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STUDIES ON EXTRACELLULAR PROTEOLYTIC ENZYMES FROM *STAPHYLOCOCCUS AUREUS*

I. PURIFICATION AND CHARACTERIZATION OF ONE NEUTRAL AND ONE ALKALINE PROTEASE

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SUMMARY

Two extracellular proteolytic enzymes from *Staphylococcus aureus*, strain V8, were separated by isoelectric focusing. One protease, with an isoelectric point at pH 4.0 (protease I) was purified 185-fold by negative adsorption on CM-Sephadex, isoelectric focusing and gel chromatography on Sephadex G-75. The molecular weight of this enzyme was about 21 000. The pH-optimum was at 7.8 using casein as the substrate, and at 5.0 and 7.0–9.0 using hemoglobin as the substrate. The activity of protease I was not significantly affected by divalent cations, metal-chelating agents, sulfhydryl reagents, or by diisopropylfluorophosphate.

The other protease with an isoelectric point at 9.4 (protease II) was purified 310-fold by negative adsorption on DEAE-Sephadex, isoelectric focusing and gel chromatography on BioGel P 60. The molecular weight was 12 500. The pH optimum was at pH 8.8. Protease II was only active in the presence of reducing agents, and was inactivated by heavy metals. Protease II exhibited esterase activity, using *N*-benzoyl-L-tyrosine ethyl ester as the substrate.

INTRODUCTION

The ability to hydrolysate casein or liquefy gelatin is widely distributed among members of the family *Micrococcaceae*. Extracellular proteases are produced by the majority of the strains in all groups of staphylococci according to Baird-Parker[1]. It has been suggested by Shooter and Wyatt[2] and by McDonald and Chambers[3] that the function of extracellular proteases from staphylococci and micrococci were to ensure the supply of amino acids for growth and synthesis.

In a previous paper the identification of three different proteolytic enzymes in culture supernatants from *Staphylococcus aureus*, strain V8, was reported[4]. One enzyme was completely inactivated by EDTA and could not be found after isoelectric

Abbreviation: BTEE, *N*-benzoyl-L-tyrosine ethyl ester.

focusing of the culture supernatant. The other two proteases were separated by isoelectric focusing. They focused at pH 4.0 and 9.4. Before further studies on the mechanisms of their formation and biological effects could be performed, the different proteases had to be purified and characterized.

The present paper describes the purification and characterization of the two EDTA-stable enzymes, which will be referred to as protease I (pI 4.0) and protease II (pI 9.4).

MATERIALS AND METHODS

Bacterial strain

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

Cultivation conditions

A detailed description of cultivation techniques has been given earlier[5]. The bacteria were grown in a casein hydrolysate–yeast extract medium (CCY_I-medium[6]) in a stirred fermentor with a working volume of 2.5 l. The cultivation time was 6 h after which the cultures were harvested by centrifugation (700 × *g*, at 4 °C).

Purification

The culture supernatant (4500 ml) was dialyzed by a continuous process described by Wadström and Vesterberg[7]. For the purification of protease I, 5 g of CM-Sephadex C 25, swelled in 0.02 M phosphate buffer, pH 6.5, was added per l of dialyzed culture supernatant. For the purification of protease II the same amounts of DEAE-Sephadex A 25 swelled in 0.02 M Tris–HCl buffer, pH 8.5, was used. After gentle stirring for 2 h at 4 °C the Sephadex grains were allowed to settle, and the supernatants were withdrawn. After negative absorption to the ion exchangers the preparations were concentrated by rotary evaporation at 30 °C, to a final volume of 200 ml. The concentrated materials were dialyzed against 0.5% glycine and subjected to isoelectric focusing in density gradient as described earlier by Vesterberg *et al.*[8]. A 450-ml column equipped with a cooling jacket was used (LKB-Produkter, S-161 25 Bromma-Stockholm, Sweden). Carrier ampholytes, Ampholine, pH 3.0–10.0 (LKB) were used to establish the pH gradient. The focusing experiments were run for 48 h at 4 °C with a potential of 500–600 V. The contents of the columns were collected in 10-ml fractions and assayed for proteolytic activity after pH determination of each fraction.

