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Role of a Metalloprotease in Activation of the Precursor of Staphylococcal Protease

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A metalloprotease was isolated from the culture medium of a mutant of *Staphylococcus aureus* strain V8. The enzyme had a molecular weight of 38,000 as estimated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and an optimum pH of 7.0 and exhibited a specificity for peptide bonds on the N-terminal side of large hydrophobic residues. The protease was fully inactivated by *o*-phenanthroline but could be reactivated by zinc ions. Cobalt may be substituted for zinc, producing an activity which corresponds to 160% of that of the native enzyme. All these data indicate that this protease is a typical bacterial neutral metalloprotease. The role of this metalloprotease in the activation of the precursor of another protease secreted by the same organism, staphylococcal protease, has been identified. Mutants which lack the metalloprotease accumulated the precursor, which can be specifically activated by the addition of the purified metalloprotease or the related enzyme thermolysin. The purification of the precursor is also reported.

Evidence that at least some extracellular proteins of bacterial cells are synthesized as precursor molecules has accumulated recently. In *Bacillus licheniformis*, the precursor form of the extracellular penicillinase contains at its N-terminal end an extra segment of amino acids which is processed before the enzyme is released into the growth medium (24, 26). Cells of *Klebsiella aerogenes* contain a cell-bound form of pullulanase which is released by the action of proteases (4). Precursor forms of periplasmic enzymes or proteins from *Escherichia coli*, such as alkaline phosphatase (14), maltose-binding protein, arabinose-binding protein, the outer membrane, and the λ-receptor proteins (20), were found to have higher molecular weights when synthesized in vitro than the mature proteins. Extracellular enzymes have also been found to have different N-terminal sequences. For example, staphylococcal nuclease, an enzyme which is not yet known to be synthesized as a precursor, can be detected in the growth medium with an additional amino acid sequence of 19 residues at the N terminus (6). This suggests that proteolytic cleavage of extracellular enzymes can also occur once the transport process has taken place.

Staphylococci are known to secrete several extracellular proteins, a number of which are proteases. One of these proteolytic enzymes, identified as staphylococcal protease, has been characterized extensively (9, 12) and its amino acid sequence has been determined (8). The presence of two additional proteolytic enzymes which can be distinguished on the basis of their sensitivity to the chelating agent ethylenediaminetetraacetate (EDTA) and their requirements for reducing agents has also been reported (1, 2). Although two procedures for the purification of the EDTA-inhibited enzyme are available (1, 21), neither of these procedures was satisfactory in our hands. In one case, low yields were obtained and, in the other case, the conditions for the purification of the enzyme were difficult to reproduce. Moreover, no criteria indicating homogeneity of the enzyme were provided in either instance.

In the present study, an improved purification procedure is reported for the EDTA-inhibited enzyme secreted by a mutant strain of *Staphylococcus aureus* V8. It is also shown that the enzyme is a metalloprotease and plays an important role in the activation of an inactive precursor of staphylococcal protease.

MATERIALS AND METHODS

Chemicals. *N*-CBZ-L-glutamyl-α-phenyl ester (ZGPE) was synthesized as reported previously (7). *N*-CBZ-L-glutamyl-α-naphthyl ester (ZGNE) was prepared by the same procedure by replacing phenol with β-naphthol. ZGNE was crystallized from chloroform-petroleum ether (bp 30 to 75°C). *N*-α-furylacryloylglycyl-leucinamide (FAGLA) was obtained from Vega-Fox Biochemicals. Spectroscopically pure zinc chloride was purchased from Johnson-Matthey. Diethylaminoethyl (DEAE)-cellulose D22 was obtained from

Whatman. Oxidized insulin B chain was prepared by the procedure of Humbel et al. (13).

