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

Permalink
https://escholarship.org/uc/item/9hh984w0

Author
SARNER, NITZA Z

Publication Date
2011-03-25

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

NITZA Z. SARNER,¹ MINA J. BISSELL,² MARIO DI GIROLAMO,³ AND LUIGI GORINI

Department of Microbiology and Molecular Genetics, Harvard Medical School, Boston, Massachusetts 02115

Received for publication 29 June 1970

¹Present address: Biophysics Department, Portsmouth Polytechnic, Portsmouth, England.
²Present address: Department of Molecular Biology, University of California, Berkeley.
³Present address: Laboratorio Internazionale di Genetica e Biofisica, Naples, Italy.

LBNL/DOE funding & contract number: DE-AC02-05CH11231

DISCLAIMER

This document was prepared as an account of work sponsored by the United States Government. While this document is believed to contain correct information, neither the United States Government nor any agency thereof, nor The Regents of the University of California, nor any of their employees, makes any warranty, express or implied, or assumes any legal responsibility for the accuracy, completeness, or usefulness of any information, apparatus, product, or process disclosed, or represents that its use would not infringe privately owned rights. Reference herein to any specific commercial product, process, or service by its trade name, trademark, manufacturer, or otherwise, does not necessarily constitute or imply its endorsement, recommendation, or favoring by the United States Government or any agency thereof, or The Regents of the University of California. The views and opinions of authors expressed herein do not necessarily state or reflect those of the United States Government or any agency thereof or The Regents of the University of California.
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 intraand 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 (10⁹ 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 relation 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 (orguanosine), biotin, and catechol (or any other orthodiphenol; 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 10⁸ 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 (≈ 10⁻⁵) grew. Dilutions were plated on Field's medium and on agarmilk 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 Ca\(^{2+}\) concentration, whereas it retains its cell-associated activity.

**Minimal medium**

The basal medium was the minimal medium (MMCP) described by Gorini and Lord (12) with the following composition (grams per liter): sodium glutamate, 3; sodium acetate, 3; glycine, 1; (NH\(_4\))\(_2\)SO\(_4\), 0.04; MgCl\(_2\), 0.2; KH\(_2\)PO\(_4\), 0.137; guanosine, 0.3; biotin, 0.0023; tris(hydroxymethyl)aminomethane (Tris), 12.1. The \(pH\) was adjusted to 7. After autoclaving, the following were added; catechol (sterilized by filtration), 0.0029 g/liter, and sterile glucose, 2 g/liter. The calcium content of this medium was determined by atomic absorption (kindly performed by K. Fuwa) and was consistently of the order of 10\(^{-6}\) M, presumably attributable to calcium contamination in the chemicals, water, and glassware used. Since strictly calcium-free conditions were not obtainable, all experiments and cultures to which no calcium was intentionally added are designated as "low-calcium" and assumed to contain a concentration of Ca\(^{2+}\) ions of the order of 10\(^{-6}\) M. To obtain the "high-calcium" cultures, 0.111 g/liter of CaCl\(_2\) was added to MMCP, which brought the concentration of Ca\(^{2+}\) to 10\(^{-3}\) M.

**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 casein 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 precipitates before being digested. Mutant CPIO 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 unpredictable length before initiation of growth were avoided by using a rather large inoculum (1/10 volume of the inoculated medium) taken from a culture in low-Ca MMCP at mid-exponential phase of growth (\(\approx\)4 x 10\(^{8}\) cells/ml). The culture thus started from a density of \(\approx\)4 x 10\(^{7}\) cells/ml, and growth was usually stopped at a density of 6 x 10\(^{8}\) cells/ml (or at the density indicated in each particular experiment). Small-size cultures (up to 500 ml) were grown in Erlenmeyer flasks (culture volume to flask volume ratio, 1: 10) shaken with a Dubnoff metabolic shaker at 88 excursions per min. Large batches (30 liters) were grown in a 60-liter New Brunswick fermentor at 200 rev/min with stirring, and 1.8 liters of air per min
(at the Enzyme Center, Tufts University Medical School). Growth was followed by measuring increase in optical density (OD) at 490 nm in a Beckman spectrophotometer. One unit of OD corresponds to a bacterial density of $4 \times 10^8$ cells/ml or to bacterial dry weight of $\approx 0.8$ mg/ml.