The fractions from the isoelectric focusing experiment containing Protease I, were combined and dialyzed against 40 vol. of distilled water for 24 h. The dialysed material was concentrated to a volume 3 ml by powdered polyethyleneglycol 20 M. This was layered on a column of Sephadex G-75 (2.5 cm × 150 cm). The enzyme was eluted from the column with 0.3 M NaCl in 0.1 M phosphate buffer, pH 7.0 (20 ml/h). Samples (7.5 ml) were collected at 4 °C and assayed for proteolytic activity and the absorption at 280 nm of each fraction was measured in a Zeiss spectrophotometer model PMQ II. The peak of proteolytic activity was recycled through the column as described above. This purified preparation was used for the characterization studies.

The proteolytic activity found in the alkaline region of the column after isoelectric focusing was dialyzed against distilled water to remove the sucrose and the carrier ampholytes, and was then concentrated by polyethyleneglycol to a final volume of 3 ml and applied to a column of Bio Gel P 60 (2.5 cm × 110 cm). The enzyme was eluted from the column with 0.2 M ammonium acetate buffer, pH 7.0, at a rate of 20 ml/h. Fractions (7.5 ml) were collected at 4 °C and assayed for proteolytic activity. The fractions containing maximal proteolytic activity were combined and lyophilized. The dried material was dissolved in 2.5 ml of ammonium acetate, pH 7.0, and recycled through Bio Gel P 60 as described above. This purified enzyme was used for the characterization.

Determination of molecular weights by gel chromatography

The molecular weights of the enzymes were estimated according to Andrews[9] by gel chromatography on Sephadex G-75. The void volume of the column (2.5 cm × 150 cm) was determined with Blue Dextran 2000 (Pharmacia, Uppsala, Sweden). Cytochrome *c*, α-chymotrypsinogen A, ovalbumin, (purchased from Sigma Chemical Co., St. Louis, Mo., U.S.A.) and myoglobin (Nutritional Biochemicals Corporation, Cleveland, Ohio, U.S.A.) were used for calibration of the column.

Enzyme assay

Proteolytic activity was determined according to Kunitz[10] as previously described[5] using heat-denatured casein as a substrate. The standard assay procedure was carried out in 0.1 M phosphate buffer, pH 7.5, in the presence of 1.0 mM $CaCl_2$ and 1.0 mM cysteine.

Heat-denatured hemoglobin (1% w/v) was used as the substrate under test conditions identical to those described for casein.

Esterase activity was determined according to Hummel[11], using *N*-benzoyl-L-tyrosine ethyl ester (BTEE, purchased from Fluka AG, Buchs, Switzerland) as a substrate. Hydrolysis of the ester bond was followed by the increase in absorbance at 256 nm, using a Beckman DBG recording spectrophotometer. The assay was carried out at pH 7.8, in the presence of 0.01 M $CaCl_2$. One unit of activity is equal to the hydrolysis of 1 μmole of substrate per min.

Protein determination

The protein contents of samples taken during the purification procedure were estimated with the Folin reagent according to Lowry *et al.*[12]. The samples were dialyzed against distilled water for 24 h before the assay.

Antiserum was produced in rabbits. 5-fold concentrated culture supernatant was dialysed against 0.9% NaCl and mixed with equal amounts of Freund's complete adjuvans (Difco). 2 ml was injected intramuscularly at 7-day intervals. Three days after the third injection the blood serum was tested for specific anti-proteases.

RESULTS

Purification

The two proteases studied constitute about 40% of the total proteolytic activity in culture supernatants from *S. aureus*, strain V8 when grown in the CCY_I-

TABLE I

PURIFICATION OF PROTEASE I AND II

Fraction	*Total activity (units)*		*Total protein (mg)*		*Spec. act. (units/mg protein)*		*Yield (%)*		*Purification*	
	Protease I	*Protease II*	*Protease I*	*Protease II*	*Protease I*	*Protease II*	*Protease I*	*Protease II*	*Protease I*	*Protease II*
Culture supernatant (4500 ml)	4500	13 500	2800	2800	1.6*	4.8*				
Isoelectric focusing	625	4 554	39	61	16	74	14	33	10	15
First gel filtration **	597	1 880	2.5	3.0	235	617	13	14	147	128
Second gel filtration**	298	1 690	1.0	1.12	298	1500	7	12.5	186	310

* Figures based on the rough estimation of the proportion of the different proteases in the crude culture supernatant.
** For protease I, Sephadex G-75 was used, and for protease II Biotec R60.

medium[4]. The rest of the proteolytic activity was supposed to be a third protease, which was inactivated by EDTA and which could not be found after isoelectric focusing of the crude culture supernatant. The gradual decrease in proteolytic activity during the purification steps prior to the gel filtration was mainly due to the inactivation of the EDTA-sensitive Protease III.

