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STUDIES ON EXTRACELLULAR PROTEOLYTIC ENZYMES FROM *STAPHYLOCOCCUS AUREUS*

II. ISOLATION AND CHARACTERIZATION OF AN EDTA-SENSITIVE PROTEASE

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SUMMARY

An EDTA-sensitive proteolytic enzyme was separated by gel chromatography on Sephadex G-75 from two EDTA-insensitive proteases in culture supernatants of *Staphylococcus aureus*, strain V8. The protease was irreversibly inactivated by EDTA, and was insensitive to sulfhydryl reagents and DFP. Calcium was shown to be essential for the stability of the protease. Optimum pH for hydrolysis of casein was 7.4. The molecular weight was 28 000 as determined by gel chromatography.

INTRODUCTION

The proteolytic activity of the culture fluid of *Staphylococcus aureus*, strain V8 was 50% inactivated by EDTA[1]. The EDTA-insensitive part of the proteolytic activity was shown to consist of two different proteases, protease I and II[1]. These two enzymes have recently been purified and characterized[2]. The EDTA-sensitive portion of the extracellular protease activity was not found in cultures grown in Ca^{2+}-deficient media[1], and it was suggested that this part of the protease activity is the manifestation of a third protease. The present study was undertaken to find out whether the EDTA-sensitive activity is an individual protease or a part of protease I or II. The EDTA-sensitive proteolytic activity will be referred to as protease III.

MATERIAL AND METHODS

Bacterial strain

S. aureus, strain V8. Stock cultures were maintained on nutrient agar slants at 4 °C, and were transferred monthly.

Cultivation conditions

The bacteria were cultivated in 1-l baffled Erlenmeyer flasks on a rotary shaker

(120 rev./min, 50 mm displacement). The culture medium (100 ml) was a casein hydrolysate medium (CC-medium[1]) to which was added Ca^{2+} to a final concentration of 1.0 mM. The cultures were harvested by centrifugation (7000 × *g*, at 4 °C) after 7 h of growth at which time the proteolytic activity in the culture fluid had reached its maximum.

Enzyme assay

The proteolytic activity was determined according to Kunitz[3] modified as previously described[4], using heat denatured casein as the substrate (purchased from Nutritional Biochemicals Corporation, Cleveland, Ohio, U.S.A.) in 0.1 M phosphate buffer (pH 7.5). The hydrolysis was measured as the increase in the absorbance at 280 nm of $HClO_4$-soluble material. Under standard conditions Ca^{2+} (1.0 mM) and cysteine (1.0 mM) were added to the test solution. One unit was defined as the amount of enzyme giving an increase in $A_{280\ nm}$ of 1.0 in 30 min at 37 °C.

The amount of EDTA-sensitive protease was determined by testing each sample before and after treatment by EDTA (5.0 mM) for 30 min at 20 °C[1].

Isolation of protease III

200 ml of culture supernatant was dialyzed against 30 vol. of 1.0 mM $CaCl_2$ for 24 h at 4 °C. The dialyzed material was concentrated by rotor evaporation at 25 °C to a final volume of 5 ml and layered on a column of Sephadex G-75 (2.5 cm × 150 cm). The column was eluted at a rate of 20 ml/h with 0.1 M Tris–HCl buffer, pH 7.2, containing Ca^{2+} at a concentration of 1.0 mM. Samples (6 ml) were collected at 4 °C. The peak of protease III was recycled through the column under identical conditions. The fractions containing protease III, as judged by the sensitivity to EDTA, were combined and used for the characterization studies.

Molecular weight determination

The molecular weight of protease III was determined according to Andrews[5] by gel filtration on Sephadex G-75. The column (2.5 cm × 150 cm) was calibrated with Dextran blue 2000 (Pharmacia, Uppsala, Sweden), α-chymotrypsinogen A, ovalbumin (Sigma Chemicals Co., St. Louis, Mo., U.S.A.) and myoglobin (Nutritional Biochemicals Corporation, Cleveland, Ohio, U.S.A.). The elution buffer was 0.1 M Tris–HCl, pH 7.0, with 1.0 mM Ca^{2+}. The marker proteins were detected by measuring the absorption at 280 nm.