**Separation of cells from the culture fluid**

*Coccus P* produces two proteolytic enzymes: proteinase C, excreted into the medium, and proteinase B, loosely associated with the cells. To prevent contamination of one with the other, the cells were completely and quickly separated from the medium. When the incubation was stopped, the culture was immediately chilled and centrifuged at 11,000 x $g$ for 10 min, and the supernatant fluid was filtered through sterilized filters (0.45-µm pore size; Millipore Corp., Bedford, Mass.). The Ca$^{2+}$ concentration in the supernatant fluid was then immediately raised to $10^{-3}$ 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 standardized procedure.

**Preparation of supernatant fluid concentrates**

For the analysis of all supernatant proteins, concentration by evaporation at 35 C in a rotary evaporator was adopted. To avoid loss of activity at high salt concentrations, evaporation was performed in four steps to about one-eighth the original volume, each step interspaced by dialysis against 60 times (v/v) 0.08 M Tris-hydrochloride buffer containing $10^{-3}$ M CaCl$_2$ 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 centrifuged out (at 17,000 x $g$ for 20 min) before the supernatant was dialyzed and further concentrated to 9 ml. After a final dialysis, the resulting preparation was subjected to gel filtration.

**Preparation of cell lysate and protoplasts**

The cells were suspended at an OD of 15 to 20 ($6 \times 10^9$ to $8 \times 10^9$ cells/ml) in 0.08 M Tris-hydrochloride buffer (pH 7.0); CaCl$_2$ ($10^{-3}$ M). Lysozyme (0.2 mg/ml; Nutritional Biochemicals Corp.) and merthiolate (0.2 mg/ml; Lilly) were added, and the preparation was incubated for several hours at 37 C. For protoplast preparations, sucrose (350 mg/ml) was added for protection, and the incubation time was limited to 1 hr. The viscosity of the lysate was reduced by addition of deoxyribonuclease (1.8 µg/ml; Nutritional Biochemicals Corp.) and ribonuclease (0.78 µg/ml; Nutritional Biochemicals Corp.), and the debris was centrifuged out. For experiments involving chromatography, the lysate proteins were precipitated with ammonium sulfate (90% saturation), redissolved in Tris buffer as above (protein recovery 80%); the solution was dialyzed, centrifuged, and finally applied to a Sephadex column. For experiments in which the lysate was used only for determination of proteolytic activity, the cells were lysed directly in cacodylate buffer of the same composition as that used for preparing the standard skim milk.
Gel filtration

Sephadex G-100 (40-120 mesh, Pharmacia) was packed by gravity in a jacketed vertical column (95 x 2.5 cm) and equilibrated overnight with 0.08 M Tris-hydrochloride buffer containing 10^{-3} M CaCl_2. 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 protein content of each fraction was estimated spectrophotometrically (absorption at 280 nm, Beckman) and proteolytic 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 CaCl_2 (10^{-3} M, final concentration) were added to a growing culture (1 liter) at 2 x 10^8 cells/ml. Growth was stopped at 7 x 10^8 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^{-3} M CaCl_2. 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 geliltration. 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% Coomasie 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 11% powdered skim milk (Difco) in cacodylate buffer (6.6 x 10^{-2} M cacodylic acid, 40 ml; 6.6 x 10^{-2} M triethanolamine, 60 ml; 3 M CaCl_2, 1 ml). This mixture was homogenized for 30 sec in a Waring Blender 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

\[ T = \frac{K}{E} + a \quad (11) \]

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 I 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 wildtype 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 cellfree supernatant fluid must be concentrate extensively before chromatography.

Wild-type cells grown in low-Ca MMCP were inoculated at a density of 1.5 x 10⁸ cells/ml into two 30-liter cultures of MMCP (low- and high-Ca concentration). The two cultures were grown to 8 x 10⁸ 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. Ia and b from the high-Ca and the low-Ca wild-type cultures, respectively. Two ellseparated 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 higher low-Ca cultures were pooled, concentrated, and run again through the Sephadex column, we obtained the effluent profile given in the insert of Fig. Ib. 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 superantant fluid shows another major peak, A, which is roughly similar in the two profiles. Although it appears as a narrow peak in Fig. I, 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 $10^{-3}$ 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 CPIO 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 Ca$^{2+}$. The proteins, precipitated from the lysate by addition of ammonium sulfate, were redissolved in buffer containing $10^{-3}$ M Ca$^{2+}$, dialyzed, and applied to a Sephadex G-100 column. The elution profiles of the proteins from wild-type and CPIO mutant cells grown in high-Ca MMCP are given in Fig. 2a and b, respectively. The profiles obtained from the same strains grown in low-Ca medium were similar. As expected, no individual peaks for protein B or C are recognizable in the presence of all the cell proteins. However, two separate regions of proteolytic activity are found in the chromatogram of wild-type cells and only one in that of CPIO mutant cells. In both strains, the presence of these proteolytic regions does not depend on the level C$^{2+}$ in the culture. The position in the chromatogram of the cell lysate,
where enzyme C should be found, was determined by a separate gel filtration with a sample of lysate to which radioactive enzyme C was added. Furthermore, given the position of enzyme C, the region where peak B should be was inferred by its distance from C, found in the chromatogram of the supernatant fraction (Fig. 1a). The two regions where proteolytic activity was found in extracts of wild-type cells correspond to those of enzymes B and C found in the supernatant fluid. The only activity found in the cells of CP 10 mutant was the region corresponding to enzyme B.