After dialysis and adsorption to CM-Sephadex of the culture fluid, about 65% of the initial proteolytic activity remained in the supernatant. Isoelectric focusing of this material resulted in a 80% loss of the proteolytic activity, which appeared in two different peaks, one at pH 4.0 (protease I) and the other at pH 9.4 (protease II) (Fig. 1).

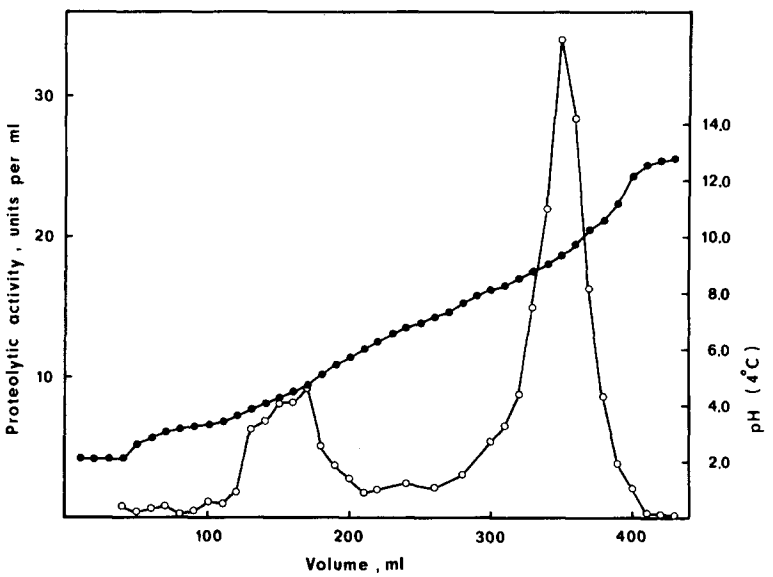


Fig. 1. Isoelectric focusing of CM-Sephadex treated culture supernatant. ○—○, proteolytic activity; ●—●, pH.

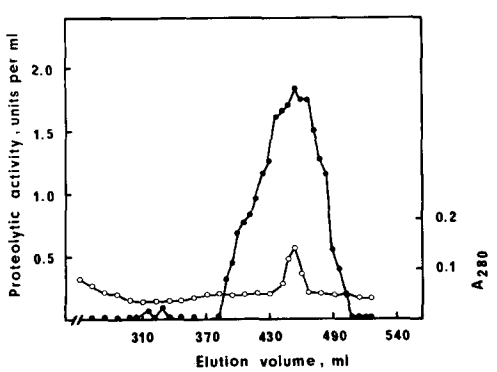


Fig. 2. Recycling of protease I through a Sephadex G-75 column (2.5 cm × 150 cm). The column was eluted with 0.3 M NaCl in 0.1 M phosphate buffer (pH 7.0). The flow rate was 20 ml/h. ●—●, proteolytic activity; ○—○, $A_{280\ nm}$.

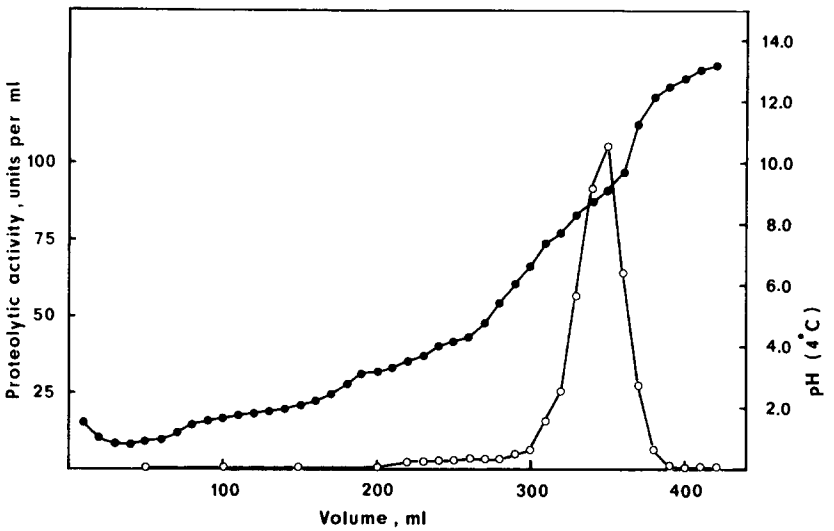


Fig. 3. Isoelectric focusing of DEAE-Sephadex treated culture supernatant. ○—○, proteolytic activity; ●—●, pH.

