a rotary evaporator was
done. To avoid loss of activity at high salt concen-

trations, evaporation was performed in four steps to about one-eighth the original volume, each step inter-

spaced by dialysis against 60 times (v/v) 0.08 M Tris-

hydrochloride buffer containing 10⁻³ M CaCl₂ at pH 7.0 for 12 hr at 4 C. When the volume was about 800-

fold (to 40 ml), a small, reddish precipitate was centri-

fuged out at (17,000 × g for 20 min) before the super-
natant was dialyzed and further concentrated to 9 ml. After a final dialysis, the resulting preparation was sub-

jected to gel filtration.

Preparation of cell lysate and proteolysates. The cells were suspended at an OD of 15 to 20 (6 × 10⁹ to 8 × 10⁹

cells/ml) in 0.08 M Tris-hydrochloride buffer (pH 7.0); CaCl₂ (10⁻⁴ M). Lysozyme (0.2 mg/ml; Nutritional Bio-

chemicals Corp.) and merthiolate (0.2 mg/ml; Lilly) were added, and the preparation was incubated for sev-

eral hours at 37 C. For proteolysis preparations, sucrone (350 mg/ml; Sigma) and protease inhibitor cocktail

(100 mg/ml) were added, and the preparation was incubated for 1 hr to 1 day to allow the proteolysates to form.

Gel filtration. Sephadex G-100 (40-120 mesh, Phar-

macia) was packed by gravity in a jacketed vertical column (95 × 2.5 cm) and equilibrated overnight with 0.08 M Tris-hydrochloride buffer containing 10⁻⁴ M

CaCl₂. The chromatography was carried out at a flow rate of 0.5 ml/min with water cooled to 4 C circulating in the jacket. Five ml samples were collected. The pro-

tein content of each fraction was estimated spectropho-

tometrically (absorption at 280 nm, Beckman) and pro-

teolytic activity was determined.

Preparation of radioactive enzyme C. A 0.2-µg amount of ¹⁴C-leucine (New England Nuclear Corp.; specific activity, 183.5 Ci/mole) per ml, 4 µg of cold

leucine per ml, and CaCl₂ (10⁻³ M, final concentration) were added to a growing culture (1 liter) at 2 × 10⁸

cells/ml. Growth was stopped at 7 × 10⁹ cells/ml; the culture medium was separated and filtered as described
before, and the proteins were precipitated with 1.5 volume of absolute ethanol at -7 C with stirring overnight. The precipitate was dissolved in 0.08 M Tris-hydrochloride buffer (pH 7.8) containing 10⁻⁴ M CaCl₂. The solution was dialyzed against 2,000 times its volume of the same buffer at 4 C for 24 hr with one change of buffer. The solution was then concentrated further by rotary evaporation and was finally fractionated through a Sephadex G-100 column.

**Cellulose acetate electrophoresis.** Electrophoresis was performed by the method of Smith (18) on the concentrated proteolytically active fractions separated by gel filtration. Electrophoresis buffer was 0.08 M Tris-hydrochloride, pH 7.2. The time of each run was 3.5 hr. The bands were fixed with 10% acetic acid and stained with 0.01% Coomassie Blue for 10 min.

**Enzyme assay.** Proteolytic activity was measured by milk coagulation, as described by Gorini and Lanza­vecchia (11). The assay solution consists of 1% powdered skim milk (Difco) in cacodylate buffer (6.6 x 10⁻⁴ M cacodylic acid, 40 ml; 6.6 x 10⁻⁴ M triethanolamine, 60 ml; 3 M CaCl₂, 1 ml). This mixture was homogenized for 30 sec in a Waring Blendor and allowed to stand at room temperature for 10 min; some undispersed material was then removed by decantation. This reconstituted standard milk must be used within 2 hr. Coagulation time was determined in a 37 C water bath on 5 ml of the milk solution to which 0.5 ml of solution to be tested was added. The enzyme concentration, E, is related to the coagulation time, T, by the formula

\[ T = K (1/E) + a \]  

(11)

for which the slope, K, and the intersection with the time axis, a, must be experimentally determined for each new batch of skim milk. In the present work, a was usually found to be about 100 sec, and the milk solution without enzyme (a sample of which should accompany each determination) did not precipitate before 38 hr of incubation.