Bacterial strains and isolation of mutants. *S. aureus* strain V8 was used as the parental organism. Mutants which had lost the capacity to produce one or more of the proteases were isolated after mutagenesis with nitrosoguanidine. Washed cells of strain V8, in the log phase, were exposed to nitrosoguanidine at a concentration of 100 μg/ml in 50 mM citrate buffer, pH 5.0, for 90 min. The cells were collected by centrifugation, washed twice in saline, and 0.1-ml aliquots of the appropriate dilutions were plated on the casein-agar medium described by Martley et al. (16). Mutants deficient in the metalloprotease gave a zone of proteolysis different from that observed with the parent organism. Whereas the zones given by the latter were clear and surrounded by a halo of precipitation, the mutant produced only a white, opaque zone. For the identification of the activity associated with staphylococcal protease in these zones, the plate staining procedure described by Miller and MacKinnon (18) was used. The fact that staphylococcal protease specifically cleaves glutamyl bonds (12) led to the use of ZGNE as substrate. The plates were incubated overnight and stained for enzymatic activity with a mixture containing 0.2 M tris(hydroxymethyl)aminoethane (Tris)-hydrochloride buffer, pH 7.5, 2 ml of ZGNE solution (10 mg/ml in dimethylformamide), 200 mg of Fast Garnet GBC (Sigma Chemical Co.), and 8 ml of dimethylformamide in 100 ml. Zones of proteolysis containing no glutamyl-specific protease showed only a slight reddish color, whereas the zones produced by the parent organism showed a dark red color.

Growth conditions. The conditions used for the production of protease were the same as described earlier (9), with the exception that the casein concentration was reduced to 1%. The growth medium employed for the isolation of the precursor of staphylococcal protease had the same ingredients, but $CaCl_2$ was omitted and 0.4 mM EDTA was added. In this case, the medium was predigested overnight with Pronase (50 μg/ml), and the proteolytic activity of Pronase was inactivated by sterilization for 20 min. Digestion of the medium was necessary in this experiment because casein is only partially hydrolyzed during the growth of the culture and interfered with the purification of the precursor.

Enzyme assays. Routine determination of the proteolytic activity was performed with casein as the substrate (9). When it was necessary to distinguish the activities associated with the staphylococcal protease or the metalloenzyme, the specific peptide substrates were used. Staphylococcal protease was assayed using ZGPE as described previously (7). The activity of the metalloprotease was determined with FAGLA as substrate. A cuvette containing 3 ml of a 1 mM solution of the appropriate substrate in 0.1 M *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES) buffer, pH 7.2, was placed in a spectrophotometer, and the activity was assayed by monitoring the decrease in absorbance at 345 nm due to hydrolysis of the Gly-Leu bond (10). Activity is defined as the number of micromoles of substrate hydrolyzed per minute. The precursor of staphylococcal protease was assayed after its activation by the metalloprotease. Portions from the growth medium or column fractions (0.1 to 0.3 ml) were incubated in the presence of 10 μg of metalloprotease in 10 mM Tris buffer, pH 7.5, and 5 mM $CaCl_2$. The final volume of the mixture was 0.5 ml. After incubation at 37°C for 30 min, 10- to 50-μl samples were withdrawn and assayed for staphylococcal protease activity with ZGPE as substrate.

Enzyme purification. The procedure for the purification of the metalloprotease was essentially the same as for the purification of staphylococcal protease (9). All buffers contained 10 mM Tris, pH 7.5, and 5 mM $CaCl_2$. Briefly, the spent culture medium was saturated with ammonium sulfate, and the precipitated protein was collected by centrifugation. The pellet was dissolved in a small volume of buffer, and the enzyme was reprecipitated with cold (−30°C) acetone (130 ml of acetone per 100 ml of enzyme solution). After a brief centrifugation, the precipitated protein was dissolved in the Tris-calcium buffer and dialyzed overnight against the same buffer. The dialysate was centrifuged to remove insoluble material and then applied on a column of DEAE-cellulose (2.5 by 20 cm) previously equilibrated with the same buffer. The enzyme was eluted with a gradient of 0 to 0.6 M KCl in the Tris-calcium buffer. The precursor of staphylococcal protease was purified by a similar procedure with the exception that all buffers were prepared without $CaCl_2$ and contained 2 mM EDTA. Furthermore, the precipitation step with acetone was omitted.