**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 $10^{-6}$ 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 CPIO strains, irrespective of Ca$^{2+}$ concentration. CPIO 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 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\(^{2+}\) concentration was reduced to 5 x 10\(^{-6}\) M. The control, in which the original 10\(^{-3}\) M Ca\(^{2+}\) concentration was reinstated by addition of CaCl\(_2\), lost less than 15% of its enzymatic activity after 2 days at 37 \(^\circ\)C, whereas the solution containing 5 x 10\(^{-6}\) M Ca\(^{2+}\) 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 at any intermediate time if 10\(^{-3}\) M Ca\(^{2+}\) were added. It is concluded that, in the absence of Ca\(^{2+}\), enzyme C undergoes rapid autodigestion.

Removal of Ca\(^{2+}\) is not the only cause of loss of enzyme C activity. It was observed that further incubation of a high-Ca culture, after the stationary phase of growth was reached, resulted in a loss of supernatant enzymatic activity. Addition of CaCl\(_2\) 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 Ca\(^{2+}\). 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 Ca\(^{2+}\) concentration. Shaking the radioactive enzyme in the presence of Ca\(^{2+}\) (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 Ca\(^{2+}\) 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.

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 flourophosphate, 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 mercaptoethanol followed by N-ethyl-maleimide treatment. Moreover, the number of cysteine residues found upon amino acid analysis (standard procedure or 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 looked for in the CPIO 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 Ca²⁺ 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 Ca²⁺ is added does contain 10⁻⁶ M Ca²⁺. 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 Ca²⁺ in enzyme excretion which is examined in a separate paper (5).

Pertinent to this study is the influence of Ca²⁺ 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 Ca²⁺; the other occurs in the presence of Ca²⁺, 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 Ca²⁺ may involve a denaturation step which is ratelimiting 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 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 Ca²⁺
indicates that this ion stabilizes an active form of the molecule, rather than being a constituent of the prosthetic 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.

Acknowledgements

This work was supported by American Cancer Society grant E-226H, Public Health Service grant 5 ROI AI 02011-12 from the National Institute of Allergy and Infectious Diseases, and by a fellowship to Mina Bissell from William F. Milton Fund Part 1924.

Literature Cited

**TABLE 1.** Proteolytic activity in cultures of wild-type *Coccus P* and of strain CP10\(^a\)

<table>
<thead>
<tr>
<th>Strain</th>
<th>(\text{Ca}^{++}) in the growth medium (m)</th>
<th>Units of proteolytic activity (units/100 ml)</th>
<th>Cell-associated activity (% of total activity)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>In cell lysate</td>
<td>In culture supernatant fluid</td>
</tr>
<tr>
<td>Wild type</td>
<td>(10^{-3})</td>
<td>30</td>
<td>10,500</td>
</tr>
<tr>
<td>Wild type</td>
<td>(10^{-6})</td>
<td>31</td>
<td>(\leq 1)</td>
</tr>
<tr>
<td>CP10</td>
<td>(10^{-3})</td>
<td>34</td>
<td>(\leq 1)</td>
</tr>
<tr>
<td>CP10</td>
<td>(10^{-8})</td>
<td>35</td>
<td>(\leq 1)</td>
</tr>
</tbody>
</table>