It has been shown for the proteinase of Coccus P (11) that the assay based on milk coagulation is strictly equivalent to the more classical one based on hydrolysis of standard proteins. We have confirmed that the coagulation time obtained with the extracellular enzyme C is inversely proportional to the amount of hydrolysis of casein. For practical reasons, one unit of enzyme C was chosen to be the amount which coagulates the standard reconstituted milk described above in 19 hr (one-half of the time for which the milk can be incubated without danger of spontaneous precipitation). We have determined that this amount of enzyme C acting on casein (Hammersten) for 30 min at 37 C liberates trichloroacetic acid-soluble products which gave an absorbancy of 0.002 per cm at 280 nm under conditions described by Kunitz (14). For the activity of the cell-associated enzyme B, this same unit of milk coagulation time was adopted, although the relationship of milk coagulation to casein hydrolysis was not determined.

**RESULTS**

**Behavior of wild-type and CP10 mutant with respect to proteolytic activity.** On a milk-agar plate, the wild-type colonies release proteolytic activity as demonstrated by a halo around the colony; strain CP10 shows no such halo. Table 1 gives the result of a similar experiment performed in liquid medium (MMCP). Low- and high-Ca media were used, and proteolytic activity was measured on both the cell-free supernatant fluid and the lysate of the washed cells. In the wild-type supernatant fluid, activity is observed only in the presence of high Ca²⁺, whereas no activity is found in the supernatant fluid from CP10 regardless of Ca²⁺ concentration. However, the cell lysate demonstrated a proteolytic activity which is constant and is observed regardless of Ca²⁺ concentration and the strain used. This suggests two types of proteolytic activity in Coccus P, only one of which, the cell-associated activity, is present in CP10. This explains the fact that, after prolonged incubation (48 hr), a small ring of proteolysis did appear around the colonies of CP10, presumably indicating late leakage of the cell-associated activity.

**Chromatography of the proteins in the culture supernatant fluid.** The amount of protein excreted by Coccus P into the culture fluid is very small. Given the micromoles of radioactive leucine incorporated, the molecular weight and the leucine content of the enzyme, it can be roughly calculated that near the end of the exponential phase the culture supernatant fluid contains only 4 to 5 µg of enzyme protein per ml. Therefore, the cell-free supernatant fluid must be concentrated extensively before chromatography.

Wild-type cells grown in low-Ca MMCP were inoculated at a density of 1.5 x 10⁶ cells/ml into two 30-liter cultures of MMCP (low- and high-Ca concentration). The two cultures were grown to 8 x 10⁹ cells/ml. The culture fluid was separated from the cells and concentrated about 2,800-fold. Sephadex G-100 elution profiles of these concentrated supernatants fluids are presented in Fig. 1a and b from the high-Ca and the low-Ca wild-type cultures, respectively. Two well-separated regions of proteolytic activity are found. In the high-Ca profile a, one of these regions is far more prevalent and coincides with a narrow, symmetric peak of protein (C). The second proteolytic region (B) is much less active and does not coincide with any individually recognizable peak of protein. However, when all fractions (excluding one at each end) containing proteolytic activity in the B region of either high- or low-Ca cultures were pooled, concentrated, and run again through the Sephadex column, we obtained the effluent profile given in the insert of Fig. 1b. In this second fractionation, a peak of protein coincidental with one peak of proteolytic activity is evident and, moreover, no fraction of proteolytic activity eluting near fraction 61 (peak...
C) is visible. This indicates that B is not an aggregate of C but is an additional peak of proteolytic activity. In the low-Ca profile b, the same two regions of proteolytic activity are found: the activity of B is of the same order as in profile a, but that of C is enormously reduced.

In addition to the two proteolytic activities, the supernatant fluid shows another major peak, A, which is roughly similar in the two profiles. Although it appears as a narrow peak in Fig. 1, nothing can be deduced about its homogeneity, because it is eluted with the void volume of the column. It is degraded completely by Pronase and partially by trypsin or chymotrypsin, but not by the peak C proteinase. It is, therefore, a protein or an aggregate of one or more protein molecules. Since it lacks proteolytic activity, it has not been further characterized. There is also a low background of 280-nm absorption that might be due to contamination by intracellular proteins.

Electrophoresis of peaks B and C. Peak B taken from the second fractionation (insert in Fig. 1b) and the central part of peak C from the fractionation of high-Ca supernatant fluid (Fig. 1a) were analyzed by electrophoresis on cellulose acetate strips. Each peak gave a single band migrating in opposite directions, confirming that B and C are single, different proteins. Their molecular weights are approximately 58,000 for B and 31,000 for C, deduced from their elution position in gel filtration by the method of Andrews (2). The specific activity is approximately 221 units/mg of protein in the case of enzyme B and 35,000 units/mg of protein in the case of enzyme C.