Substrate specificity. The substrate specificity of the enzyme was determined by two methods. One method consisted of the analysis of the peptides obtained after digestion of oxidized insulin B chain. In these experiments, 5 mg of the B chain were dissolved in 1 ml of ammonium bicarbonate (0.1 M), 0.1 mg of protease was added, and the mixture was incubated at 37°C for 18 h. The digest was lyophilized, and the peptides were fractionated by paper electrophoresis at pH 3.7 and/or pH 6.4. In the second method, the cyanate procedure was used to identify the liberated N-terminal amino acid residues after digestion of casein. One-milliliter samples of casein (Hammersten) (7.5 mg/ml, adjusted to pH 7.2 with 0.1 N NaOH) were incubated overnight with 0.2 mg of protease. After incubation, 2 μmol of norleucine in 0.1 ml was added as an internal standard. The quantitative estimation of the N-terminal amino acids was carried out as described by Stark and Smyth (22). The values of each amino acid obtained with the amino acid analyzer are reported as the number of micromoles of residues per micromole of norleucine. Undigested casein was used as a control.

Protein determination. Determination of the protein content of the solutions was by the method of Lowry et al. (15) with bovine serum as the standard.

Disc electrophoresis. Polyacrylamide gel electrophoresis was performed by the procedure of Davis (5). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was carried out by the method of Weber and Osborn (25). The proteins used as standards in the determination of the molecular weight were bovine serum albumin (molecular weight, 68,000), glutamate dehydrogenase (53,000), pepsin (35,000), chymotrypsinogen (25,700), and chymotrypsin chains (11,000 and 13,000, respectively).

RESULTS

Purification of the metalloprotease. The metalloenzyme was purified from the culture medium of a mutant of strain V8 (V8-10B). This mutant differs from the parent organism by the procedure of a more stable metalloprotease and of little staphylococcal protease. Chromatography on DEAE-cellulose of the enzyme preparation obtained after precipitation with acetone resolved three fractions with proteolytic activity (Fig. 1). The first large peak contained mainly pigmented material and little protein. A small amount of proteolytic activity, as detected with casein as substrate, was present in this peak. This activity was strongly enhanced by the addition of β-mercaptoethanol and might probably be due to the action of the sulfhydryl protease reported previously (2). The second peak contained the activity of staphylococcal protease as determined specifically with ZGPE as substrate. The metalloprotease, as assayed with FAGLA, was eluted in the third peak. Although this fraction gave a single protein band on the polyacrylamide gel (Fig. 2A), it nevertheless contained traces of staphylococcal protease activity, readily detectable due to the high sensitivity of the assay method for this enzyme. This contamination was removed, however, by rechromatography of the fraction 39 to 47 on DEAE-cellulose with a shallower KCl gradient (0 to 0.4 M). Table 1 summarizes the results of the purification of the metalloprotease of *S. aureus*. The increased yield of the enzyme after the ammonium sulfate precipitation step might be attributable to the presence of an inhibitor, possibly from the growth medium, which was eliminated by the acetone precipitation step.

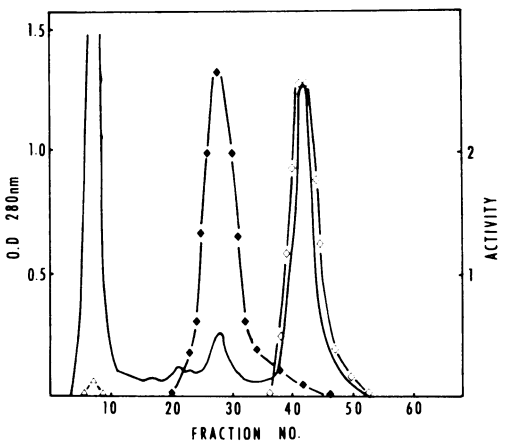

FIG. 1. *Chromatography on DEAE-cellulose. The enzyme obtained after the acetone precipitation step was applied to the column. Flow rate, 36 ml/h. Substrates: casein (△); ZGPE (◆); FAGLA (◇). OD_{280nm}, Optical density at 280 nm.*