\(^a\) Figures are adjusted for 100 ml of culture at density of \(4 \times 10^8\) cells/ml and give the activity contained in the total amount of cells or supernatant fluid. The high-calcium cultures were derived from the low-calcium ones by adding \(10^{-5}\) M CaCl\(_2\) to a portion of them withdrawn when a density of \(10^8\) cells/ml was reached. From experiment to experiment the activity in the cell lysate may vary from 25 to 50 units.
FIG. 1. G100-Sephadex elution profiles of cell-free supernatant fluids. The [Ca²⁺] in the media of the two cultures is either high (10⁻⁴ M; chromatogram a) or low (10⁻⁶ M; chromatogram b). The [Ca²⁺] in the two cell-free supernatant fluids is equalized to 10⁻⁵ M and kept at this level thereafter. The supernatants, concentrated 2,800-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.
Fig. 2. G100-Sephadex elution profiles of cell lysates. Profile a was obtained with the wild-type cells, profile b with the cells of mutant CP10. Six-liter cultures were grown in high-Ca MMCP (Ca$^{2+}$ $10^{-3}$ M). The cells were lysed and the proteins were precipitated with ammonium sulfate, redissolved, and applied to the column: 8 ml (70 mg of protein) from wild-type cells and 15 ml (130 mg of protein) from CP10 cells. The solid-line profile is the 280-nm absorption (ordinates at left); the dotted line profile is the milk-coagulating activity (ordinates at right). Numbers in the abscissae indicate the serial fraction of the column eluate.
TABLE 2. Enzyme C in high-Ca and low-Ca cultures and its distribution between cells and supernatant fluid\(^a\)

<table>
<thead>
<tr>
<th>Prep steps</th>
<th>Proteolytic activity</th>
<th>Recovery (%)</th>
<th>Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>High-Ca culture</td>
<td>Low-Ca culture</td>
<td></td>
</tr>
<tr>
<td>Supernatant</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Crude supernatant</td>
<td>3,722,000</td>
<td>(1,660)</td>
<td>60</td>
</tr>
<tr>
<td>After concentration</td>
<td>2,235,000</td>
<td>(1,000)</td>
<td>30</td>
</tr>
<tr>
<td>After column</td>
<td>1,117,500</td>
<td>500</td>
<td></td>
</tr>
<tr>
<td>Cells</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Initial activity</td>
<td>(525)</td>
<td>(300)</td>
<td>40</td>
</tr>
<tr>
<td>After precipitation</td>
<td>(210)</td>
<td>(120)</td>
<td></td>
</tr>
<tr>
<td>After column</td>
<td>105</td>
<td>60</td>
<td></td>
</tr>
</tbody>
</table>

\(^a\) Total amount of C activity adjusted for equal volumes of culture (30 liters) at equal density (8 \(\times\) 10\(^6\) 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.

\(^b\) Low-Ca/high-Ca.

\(^c\) Cells/supernatant fluid, high-Ca medium.

\(^d\) Cells/supernatant fluid, low-Ca medium.
### TABLE 3.

**Enzyme B distribution between cells and supernatant fluid**

<table>
<thead>
<tr>
<th></th>
<th>Cells</th>
<th>Supernatant Fluid</th>
<th>Ratio&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Prepn steps</td>
<td>Proteolytic activity</td>
<td>Prepn steps</td>
</tr>
<tr>
<td>Crude lysate</td>
<td>6,000</td>
<td>Initial activity</td>
<td>?</td>
</tr>
<tr>
<td>After precipitation</td>
<td>1,300</td>
<td>After concn</td>
<td>(210)</td>
</tr>
<tr>
<td>After column</td>
<td>1,250</td>
<td>After column</td>
<td>200</td>
</tr>
</tbody>
</table>

<sup>a</sup>Total amount of B activity in a 30-liter culture of the wild-type strain in high-Ca medium at a density of $8 \times 10^6$ 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.

<sup>b</sup>Cells/supernatant.
TABLE 4. Release of enzyme B from protoplasts

<table>
<thead>
<tr>
<th>Strain</th>
<th>Ca²⁺ in growth medium (M)</th>
<th>Protein content and proteolytic activity</th>
<th>Intact protoplasts</th>
<th>In total lysate</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>In supernatant fluid</td>
<td>In pellet</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Protein (mg/ml)</td>
<td>Enzyme (units/ml)</td>
<td>Enzyme (ml)</td>
</tr>
<tr>
<td>Wild type</td>
<td>10⁻³</td>
<td>0.20</td>
<td>4.0</td>
<td>&lt;1</td>
</tr>
<tr>
<td>Wild type</td>
<td>10⁻⁶</td>
<td>0.27</td>
<td>3.7</td>
<td>&lt;1</td>
</tr>
<tr>
<td>CP10</td>
<td>10⁻³</td>
<td>0.25</td>
<td>4.0</td>
<td>&lt;1</td>
</tr>
<tr>
<td>CP10</td>
<td>10⁻⁶</td>
<td>0.32</td>
<td>4.5</td>
<td>&lt;1</td>
</tr>
</tbody>
</table>