Gel chromatography on Sephadex G-75 of protease I, prepared by isoelectric focusing, resulted in a 15-fold purification of the enzyme (Table I). Fig. 2 illustrates the elution pattern of protease I after recycling through the Sephadex G-75 column. The rechromatography increased the specific activity of the enzyme from 235 units/mg of protein to 300 units/mg. After the recycling of protease I only about 50% of the activity recovered after the isoelectric focusing was left.

DEAE-Sephadex treatment of the culture supernatant removed considerable amounts of acidic material, including protease I. The total proteolytic activity decreased to about 50%. The application of the DEAE-Sephadex treated material to isoelectric focusing decreased the proteolytic activity to about 10% of the original. The proteolytic activity (protease II) appeared in one single peak at pH 9.4 (Fig. 3).

Gel chromatography on Bio Gel P 60 of protease II resulted in an 8-fold purification (Table I). Rechromatography of this protease increased the specific activity 2.4-fold, *i.e.* a 20-fold purification of the material obtained by isoelectric focusing. Fig. 4 represents the elution pattern after recycling of protease II through the Bio Gel P 60 column. One single peak of protein was observed which exhibited the protease activity. The recovery was about 90%. The specific activity of the purified protease II was about 1500 units/mg of protein. Chromatography of protease II on a Sephadex column resulted in 50% loss of the proteolytic activity.

The proportion of the three proteases, I:II:III, in the crude culture supernatants was approximately 1:3:6. The amounts of the individual proteases in mixed samples could be estimated thanks to their different sensitivity to EDTA and cystein (see below). Only protease III was inactivated by EDTA and both protease I and III showed full activity in the absence of cystein while protease II was inactive. The activity of protease I per mg of protein in the crude culture supernatant was approximately 2 units and for protease II about 6 units. Based on these figures a rough estimate of the degree of purification for protease I was 150 times and for protease II 250 times (Table I).

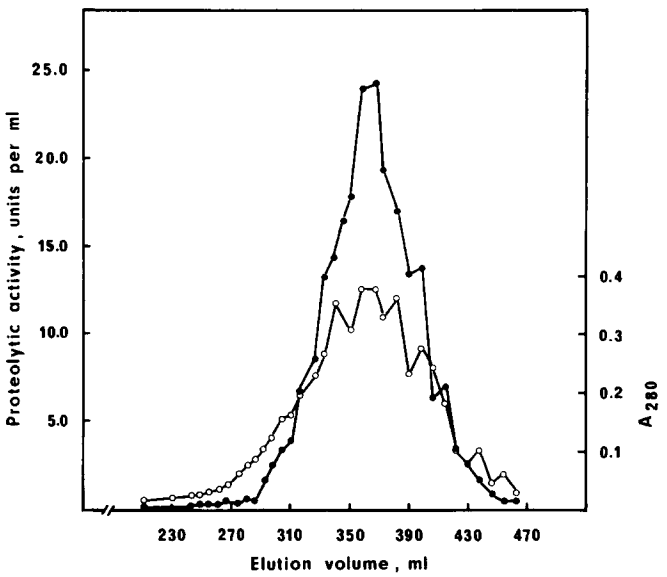


Fig. 4. Recycling of protease II through a BioGel P 60 column (2.5 cm × 110 cm). The column was eluted with 0.2 M ammonium acetate buffer (pH 7.0). The flow rate was 20 ml/h. ●—●, proteolytic activity; ○—○, $A_{280\ nm}$.

The purified enzymes gave only one band of precipitation in immunodiffusion (Fig. 5). No reaction of identity was seen between the two proteases. Four bands of precipitation were visible with 20-times concentrated culture supernatant.