Effect of inhibitors. Incubation for 2 hr at 37°C with \(10^{-8}\) M diisopropyl fluorophosphate (DFP) completely inactivated enzyme C but did not affect enzyme B.

N-ethyl-maleimide and p-chloromercuribenzoate at 10 to 100 times the molar concentration of enzyme C did not inhibit its activity. Pretreatment with mercaptoethanol (1.5% for 4 hr at room temperature) did not change the effect of sulfhydryl inhibitors. Enzyme B was not tested.

Chromatography of the intracellular protein. Wild-type and CP10 mutant strains were grown in 5 liters of high-Ca or low-Ca MMCP medium and harvested when the culture reached a density of \(8 \times 10^8\) cells/ml. The washed cells were lysed in the presence of \(10^{-3}\) M Ca²⁺. The proteins, precipitated from the lysate by addition of ammonium sulfate, were redissolved in buffer containing \(10^{-3}\) M Ca²⁺, dialyzed, and applied to a Sephadex G-100 column. The elution profiles of the proteins from wild-type and CP10 mutant cells grown in high-Ca MMCP are given in Fig. 2.

![Fig. 1. G100-Sephadex elution profiles of cell-free supernatant fluids. The [Ca²⁺] in the media of the two cultures is either high (10⁻³ M; chromatogram a) or low (10⁻⁴ M; chromatogram b). The [Ca²⁺] in the two cell-free supernatant fluids is equalized to 10⁻³ M and kept at this level thereafter. The supernatants, concentrated 2,000-fold, are applied to the column: 8.8 ml (108 mg of protein) obtained from the high-Ca culture and 8.2 ml (56 mg of protein) from the low-Ca culture. The solid line is the profile of the 280-nm absorption (ordinates at left); the dotted line is that of the milk-coagulating activity (ordinates at right). The numbers in the abscissae indicate the serial fractions of the column eluate. Insert, profile obtained in a second Sephadex run of the concentrated, pooled fractions containing B activity from the low-Ca culture.](http://jb.asm.org/)
Distribution of enzymes B and C between the cells and the culture fluid. Table 2 presents the values of proteolytic activities measured in the region C of the chromatograms discussed above, with all values adjusted to standard cell density and culture volume. In the wild-type strain, the amount of enzyme C found in the supernatant fluid of a culture containing $10^{-5}$ M Ca$^{2+}$ is only 0.04% of that present in the same volume of supernatant fluid of culture at equal cell density but containing $10^{-3}$ M Ca$^{2+}$. A small amount of C activity is found associated with the wild-type cells grown in high-Ca medium. This represents 0.0014% of the total C activity in the culture, which could be contamination from the supernatant fluid. However, an equivalent amount is also found associated with the low-Ca cells, even though the total activity is 2,500 times less than in the high-Ca culture.

Table 3 reports the amount of enzyme B found in cells and supernatant of a high-Ca culture of wild-type strain. It is seen that, unlike enzyme C, most of B is associated with the cell. Analogous results were obtained with both wild type and CP10 strains, irrespective of Ca$^{2+}$ concentration. CP10 lysate shows only B activity (Fig. 1b).

Cellular location of enzyme B. The amount of B demonstrable in the supernatant fluid after concentration (Table 3) may be due to cell lysis. This amount can be roughly calculated to be between 5 and 16% of that associated with the cell. However, there was no evidence for such a large amount of cell lysis in our preparation. Therefore, the possibility arose that enzyme B may not be a strict intracellular enzyme as previously supposed. This was further suggested by the fact that, even though the cell-associated activity (enzyme B) remained constant during the exponential phase of growth (Table 1), its level dropped drastically when the cells were kept for several hours in saline or MMCP. Cell lysis was not the important factor, as the cell counts remained constant after this treatment. The loss of enzyme B activity when the cells were exposed to saline indicates that B is a loosely bound enzyme. The classical test for protoplast inability to retain such an enzyme was thus performed. Results given in Table 4 show that the transition from intact cells to protoplast releases the enzyme B activity into the supernatant fluid, even though the protoplasts are intact as shown by retention of most of the cell proteins. It is concluded that B has the characteristics of a periplasmic (13) enzyme.