Wild-type and mutant cells were grown in high-Ca and low-Ca MMCP to 4 × 10⁶ cells/ml. They were then washed repeatedly with water and finally resuspended in MMCP containing 10⁻¹ M Ca²⁺ at a final density of 6 × 10⁶ cells/ml. The suspension was lysed 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).
### Table 5

**Effect of Ca\(^{2+}\) removal on the proteolytic activity of enzymes B and C**

<table>
<thead>
<tr>
<th>Conditions determining Ca(^{2+}) in the solution</th>
<th>Ca(^{2+}) obtained (M)</th>
<th>Enzyme B (units/ml)</th>
<th>Enzyme C (units/ml)</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>97</td>
</tr>
<tr>
<td>After dialysis against low-Ca buffer</td>
<td>10(^{-6})</td>
<td>99</td>
<td>96</td>
</tr>
</tbody>
</table>

\(^a\) 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\(^{-3}\) M CaCl\(_2\)) with one change. At the end, the Ca\(^{2+}\) 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.

\(^b\) Ca\(^{2+}\) concentration during growth.

\(^c\) Ca\(^{2+}\) concentration during growth was 10\(^{-3}\) M.
<table>
<thead>
<tr>
<th>Time at 37 °C (hr)</th>
<th>Enzyme activity (units/ml)</th>
<th>TCA\textsuperscript{-}precipitable radioactivity (counts per min per ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>10\textsuperscript{-4} M Ca\textsuperscript{2+}</td>
<td>5 × 10\textsuperscript{-4} M Ca\textsuperscript{2+}</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>

\textsuperscript{a}A concentrated solution of radioactive enzyme C (Ca\textsuperscript{2+} 10\textsuperscript{-3} M) was diluted with 0.1 M Tris-hydrochloride buffer (pH 7.1) either with or without addition of Ca\textsuperscript{2+} 10\textsuperscript{-3} M. This achieved the two [Ca\textsuperscript{2+}] of 10\textsuperscript{-3} M and 5 × 10\textsuperscript{-3} M. All manipulations were performed at 0 °C. At the end of the exposure to 37 °C, the [Ca\textsuperscript{2+}] was raised to 10\textsuperscript{-3} M when necessary, and the samples were conserved at 0 °C until analysis.

\textsuperscript{b}Trichloroacetic acid.
TABLE 7. *Effect of Ficoll on surface denaturation of enzyme C in the presence of Ca\(^{2+}\) \(^a\)*

<table>
<thead>
<tr>
<th>Function measured</th>
<th>Per cent remaining after 5 hr at 37 C</th>
<th>Standing</th>
<th>Shaking</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>No Ficoll</td>
<td>5% Ficoll</td>
</tr>
<tr>
<td>Enzyme activity ............</td>
<td>84</td>
<td>98</td>
<td>50</td>
</tr>
<tr>
<td>TCA(^b)-precipitable radio-activity .................</td>
<td>92</td>
<td>98</td>
<td>75</td>
</tr>
</tbody>
</table>

\(^a\) 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 \(10^{-5}\) M Ca\(^{2+}\). 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).

\(^b\) Trichloroacetic acid.
<table>
<thead>
<tr>
<th>Time</th>
<th>10^{-4} M Ca^{2+} (control)</th>
<th>10^{-4} M Ca^{2+}</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No addition</td>
<td>+ 10^{-4} M citrate</td>
</tr>
<tr>
<td></td>
<td>- Ficoll</td>
<td>+ Ficoll</td>
</tr>
<tr>
<td>0 min</td>
<td>29.2</td>
<td>29.2</td>
</tr>
<tr>
<td>15 min</td>
<td>28.6</td>
<td>28.6</td>
</tr>
<tr>
<td>30 min</td>
<td>28.6</td>
<td>25.2</td>
</tr>
<tr>
<td>1 hr</td>
<td>28.6</td>
<td>8.7</td>
</tr>
<tr>
<td>1 hr 50 min</td>
<td>26.7</td>
<td>0</td>
</tr>
<tr>
<td>3 hr</td>
<td>26.7</td>
<td>0</td>
</tr>
<tr>
<td>24 hr</td>
<td>25.0</td>
<td>0</td>
</tr>
</tbody>
</table>

^a Values are units of milk-coagulating activity per milliliter. A concentrated solution of enzyme C (10^{-3} 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^{-3} M Ca^{2+}. Ficoll concentration was 5%.

^b Ethylenediaminetetraacetic acid.