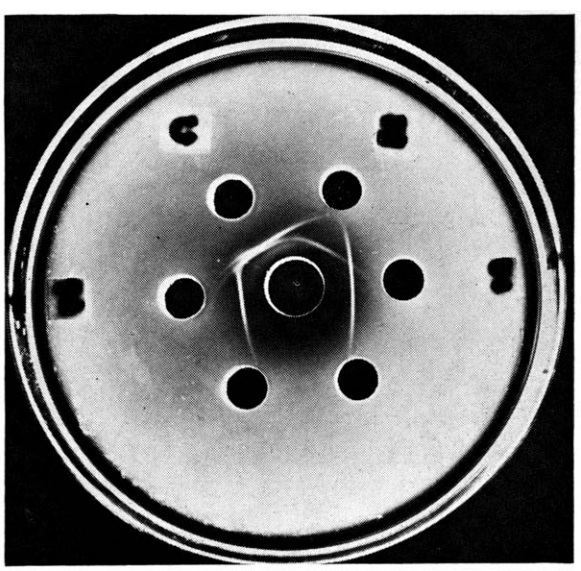


Fig. 5. Immunodiffusion analysis of purified protease I and II. Central well containing antiserum against concentrated culture supernatant. C: 20-fold concentrated culture supernatant.

Molecular weights

Gel chromatography on Sephadex G-75 of a 20-fold concentrated culture supernatant yielded two separate peaks of proteolytic activity which were identified as protease I and protease II by isoelectric focusing. The elution volumes of these two peaks correspond to a molecular weight of 45 000 for protease I and 12 500 for protease II. The determination of the molecular weights of the purified enzymes, using a buffer of a higher ionic strength for the elution, resulted in the same value for protease II, whereas the molecular weight of protease I was shown to be about 21 000.

Stability of the enzymes

Protease I was stable for 3 h at pH values between 4.0 and 11.0 at 37 °C (Table II). Heat treatment of the enzyme in 0.1 M phosphate buffer (pH 7.0) for 30 min at varying temperatures indicated that protease I retained its full activity up to 45 °C (Fig. 6). Complete inactivation was seen at 90 °C.

Incubation of protease II for 3 h at pH 7.0 resulted in a 50% inactivation of the enzyme (Table II). At alkaline pH the stability was much reduced. Only 20% of the activity remained after 3 h at pH 10.0.

Incubation of protease II at various temperatures showed that treatment for 30 min at 50 °C reduced the activity by 90% (Fig. 7).

TABLE II

INFLUENCE OF pH ON THE STABILITY AT 37 °C OF PROTEASE I AND II

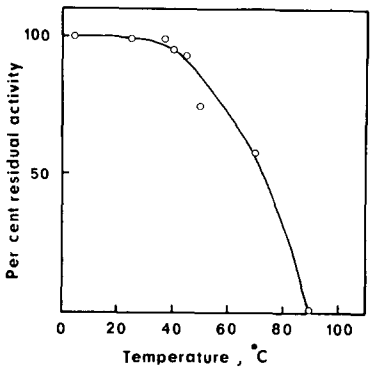
The enzymes were incubated at 37 °C in the following buffers (0.1 M), citrate–phosphate (pH 4.0–5.0); phosphate (pH 6.0–8.0); boric acid–borax (pH 9.0–10.0). At the end of 3 h the proteolytic activity was determined at pH 7.5.

pH	*Residual activity %*	
	Protease I	*Protease II*
4.0	99	30
5.0	100	40
6.0	97	49
7.0	97	53
8.0	100	26
9.0	100	26
10.0	100	20

Enzymic activity of the purified proteases

The hydrolytic activity of protease I and II was determined at 37 °C as a function of pH using casein and hemoglobin as substrates. As illustrated in Fig. 8 protease I was active against casein in the slightly alkaline region with an optimum at 7.8, whereas two optima were observed using hemoglobin as the substrate, one around pH 8.0 and the other at pH 5.0 (Fig. 9). Protease II showed maximum activity at pH 8.8 with casein and at 8.0 with hemoglobin (Fig. 10).

The effects of some divalent cations and group specific reagents on the enzymatic activity are summarized in Tables III and IV. Protease I was slightly stimulated by the presence of Mg^{2+}, Co^{2+}, Ca^{2+} and Pb^{2+}. This enzyme was not significantly



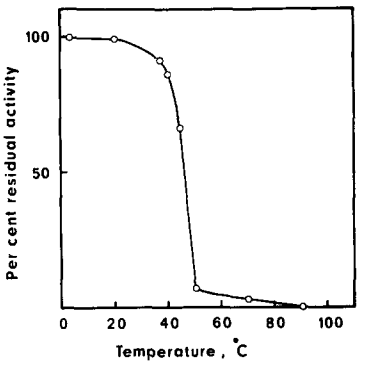


Fig. 6. Stability of protease I at different temperatures. Aliquots of the purified enzyme in 0.1 M phosphate buffer (pH 7.0) were incubated for 30 min at the temperatures indicated.