Stability of enzymes B and C. Since the amount of enzyme B was found to be almost independent of Ca$^{2+}$ concentration, whereas C was drastically reduced when Ca$^{2+}$ concentration was lowered, the stability of the enzymes in a cell-free
TABLE 2. Enzyme C in high-Ca and low-Ca cultures and its distribution between cells and supernatant fluid*

<table>
<thead>
<tr>
<th>Prepn steps</th>
<th>Proteolytic activity (High-Ca culture)</th>
<th>Proteolytic activity (Low-Ca culture)</th>
<th>Recovery (%)</th>
<th>Ratio (Low-Ca/High-Ca)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Supernatant</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Crude supernatant</td>
<td>3,722,000 (1,660)</td>
<td></td>
<td></td>
<td>4 x 10^{-4} c</td>
</tr>
<tr>
<td>After concentration</td>
<td>2,235,000 (1,000)</td>
<td></td>
<td>60</td>
<td></td>
</tr>
<tr>
<td>After column 1</td>
<td>1,117,500 (500)</td>
<td></td>
<td>30</td>
<td></td>
</tr>
<tr>
<td>Cells</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Initial activity</td>
<td>(525)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>After precipitation</td>
<td>(210)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>After column</td>
<td>105</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Total amount of C activity adjusted for equal volumes of culture (30 liters) at equal density (8 x 10^8 cells/ml)

When the activity before concentration or fractionation, or both, is not measurable, it is calculated from the after-column data assuming similar recovery for equivalent steps. Enzyme C was fractionated in the supernatant fluid by evaporation followed by Sephadex chromatography and in the cell lysate by ammonium sulfate precipitation followed by Sephadex chromatography. Enzyme C recovery from ammonium sulfate precipitation was determined in a separate experiment. The calculated values are in parentheses. The after-column data concerning the supernatant are the sum of the proteolytic activities found in all elution tubes corresponding to the C region in Fig. 1a and b. Analogously, data concerning the high-Ca cells were obtained from the C region in Fig. 2a. The data for the low-Ca cells correspond to the C region in a chromatogram not reported but with a profile analogous to Fig. 2a.

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TABLE 3. Enzyme B distribution between cells and supernatant fluid*

<table>
<thead>
<tr>
<th>Prepn steps</th>
<th>Proteolytic activity (Cells)</th>
<th>Recovery %</th>
<th>Prepn steps</th>
<th>Proteolytic activity (Supernatant fluid)</th>
<th>Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude lysate</td>
<td>6,000</td>
<td>60</td>
<td>Initial activity</td>
<td>?</td>
<td>?</td>
</tr>
<tr>
<td>After precipitation</td>
<td>1,300</td>
<td>22</td>
<td>After concn</td>
<td>(210)</td>
<td></td>
</tr>
<tr>
<td>After column</td>
<td>1,250</td>
<td>21</td>
<td>After column</td>
<td>200</td>
<td>6.25</td>
</tr>
</tbody>
</table>

* Total amount of B activity in a 30-liter culture of the wild-type strain in high-Ca medium at a density of 8 x 10^8 cells/ml

Analogous results are obtained with a culture in low-Ca medium or with mutant CP10 in either media. In the cell lysate, enzyme B was fractionated by ammonium sulfate precipitation followed by Sephadex chromatography, in the supernatant, by concentration directly followed by Sephadex chromatography. B activity in the supernatant is not measurable directly, and it cannot be calculated because B stability through the concentration procedure is unknown. The after-column data concerning the cells are the sum of the proteolytic activity found in all tubes corresponding to the B region in Fig. 2a, those concerning the supernatant fluid are from the B region in Fig. 1a. The calculated values are in parentheses.

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TABLE 4. Release of enzyme B from protoplasts*

<table>
<thead>
<tr>
<th>Strain</th>
<th>Ca^2+ in growth medium (m)</th>
<th>Protein content and proteolytic activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>In supernatant fluid</td>
</tr>
<tr>
<td></td>
<td>Protein (mg/ml)</td>
<td>Enzyme (units/ml)</td>
</tr>
<tr>
<td>Wild type</td>
<td>10^{-3}</td>
<td>0.20</td>
</tr>
<tr>
<td>Wild type</td>
<td>10^{-4}</td>
<td>0.27</td>
</tr>
<tr>
<td>CP10</td>
<td>10^{-3}</td>
<td>0.25</td>
</tr>
<tr>
<td>CP10</td>
<td>10^{-4}</td>
<td>0.32</td>
</tr>
</tbody>
</table>