Isoelectric focusing

Isoelectric focusing was carried out in a density gradient (0–50% sucrose) as described by Vesterberg *et al.*[6]. A 110-ml column (LKB Produkter, Bromma, Sweden) was used. The pH gradient was established with Ampholine, pH 3–10 (LKB-Produkter). The experiment was run for 48 h at 500 V and 2–3 mA.

Antiserum

Antiserum was prepared in rabbits as described earlier[2] by intramuscular injection of concentrated culture supernatant with Freund's complete adjuvans.

RESULTS

Purification

About 50% of the proteolytic activity in the starting material was inactivated by EDTA, representing the postulated protease III. The presence of Ca^{2+} throughout the purification was necessary to prevent total inactivation of the enzyme. Dialysis against $CaCl_2$ did not result in any decrease in the proteolytic activity. However, concentration by rotor evaporation decreased the activity of EDTA-sensitive protease by 20%. Also the EDTA-stable part of the proteolytic activity decreased by about 20%. The separation of the concentrated material by gel filtration on Sephadex G-75 is shown in Fig. 1. Three different peaks of proteolytic activity could be demon-

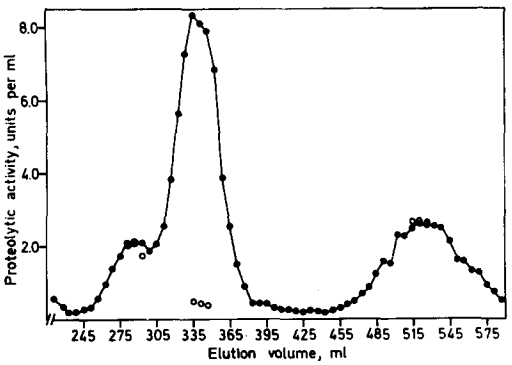


Fig. 1. Gel chromatography of concentrated culture supernatant on Sephadex G-75. ●—●, proteolytic activity; ○—○, proteolytic activity after treatment by 5.0 mM EDTA for 30 min at 20 °C.

strated. By testing the effect of EDTA on each peak, one of them could be identified as the EDTA-sensitive protease III. The other two peaks of protease activity were recognized as protease I and II by their insensitivity to EDTA and by their molecular weights[2]. The total recovery of proteolytic activity was about 50% and the amounts of protease III was only 30% of the original. Protease III was completely devoid of protease II activity, whereas small amounts of protease I appeared in the protease III peak. The contaminating protease I activity was removed by rechromatography of

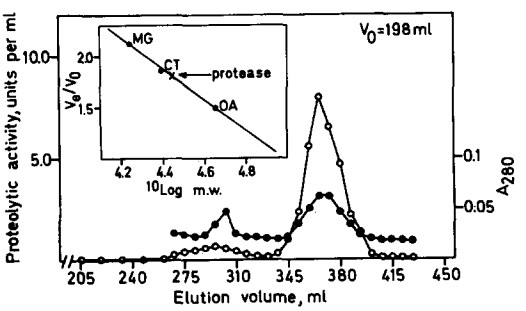


Fig. 2. Rechromatography of protease III on Sephadex G-75. The inserted diagram shows the relation of the elution volumes (V_e) of the marker proteins and their molecular weights. MG, myoglobin; CT, α-chymotrypsin; OA, ovalbumin.