Fig. 7. Stability of protease II at different temperatures. Aliquots of the purified enzyme in 0.1 M phosphate buffer (pH 7.0) were incubated for 30 min at the temperatures indicated.

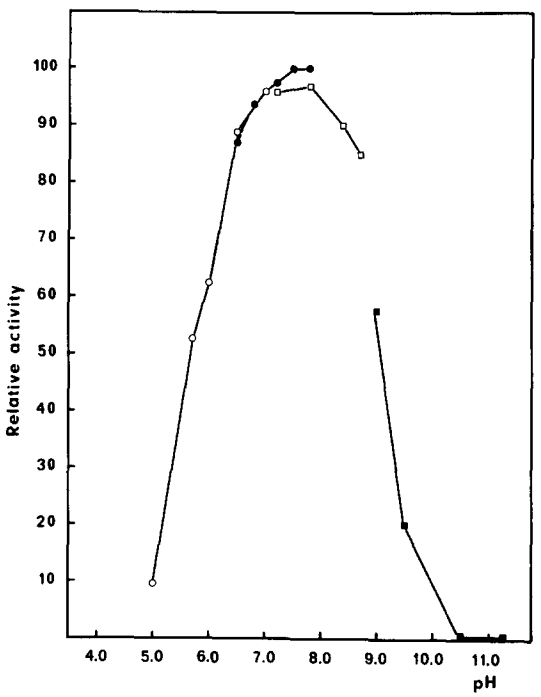


Fig. 8. pH dependence of hydrolysis of casein catalyzed by protease I. The following buffers were employed in the assay system at a concentration of 0.1 M: citrate–phosphate (○—○) (pH 5.0–7.0); phosphate (●—●) (pH 6.0–8.0); boric acid–borax (□—□) (pH 7.6–9.0); borax–NaOH (■—■) (pH 9.2–11.3).

affected by reducing, -SH-inactivating, metal-complexing agents or diisopropyl-fluorophosphate (DFP).

Protease II was inactive in the presence of Co^{2+}, Hg^{+}, Ag^{2+} and Zn^{2+} at a concentration of 1.0 mM. Also Cu^{2+} and Mn^{2+} reduced the activity of protease II, but only by 50%. Protease II was only active in the presence of reducing agents such

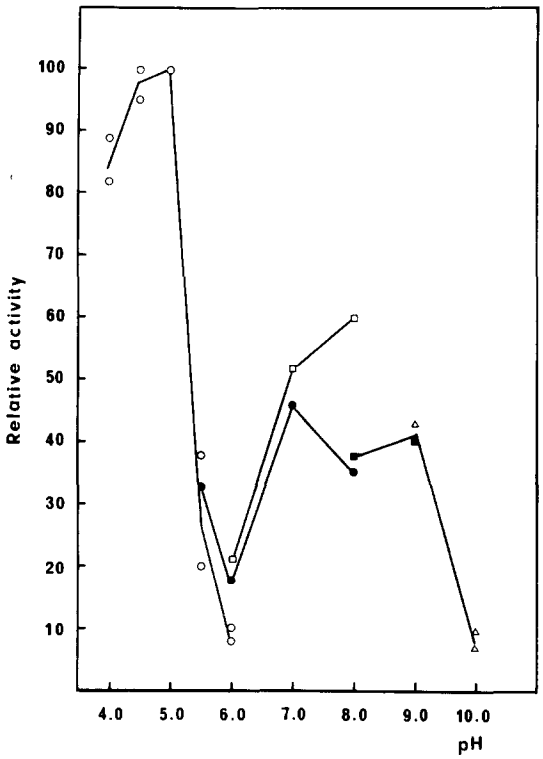


Fig. 9. pH dependence of hydrolysis of hemoglobin catalyzed by protease II. The following buffers were employed in the assay system at a concentration of 0.1 M: succinate (○—○) (pH 4.0–6.0); Tris–maleate (●—●) (pH 5.5–8.0); phosphate (□—□) (pH 6.0–8.0); boric acid–borax (■—■) (pH 7.6–9.0); borax–NaOH (△—△) (pH 9.2–11.3).