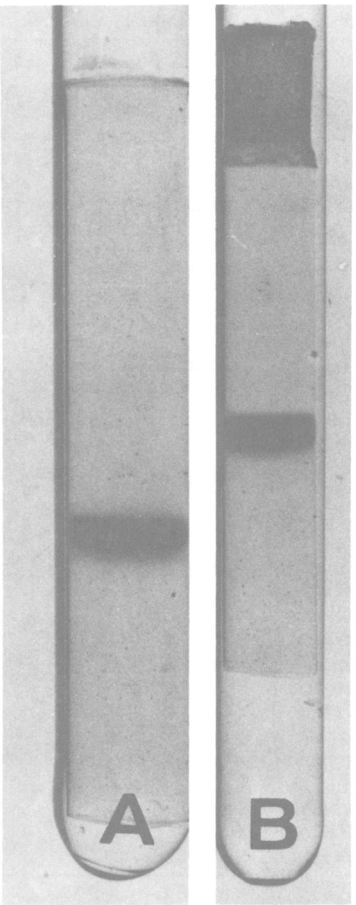

FIG. 2. *Polyacrylamide gel electrophoresis of purified metalloprotease (A) and precursor of staphylococcal protease (B). Approximately 100 μg of the purified preparation was applied. The direction of migration is downward, and the gel concentration is 10%.*

Properties of the metalloprotease. For the estimation of the molecular weight of the metalloprotease by sodium dodecyl sulfate-gel electrophoresis, the protease sample was dissolved in 5 mM *o*-phenanthroline and 0.1% sodium dodecyl sulfate followed by boiling for 5 min. The addition of *o*-phenanthroline prevented autodigestion. The enzyme migrated as a single band, and a plot of the logarithm of the molecular weights of the standard proteins against mobilities gave a straight line. By interpolation, the molecular weight of the protease was estimated to be 38,000. The amino acid composition of the purified enzyme is shown in Table 2, together with that of thermolysin, which has been included for comparison. There are noteworthy differences in the numbers of residues of aspartic acid, glu-

TABLE 1. *Purification of the metalloprotease of S. aureus*

Stage	Volume (ml)	Total units	Protein (mg/ml)	Sp act[a] (μmol/min per mg)	Yield (%)
Culture medium	2840	199	7.6	0.009	100
$(NH_4)_2SO_4$ precipitation	100	135	—[b]	—	68
Acetone precipitation	27	175	7.5	0.866	88
DEAE-cellulose pool	98	127	1.45	0.902	64

[a] Assayed with FAGLA as substrate.
[b] —, Not determined because of the presence of $(NH_4)_2SO_4$.

TABLE 2. *Amino acid composition of S. aureus metalloprotease*

Amino acid	*S. aureus* metalloprotease[a]	Thermolysin[b]
Aspartic acid	58	44
Threonine[c]	23	25
Serine[c]	22	26
Glutamic acid	41	21
Proline	5	8
Glycine	36	36
Alanine	28	28
Cysteine[d]	0	0
Valine	18	22
Methionine	5	2
Isoleucine	12	18
Leucine	19	16
Tyrosine	22	28
Phenylalanine	12	10
Lysine	20	11
Histidine	9	8
Arginine	7	10
Tryptophan[e]	2	3

[a] Average of triplicate 48-h hydrolyses.
[b] Based on amino acid sequence (23).
[c] Extrapolated to zero-time hydrolysis.
[d] Determined as cysteic acid after oxidation with performic acid.
[e] Determined after hydrolysis with mercaptoethanesulfonic acid (19).