* Wild-type and mutant cells were grown in high-Ca and low-Ca MUCP to 4 x 10^9 cells/ml. They were then washed repeatedly with water and finally resuspended in MUCP containing 10^{-3} M Ca^{2+} at a final density of 6 x 10^9 cells/ml. The suspension was lyzed by the standard procedure described in Materials and Methods or in the presence of sucrose (350 mg/ml) to preserve the protoplasts. The lysozyme treatment was performed at 37 C for 1 hr. The intact protoplasts were separated by centrifugation; pellet and supernatant fluid were analyzed separately. The lysate was analyzed directly. The proteins were determined by the method of Lowry et al. (15).

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system was tested. The Ca^{2+} concentration in solutions of enzymes B and C (originally containing 10^{-3} M Ca^{2+}) was lowered by extensive dialysis against low-Ca buffer. Table 5 gives the proteolytic activity remaining after dialysis as percentage of the original activity in the untreated sample. Enzyme B activity was not affected by Ca^{2+} removal, whereas enzyme C activity was lost. This confirms the earlier findings (9) that the extracellular proteinase of Coccus P is quickly and irreversibly inactivated upon removal of Ca^{2+} by ion-complexing agents. The mechanism of this inactivation was further investigated with radioactive enzyme C prepared as described in Materials and Methods. The concentrated enzyme peak was diluted so that the Ca^{2+}
incubation of a high-Ca culture, after the stationary phase of growth was reached, resulted in a loss of supernatant enzymatic activity. Addition of CaCl₂ to counteract the possible presence of complexing agents or adjustment of pH did not eliminate the loss. Experiments with purified enzyme C showed that shaking of the culture produced loss of enzymatic activity even in the presence of Ca²⁺. This was not due to oxidation, as the enzyme lost activity by shaking under a nitrogen atmosphere. Shaking with a wetting agent, Antifoam A (Dow Chemicals; 1 ml of a 5% suspension per liter) resulted in a complete loss of activity, which indicates that the enzyme is susceptible to surface denaturation irrespective of Ca²⁺ concentration. Shaking the radioactive enzyme in the presence of Ca²⁺ (10⁻³ M) resulted in an activity loss which was accompanied by only a 50% loss of trichloroacetic acid-precipitable radioactivity (Table 7), as opposed to a 90% loss when Ca²⁺ concentration was lowered (Table 6).

Since aeration, and therefore shaking, is necessary for growth, a search was instituted for a compound that would prevent surface denaturation. Proteins such as gelatin or albumin, which have a protective function for other enzyme systems, could not be used in the case of a proteolytic enzyme. It was found that Ficoll (Pharmacia), a nonionic polymer of sucrose, was useful in this respect. A 5% concentration of Ficoll was sufficient to prevent surface denaturation of the proteinase (Table 7) without impairing growth. To see whether Ficoll also counteracts autodigestion, it was added simultaneously with different Ca-complexing agents which establish a gradient of increasing instability, depending on their strength (9). Table 8 shows that Ficoll delays (but does not prevent) autodigestion.

**Table 5. Effect of Ca²⁺ removal on the proteolytic activity of enzymes B and C**

<table>
<thead>
<tr>
<th>Conditions determining Ca²⁺ in the solution</th>
<th>Ca²⁺ obtained (mL)</th>
<th>Enzyme B (units/mL) 10⁻³ M</th>
<th>Enzyme C (units/mL) 10⁻³ M⁺</th>
</tr>
</thead>
<tbody>
<tr>
<td>At zero time in high-Ca buffer</td>
<td>10⁻³</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>After dialysis against high-Ca buffer</td>
<td>10⁻³</td>
<td>100</td>
<td>95</td>
</tr>
<tr>
<td>After dialysis against low-Ca buffer</td>
<td>10⁻⁴</td>
<td>99</td>
<td>&lt; 1</td>
</tr>
</tbody>
</table>

* Dialysis lasted for 24 hr at 4 C and was performed against 1,000 times volume of buffer (0.1 M Tris-hydrochloride, pH 7, with 10⁻⁴ M or 10⁻⁵ M CaCl₂) with one change. At the end, the Ca²⁺ concentration was raised to 10⁻³ M, and the samples were tested on milk and casein for proteolytic activity. The enzyme solutions are the fractions under peaks B and C shown in Fig. 1a and b (insert). The values are units of activity per milliliter in per cent of the activity at zero time.  
* Ca²⁺ concentration during growth.  
* Ca²⁺ concentration during growth was 10⁻³ M.