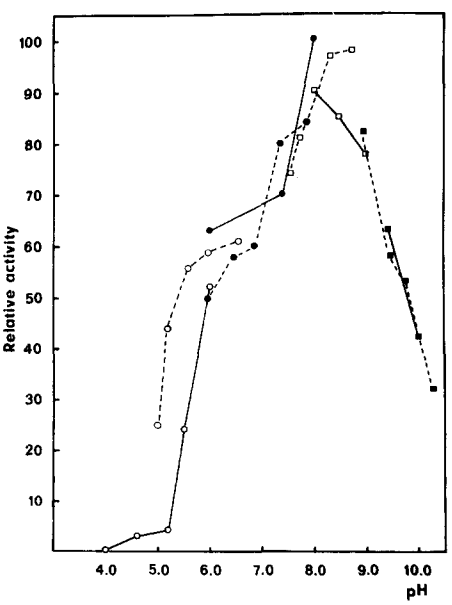


Fig. 10. pH dependence of hydrolysis of casein (———) and hemoglobin (– – –) catalyzed by protease II. The following buffers were employed in the assay system at a concentration of 0.1 M: citrate–phosphate (○—○) (pH 4.0–6.6); phosphate (●—●) (pH 6.0–8.0); boric acid–borax (□---□) (pH 7.6–9.0); borax–NaOH (■---■) (pH 9.2–10.5).

TABLE III

EFFECT OF DIVALENT CATIONS ON THE ENZYMATIC ACTIVITY

The enzymes were dialyzed thoroughly against distilled water at 4 °C. The enzymatic activity was determined in the presence of the metal ions at a concentration of 1.0 mM. All metals were added as the chloride form except for Ag^+ which was in the nitrate form.

Addition	*Relative activity (%)*	
	Protease I	*Protease II*
None	100	100
Hg^{2+}	105	6
Pb^{2+}	122	116
Ag^+	110	8
Zn^{2+}	106	10
Mg^{2+}	126	117
Mn^{2+}	114	61
Ca^{2+}	120	126
Co^{2+}	128	0
Cu^{2+}	104	47

TABLE IV

EFFECTS OF SOME GROUP-SPECIFIC REAGENTS ON THE ENZYMATIC ACTIVITY OF PROTEASE I AND II

The activity of the enzymes was determined in the presence of each reagent at the concentrations given in the parenthesis. The influence of DFP was tested by incubation of the enzymes at 20 °C for 30 min in the presence of the reagent at a concentration of 1.0 mM.

Addition	*Relative activity (%)*	
	Protease I	*Protease II*
None	100	5
Cysteine (1.0 mM)*	100	100
2-Mercaptoethanol (10.0 mM)	96	104
Sodium thioglycollate (10.0 mM)	92	36
Na_2SO_3 (10.0 mM)	119	15
EDTA (5.0 mM)	100	1
Sodium citrate (10.0 mM)	100	5
Sodium citrate + cysteine	100	89
EDTA + cysteine	100	93
DFP (1.0 mM)	90	70

* *I.e.* standard conditions.

as cysteine, 2-mercaptoethanol and sodium thioglycollate. Sodium citrate, EDTA and Na_2SO_3 had no apparent effect on the activity. Incubation with DFP (1.0 mM) for 30 min at 20 °C reduced the activity of protease I by 10% and the activity of protease II by 30%.

Fig. 11 shows that the activity of both protease I and protease II was reduced by the presence of NaCl. 50% reduction of the activity was obtained at a salt concentration of 0.3 M for protease I and 0.5 M for protease II.

Esterase activity of protease I and II

Protease I exhibited no esterase activity using BTEE as the substrate. The

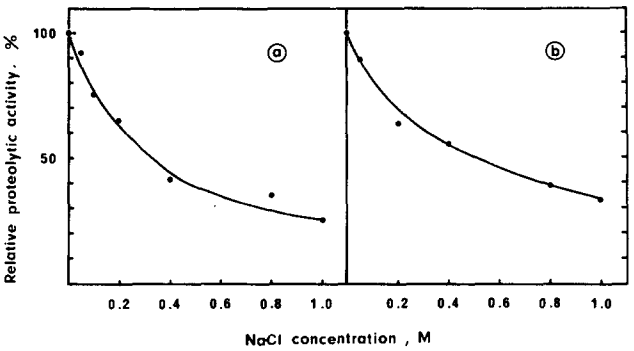


Fig. 11. Influence of NaCl on the enzymatic activity of protease I (a) and protease II (b). Proteolytic activity was determined under standard conditions in the presence of NaCl at the concentrations indicated.

purified protease II hydrolyzed BTEE at a rate corresponding to a specific activity of 8 units/mg of protein (hydrolysis of 8 μmoles of BTEE per min).