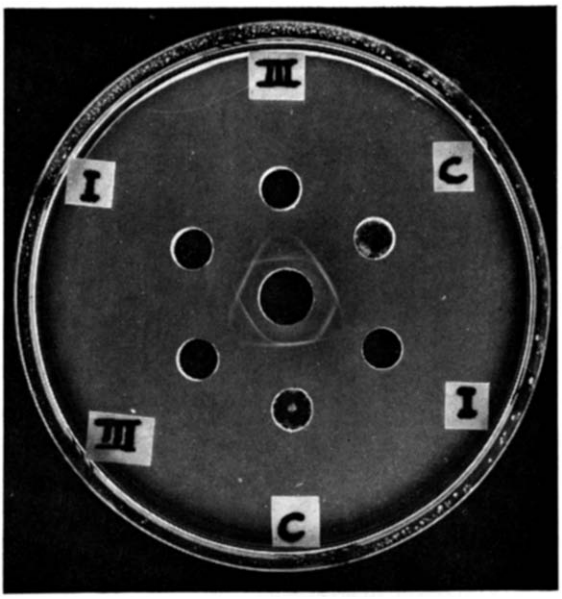


Fig. 3. Immunodiffusion analysis of purified protease III. The central well containing antiserum against crude culture supernatant surrounded by: C, concentrated culture supernatant; III, purified protease III; and I, purified protease I, from the same strain of *S. aureus*.

the middle fractions of the protease III peak (Fig. 2). The overall recovery was about 30% and the specific activity of the purified enzyme was 120 units/mg of protein. The purified enzyme gave only one band of precipitation in immunodiffusion according to Ouchterlony with serum prepared with crude culture filtrate (Fig. 3).

Properties of the purified protease

Molecular weight determinations according to Andrews[5] gave a value of 28 000 ± 800 (Fig. 2).

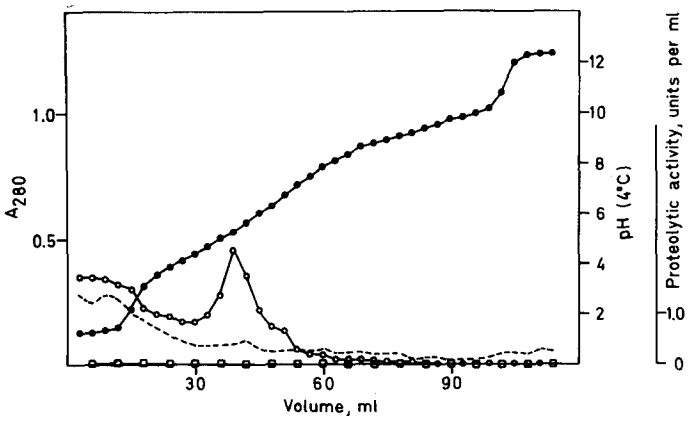
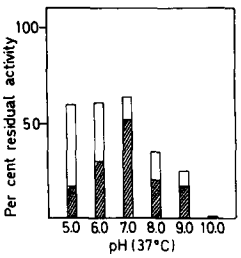


Fig. 4. Isoelectric focusing of purified protease III. □—□, proteolytic activity; ●—●, pH at 4 °C; ○—○, absorbance at 280 nm; — — —, absorbance at 280 nm in a control experiment run without the enzyme.



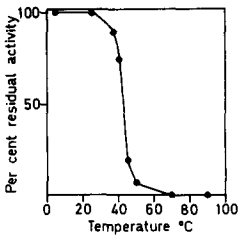
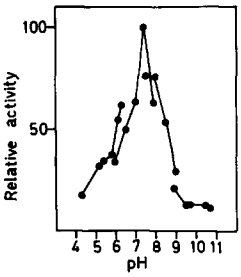


Fig. 5. Influence of pH on the stability of protease III at 37 °C. The enzyme was dissolved in 0.1 M buffers of different pH. Succinate (pH 5.0–6.0), Tris–maleate (pH 5.0–8.0) and boric acid–borax (pH 8.0–10.0). The bars represents the remaining activity after 24 h incubation in the presence of Ca^{2+} (1.0 mM). The hatched areas represents the activity of samples incubated without added Ca^{2+}.

Fig. 6. Stability of protease III at different temperatures. The enzyme was incubated for 30 min at the temperatures indicated in 0.1 M Tris–HCl buffer (pH 7.0).

Isoelectric focusing of the purified protease resulted in total inactivation. The inactive protease focused at pH 5.0 (Fig. 4). Attempts to reactivate the protease by dialysis against 10 mM Ca^{2+} in 0.1 M Tris–HCl buffer (pH 7.0) were without success.