concentration was reduced to 5 x 10⁻⁶ M. The control, in which the original 10⁻³ M Ca²⁺ concentration was reinstated by addition of CaCl₂, lost less than 15% of its enzymatic activity after 2 days at 37 C, whereas the solution containing 5 x 10⁻⁶ M Ca²⁺ lost all of its enzymatic activity within 2 hr at room temperature (Table 6). This loss was accompanied by a 90% loss of trichloroacetic acid-precipitable radioactivity (Table 6). This process could be arrested by an intermediate time if 10⁻³ M Ca²⁺ were added. It is concluded that, in the absence of Ca²⁺, enzyme C undergoes rapid autodigestion.

Removal of Ca²⁺ is not the only cause of loss of enzyme C activity. It was observed that further

**Table 6. Autodigestion of enzyme C upon removal of calcium**

<table>
<thead>
<tr>
<th>Time at 37 C (hr)</th>
<th>Enzyme activity (units/mL)</th>
<th>TCA⁺-precipitable radioactivity (counts per min/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>10⁻³ M Ca²⁺</td>
<td>5 x 10⁻⁴ M Ca²⁺</td>
</tr>
<tr>
<td>0</td>
<td>73.5</td>
<td>65.8</td>
</tr>
<tr>
<td>2</td>
<td>62</td>
<td>0</td>
</tr>
<tr>
<td>24</td>
<td>62</td>
<td>0</td>
</tr>
<tr>
<td>Percent remaining after 2 hr</td>
<td>85</td>
<td>0</td>
</tr>
</tbody>
</table>

* A concentrated solution of radioactive enzyme C (Ca²⁺ 10⁻³ M) was diluted with 0.1 M Tris-hydrochloride buffer (pH 7.1) either with or without addition of Ca²⁺ 10⁻³ M. This achieved the two [Ca²⁺] of 10⁻³ M and 5 x 10⁻⁶ M. All manipulations were performed at 0 C. At the end of the exposure to 37 C, the [Ca²⁺] was raised to 10⁻³ M when necessary, and the samples were conserved at 0 C until analysis.
* Trichloroacetic acid.

**Table 7. Effect of Ficoll on surface denaturation of enzyme C in the presence of Ca²⁺**

<table>
<thead>
<tr>
<th>Function measured</th>
<th>Per cent remaining after 5 hr at 37 C</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Standing</td>
</tr>
<tr>
<td>Enzyme activity . . . . . . . .</td>
<td>84</td>
</tr>
<tr>
<td>TCA⁺-precipitable radioactivity . .</td>
<td>92</td>
</tr>
</tbody>
</table>

* The enzyme used was taken from the peak-C of the Sephadex column (Fig. 1a). Experiments were carried out at 37 C in the presence of 10⁻³ M Ca²⁺. After 5 hr, duplicate samples were assayed together with a set of zero-time samples, which were kept in ice. The shaking apparatus was a standard Dubnoff metabolic shaker (88 excursions per min).
* Trichloroacetic acid.
**DISCUSSION**

It is found that Coccus P produces two proteolytic enzymes (B and C) separable by gel filtration and by electrophoresis on cellulose acetate. They have different molecular weights and widely different specific activities; they move in opposite directions in electrophoresis, and Ca\(^{2+}\) is essential for stability of enzyme C only. Enzyme C is inhibited by diisopropyl fluorophosphate, and its substrate specificity [splits off the N-terminal glycine of synthetic tripeptides, method of Matheson et al. (16, 17) used for testing] is in agreement with the results reported previously for the excreted proteinase (8). Enzyme B does not hydrolyze the tripeptides split by enzyme C. These differences, to which it should be added that antiserum to C does not cross-react with B (M. Bissell and L. Gorini, *unpublished data*), exclude the possibility that one of the enzymes derives from the other by some slight modification occurring after the synthesis of a polypeptide chain common to both. Enzyme C does not contain any essential -S-S- bridge, because it is not affected by mercuric-ethanol followed by N-ethyl-maleimide treatment. Moreover, the number of cysteine residues found upon amino acid analysis (standard procedure for Beckman/Spinco Analyzer model 120) is consistent with a maximum of one -S-S- bridge.