DISCUSSION

The presence of three different proteases in culture supernatants from *S. aureus*, strain V8 was reported earlier[4]. In the present work, two proteolytic enzymes were separated by isoelectric focusing and gel chromatography. The purified enzymes differed by both physicochemical and antigenic properties. Although there is good evidence for protease III being a separate protease and not only an EDTA-sensitive portion of protease I or II, it has to be separated from these two enzymes and the sensitivity to EDTA has to be demonstrated for the purified protease.

The pH optimum of protease I was 7.8 with casein and it should consequently fall into the group of neutral proteases[13]. However, protease I was also active at acidic pH as shown in the experiment with hemoglobin as the substrate. The difference between the pH optimum curves with casein and hemoglobin must be attributed to differences in the solubility of the substrates at different pH. The absence of activity against casein at pH 5.0 was probably due to precipitation of the casein. The degree of purity of the enzyme preparation used for the pH optimum experiments was rather high and it seems unlikely that the double pH optimum is a manifestation of two enzymes. Two pH optima (pH 4.0 and 8.5) were shown for an extracellular protease of a cytophaga species by Christison and Martin[14], using hemoglobin as the substrate. Only one optimum at pH 8.0 was observed with casein. Tirunarayanan and Lundblad[15] who studied the proteolytic activity of one staphylococcal strain observed two pH optima, at pH 4.5 and 8.5 against hemoglobin and only one optimum at pH 8.5 with casein. The proteolytic activity appeared as one peak in gel chromatography (Sephadex G-100) but was separated by electrophoresis into one component mainly active against hemoglobin at pH 8.5 and another active at pH 4.5. Their results however, give very little evidence that these are two different proteases. Protease I appeared as one protein in both gel chromatography and electrophoresis (*i.e.* isoelectric focusing).

Apart from the activity at neutral pH against casein protease I fits well into the group of microbial acidic proteases, which are insensitive to sulfhydryl reagents, metal chelating agents, heavy metals and DFP[16]. Also the low isoelectric point of protease I makes it belong to this group of microbial proteases. Most acidic proteases are fungal in origin but there are some reports on bacterial acid proteases from clostridia[17] and lactobacilli[18].

The pH optimum of protease II against casein was in the alkaline region (pH 8.4–8.8) but the enzyme exhibited approximately 50% of its peak activity at pH 6.0. A similar broad range of activity was also found with highly purified alkaline proteases from *B. subtilis*[19]. The loss of activity in the absence of reducing agents indicated that protease II must possess free SH-groups to be active. The inhibitory effect of Hg^{2+}, Zn^{2+} and Ag^{+} supported this assumption. On the basis of these findings protease II should be classified as a cysteine enzyme[20].

According to its slightly alkaline pH optimum, protease II should belong to the group of alkaline proteases. Protease II, however, differs in several respects from other microbial alkaline proteases, mainly by its appearance as a cysteine enzyme. Microbial alkaline proteases are generally serine enzymes and are inhibited by DFP[16]. There are very few reports on microbial cysteine proteases. So far only proteases from streptococci have been classified as cysteine enzymes[21,22].

The molecular weight of protease II was very low compared to other known proteases. One protease from Chinese gooseberry which was also a cysteine enzyme was shown to have a molecular weight of 12 800 (ref. 23). Among microbial proteases only the AL-1 protease from *Myxobacter* has a molecular weight of less than 14 000 (ref. 24). The low value of protease II could be due to retardation in the Sephadex gel, as has been reported for several other basic proteins[25]. However, preliminary experiments using Bio Gel P 60 molecular weight determination, showed approximately the same value (13 000). The loss of protease II activity in Sephadex experiments might however depend on adsorbtion to the gel since chromatography on Bio Gel under identical conditions gave much higher yields.

Esterase activity using BTEE as a substrate was exhibited only by protease II. The activity was about 20% of the activity of α-chymotrypsin A, per mg of protein. Preliminary experiments showed that protease III was not active against BTEE, and consequently the test for esterase activity on this substrate can be used as a convenient method for the determination of protease II activity.

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