The stability of the enzyme at different pH at 37 °C is represented in Fig. 5. At pH 5.0 and at pH values above 8.0 only 20% of the activity remained after 3 h while at pH 7.0 the inactivation was only 50%. Ca^{2+} showed a marked protective effect on the protease at pH below 7.0. At pH 5.0, 60% of the activity remained after 3 h in the presence of Ca^{2+} compared to 17% in the absence of these ions. Heat treatment of the enzyme in 0.1 M phosphate buffer (pH 7.0) for 30 min at varying temperatures indicated that the enzyme retained its full activity up to 30 °C, but lost the activity almost completely above 50 °C (Fig. 6).



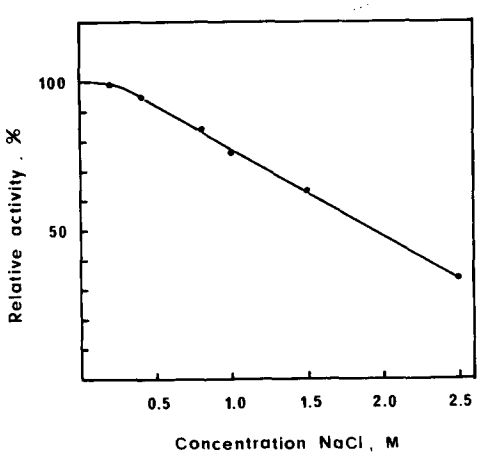


Fig. 7. Influence of pH on the hydrolysis of casein catalyzed by protease III. The following buffers were employed in the assay system at a concentration of 0.1 M: succinate (pH 4.3–6.5); phosphate (pH 6.0–8.0); boric acid–borax (pH 7.6–9.0); and borax–NaOH (pH 9.2–11.0).

Fig. 8. Influence of NaCl concentration on the enzymatic activity. Protease activity was determined under standard conditions in the presence of NaCl at the concentrations indicated.

Enzymatic activity

The enzymatic activity was determined as a function of pH. As illustrated in Fig. 7 the optimum is at pH 7.5. The influence of ionic strength on the enzymatic activity is illustrated in Fig. 8. The presence of NaCl at concentrations above 0.1 M decreased the enzyme activity.

TABLE I

EFFECT OF DIVALENT CATIONS ON THE ENZYMATIC ACTIVITY

Protease III was dialyzed against 1000 vol. of distilled water for 18 h at 4 °C and the enzymatic activity was determined in 0.1 M phosphate buffer (pH 7.5) in the presence of each metal at a concentration of 1.0 mM. The metals were added as the chloride form.

Addition	*Relative activity (%)*
None	100
Hg^{2+}	88
Pb^{2+}	45
Zn^{2+}	160
Mg^{2+}	174
Mn^{2+}	63
Ca^{2+}	208
Cu^{2+}	36

TABLE II

EFFECTS OF SOME GROUP-SPECIFIC REAGENTS ON THE ENZYMATIC ACTIVITY

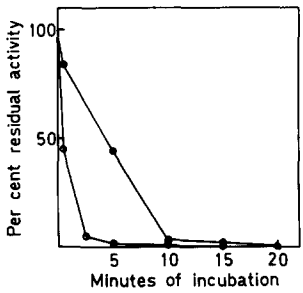
Each compound was added to samples of protease III diluted in 0.1 M phosphate buffer (pH 7.0). The mixture, after kept standing at 20 °C for 30 min, was assayed for proteolytic activity by the standard method.

Addition	*Relative activity (%)*
None	100
Na_2SO_3 (10.0 mM)	105
Sodium thioglycollate (10.0 mM)	105
Cysteine (1.0 mM)	100
2-Mercaptoethanol (10.0 mM)	98
DFP (1.0 mM)*	100
Sodium citrate (10.0 mM)	12
EDTA (1.0 mM)	1
1,10-phenanthrolene (1.0 mM)	88
1,10-phenanthrolene (10.0 mM)	70

* Fluka AG, Buchs, Switzerland.