An important physiological difference between the two enzymes is their localization. Enzyme B is periplasmic and, therefore, is loosely bound to the cell. It is easily extractable by saline or by the culture medium itself and may leak out into the supernatant fluid. Leakage was found for in the CP10 mutant which does not produce enzyme C and, therefore, would not mask a small amount of enzyme B should it appear in the supernatant fluid. It was found, on selective agar plates and in liquid culture, that no proteolytic activity was detectable in the surrounding medium until 4 to 5 hr of exponential growth, a time exceeding the standard duration of our experiments. Enzyme C, on the other hand, is an extracellular enzyme with no detectable pool inside the cell. Having established that the two are separated with respect to their synthesis and localization, the study of extracellular enzyme C excretion may be pursued without interference by enzyme B.

The presence of enzyme C in the supernatant is strictly dependent on Ca\(^{2+}\) concentration. The small amount of activity found in the supernatant fluid of low-Ca culture (0.04% of the high-Ca culture) can be reasonably assumed to be due to the fact that the medium to which no Ca\(^{2+}\) is added contains 10\(^{-4}\) M Ca\(^{2+}\). A small amount of C (0.0014% of the total) was found associated with the high-Ca wild-type cells which could be due to contamination. However, an amount of enzyme in the same order of magnitude is also found associated with the low-Ca cells; this has an important bearing on the role of Ca\(^{2+}\) in enzyme excretion which is examined in a separate paper (5).

Pertinent to this study is the influence of Ca\(^{2+}\) on the stability of enzyme C in cell-free supernatant fluids. Two different mechanisms are shown to cause a loss of enzyme C activity: one is autodigestion which is counteracted by Ca\(^{2+}\); the other occurs in the presence of Ca\(^{2+}\), is counteracted by antiwetting agents like Ficoll, and, therefore, may be attributed to surface denaturation. It was also found that Ficoll delays autodigestion, which suggests that removal of Ca\(^{2+}\) may involve a denaturation step which is rate-limiting in proteolysis. Such a possibility has previously been shown to exist for autodigestion of trypsin (10). It was shown that trypsin undergoes a reversible structural modification equivalent to an initial step of denaturation. This modification

<table>
<thead>
<tr>
<th>Time (hr)</th>
<th>0</th>
<th>15</th>
<th>30</th>
<th>1</th>
<th>1 hr</th>
<th>3 hr</th>
<th>24 hr</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 min</td>
<td>29.2</td>
<td>30.1</td>
<td>31.6</td>
<td>7.5</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>15 min</td>
<td>28.6</td>
<td>29.2</td>
<td>31.0</td>
<td>1.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>30 min</td>
<td>28.6</td>
<td>29.0</td>
<td>30.0</td>
<td>1.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>1 hr</td>
<td>28.6</td>
<td>8.7</td>
<td>14.3</td>
<td>2.5</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>1 hr 50 min</td>
<td>26.7</td>
<td>7.5</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>3 hr</td>
<td>26.7</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>24 hr</td>
<td>25.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
</tr>
</tbody>
</table>

*Values are units of milk-coagulating activity per milliliter. A concentrated solution of enzyme C (10\(^{-4}\) M Ca\(^{2+}\)) was diluted to obtain the Ca\(^{2+}\) concentration of 10\(^{-4}\) M. To a sample of this dilution, CaCl\(_2\) was added to reobtain 10\(^{-4}\) M Ca\(^{2+}\). Ficoll concentration was 5%.

* Ethylenediaminetetraacetic acid.
occurs in a fraction of the enzyme molecules which then become accessible to the active molecules. The delaying action of Ficoll indicates that the above mechanism also applies to the autodigestion of enzyme C. The fact that the enzyme is autodigested upon removal of Ca\(^{2+}\) indicates that this ion stabilizes an active form of the molecule, rather than being a constituent of the prothetic group required for activity.

It should be realized, however, that the denaturation occurring in the presence of Ca\(^{2+}\) and that occurring upon removal of Ca\(^{2+}\) are not the same process. Both provide substrate for the active enzyme molecule, but only one is reversible and counteracted by Ca\(^{2+}\). This is in agreement with our observation that the rate of disappearance of activity in the absence of Ca\(^{2+}\) increases with increasing enzyme concentration (a second-order reaction), whereas in surface denaturation, if there is any effect of increasing enzyme concentration, it is the reverse.

ACKNOWLEDGMENTS

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LITERATURE CITED