The effects of some divalent cations and group-specific reagents on the enzymatic activity are summarized in Tables I and II. The enzyme preparation used in these experiments was dialyzed against distilled water for 18 h at 4 °C. The presence of Ca^{2+} increased the activity by 100%. Ca^{2+} could be partly replaced by Mg^{2+} and Zn^{2+}. Pb^{2+}, Mn^{2+} and Cu^{2+} decreased the activity below the value of the control. Protease III was not affected by reducing agents or DFP (diisopropylfluorophosphate, purchased from Fluka AG, Buchs, Switzerland). The enzyme was completely inactivated by sodium citrate and EDTA, which preferably bind to Ca^{2+}. 1,10-Phenanthrolene reduced the activity by only 20% (Table II). The presence of Ca^{2+} in the

assay system did not reverse the inactivation by EDTA or sodium citrate, nor did dialysis of the EDTA-inactivated enzyme for 24 h against $CaCl_2$ (10 mM) result in any reactivation. However, the addition of Ca^{2+} to the enzyme before the addition of EDTA prevented inactivation or increased the amounts of EDTA required for inactivation. The inhibitory effect of EDTA (5 mM) as a function of time at 37 °C, indicated that the inactivation was complete in less than 5 min (Fig. 9). The addition of Ca^{2+} or Zn^{2+} immediately after the addition of EDTA did not prevent the inactivation. Fig. 10 shows the effect of increasing concentrations of EDTA on the proteolytic activity. The enzyme (40 units/ml) was 100% inactivated by EDTA at a concentration of 0.2 mM.



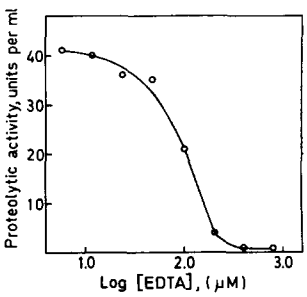


Fig. 9. Rate of EDTA inactivation of protease III at 4 and 37 °C. EDTA was added at a concentration of 5.0 mM to the enzyme dissolved in 0.05 M Tris–HCl buffer (pH 7.0). Samples were removed at intervals and immediately assayed for proteolytic activity under standard conditions. ●—●, 4 °C; ○—○, 37 °C.

Fig. 10. Inactivation of protease III by EDTA. After dialysis against distilled water for 18 h at 4 °C samples of enzyme (40 units/ml) were treated by EDTA at varying concentrations for 30 min at 20 °C. Proteolytic activity was determined under standard conditions in the presence of Ca^{2+}.

DISCUSSION

The present study gives final proof to the earlier suggestion that the EDTA-sensitive part of the proteolytic activity in culture supernatants from *S. aureus*, strain V8, is the manifestation of an individual protease separate from protease I and II[1]. Besides its sensitivity to EDTA, protease III differs from protease I and II in regard to its molecular weight, (28 000 for protease III as compared to 21 000 (monomer) or 43 000 (dimer) for protease I and 12 500 for protease II), heat stability, stability at different pH, sensitivity to divalent metal ions and sulfhydryl reagents[2].

Protease III is a very unstable enzyme. It could not be isolated in the absence of added Ca^{2+}. Gel filtration on Sephadex G-75 of crude culture supernatants of strain V8, using phosphate buffer as the elution liquid, resulted in the complete inactivation of protease III[2]. The total inactivation of the protease in isoelectric focusing was presumably due to a combination of the instability of the enzyme at acidic pH and the removal of Ca^{2+} by the Ampholine carrier ampholytes which may act as complexing agents for metal ions[7].

The fact that protease III is inactivated by low concentrations of EDTA suggests that it is a metalloenzyme. Several microbial proteases have been shown

to be metalloenzymes, most of which are zinc containing[8]. The most thoroughly studied of these zinc-metalloenzymes are the neutral proteases from different *Bacillus* species, which are all stabilized by Ca^{2+} (refs. 9–13). The stabilizing effect of Ca^{2+} has also been reported for several other proteases[14–18].

The fact that the addition of excess metal ions to the EDTA-treated enzyme did not reverse the inactivation, indicates that Ca^{2+} is removed and presumably irreversible changes occur at the ligand site. It is not very likely that the irreversible inactivation of protease III by EDTA is due to autolysis, since the inhibition was almost instantaneous. The present results suggest that Ca^{2+} is essential for the maintainance of an active conformation of the enzyme and the enzyme should consequently be classified as a calcium metalloenzyme.

Although several proteases are stabilized by calcium the only well-known calcium-metalloenzyme is the peptide peptidohydrolase from *Pseudomonas aeruginosa* which was shown to contain 1–2 moles of tightly bound Ca^{2+} per mole of enzyme[19]. The properties of an extracellular protease from a *Cytophaga* sp. reported by Christison and Martin[17] greatly suggests that this protease is also a calcium-metalloenzyme. Of particular interest is the comparison of protease III with other proteases from *micrococci*. One extracellular protease from *Micrococcus caseolyticus* was studied by Desmazeaud and Hermier[18]. This enzyme was 100% inactivated by EDTA and was partially reactivated by Ca^{2+}. The pH optimum was the same as for protease III (pH 7.4) and the molecular weight was 38 000 determined by gel chromatography. One protease of *Sarcina*, Coccus P, which was found only when Ca^{2+} was present in the medium[20], was inactivated by EDTA and was shown to have a molecular weight of approximately 31 000 (ref. 20) which is in close agreement with that of protease III. It was stated that the function of calcium in Coccus P was to stabilize the active structure of the enzyme molecule by preventing autolysis[2].

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REFERENCES

1 Arvidson, S., Holme, T. and Lindholm, B. (1972) *Acta Pathol. Microbiol. Scand. Sect. B* 80, 835–844
2 Arvidson, S. and Holme, T. (1973) *Biochim. Biophys. Acta* 302 135–148
3 Kunitz, M. (1946/47) *J. Gen. Physiol.* 30, 291–310
4 Arvidson, S., Holme, T. and Wadström, T. (1971) *Acta Pathol. Microbiol. Scand. Sect. B* 79, 399–405
5 Andrews, P. (1964) *Biochem. J.* 91, 222–233
6 Vesterberg, O., Wadström, T., Vesterberg, K., Svensson, H. and Malmgren, B. (1967) *Biochim. Biophys. Acta* 133, 435–445
7 Davies, H. (1970) *Protides of the Biological Fluids, Proc. 17th Colloq. Bruges, 1969*, pp. 389–396, Pergamon Press, Oxford and New York
8 Keay, L. (1971) *Process Biochem.* 6, 17–21
9 Feder, J., Keay, L., Garrett, L. R., Cirulius, N., Moseley, M. H. and Wildi, B. S. (1971) *Biochim. Biophys. Acta* 251. 74–78

10 Feder, J., Garrett, L. R., and Wildi, B. S. (1971) *Biochemistry* 10, 4552–4556
11 Keay, L., Feder, J., Garrett, L. R., Moseley, M. H. and Cirulius, N. (1971) *Biochim. Biophys. Acta* 229, 829–835
12 McConn, J. D., Tsuru, D., and Yasunobo, K. T. (1964) *J. Biol. Chem.* 239, 3706–3715
13 Millet, J. (1969) *Bull. Soc. Chim. Biol.* 51, 61–68
14 Morihara, K. (1963) *Biochim. Biophys. Acta* 73, 113–124
15 Nomoto, M., Narahashi, Y., and Murakami, M. (1960) *J. Biochem. Tokyo* 48, 453–463
16 Sarner, N. Z., Bissell, M. J., di Girolamo, M. and Gorini, L. (1971) *J. Bacteriol.* 105, 1090–1098
17 Christison, J. and Martin, S. M. (1971) *Can. J. Microbiol.* 17, 1207–1216
18 Desmazeaud, M. and Hermier, J. (1968) *Ann. Biol. Anim. Biochem. Biophys.* 8, 565–577
19 Morihara, K. and Tsuzuki, H. (1964) *Biochim. Biophys. Acta* 92, 351–360
20 Gorini, L. (1950) *Biochim. Biophys. Acta* 6, 237–255
21 Bissell, M. J., Tosi, R. and Gorini, L. (1971) *J. Bacteriol.* 105, 1099–1109