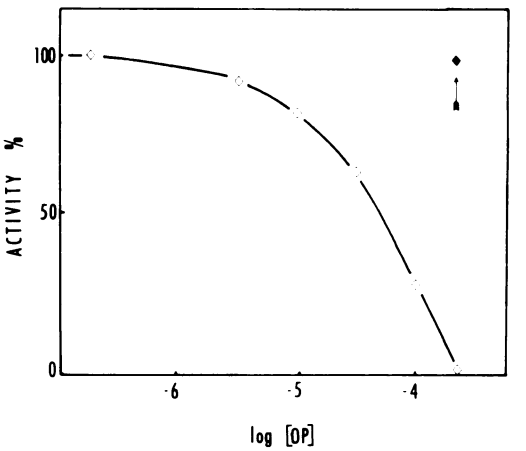

FIG. 3. *Inhibition of protease activity by o-phenanthroline (OP). Portions of enzyme (20 μl) were added to substrate, 1 mM in HEPES buffer, pH 7.2, and 10 mM $CaCl_2$ containing the indicated concentrations of the chelating agent. The point indicated by the arrow represents the activity measured if an equimolar amount of Zn^{2+} is added to the inhibited enzyme.*

tamic acid, and/or their amides, which are present in higher amounts in the staphylococcal enzyme. The metalloprotease also has a higher lysine content than thermolysin. However, like thermolysin, it is devoid of sulfhydryl groups. Although calcium ions are required during the purification procedure to maintain activity, the purified enzyme can be dialyzed against distilled water and lyophilized without an appreciable loss in activity. In the purified form, the enzyme is stable and no evidence of autodigestion was observed under normal conditions. The optimum pH for activity in HEPES buffer and with FAGLA as substrate was 7.0 (data not shown).

Metal ion requirements. When the purified enzyme was assayed in the presence of 10 mM $CaCl_2$ and increasing concentrations of *o*-phenanthroline, a gradual inhibition of the proteolytic activity was observed (Fig. 3). At a concentration of 0.1 mM of the chelating agent, the enzyme was completely inhibited, but the activity was fully restored on the addition of an equimolar amount of Zn^{2+}. These results indicate that the protease is a metalloenzyme and has a specific requirement for Zn^{2+} for activity. As demonstrated previously for thermolysin and other metalloproteases, Zn^{2+} can also be replaced by Co^{2+} in the staphylococcal enzyme (11). Although Zn^{2+} in the concentration range of 10^{-5} to 10^{-3} M gradually inhibited the enzymatic activity, Co^{2+} increased the proteolytic activity (Fig. 4). The optimum concentration of Co^{2+} was about 0.5 mM.

Substrate specificity. Digestion of the oxidized B chain of insulin and analyses of the liberated peptides revealed that the metalloprotease cleaves the peptide bonds between His-Leu (positions 5 to 6 and 10 to 11), Ala-Leu (position 14 to 15), Tyr-Leu (position 16 to 17), Gly-Phe (position 23 to 24), and Phe-Tyr (position 25 to 26). These results indicate that the enzyme has a specificity identical to that of other

metalloproteases, i.e., it cleaves peptide bonds on the N-terminal side of bulky hydrophobic residues, in agreement with earlier reports (3, 21). The specificity of the enzyme by using casein as substrate and assaying quantitatively by the cyanate method was identical to that of thermolysin. As shown in Table 3, casein digests produced by the staphylococcal metalloprotease and thermolysin liberated the same N-terminal residues, alanine, valine, isoleucine, leucine, phenylalanine, and tyrosine. The ratios of the different amino acid residues were essentially identical in both cases, but thermolysin appeared to be somewhat more efficient, cleaving more bonds.

Precursor protein of staphylococcal protease. Mutants of strain V8 which secreted no metalloprotease were also found to be deficient in staphylococcal protease. Because these two proteases are structurally different and, therefore, must be coded for by different genes, these double mutants could not have been obtained as a result of a single mutagenic event. These findings could be interpreted as suggesting a dependence of one of these enzymes (staphylococcal protease) on the activity of the other (metalloprotease) for its activation. This hypothesis was proved by the experiment shown in Fig. 5. When the mutant was grown in the casein medium, no activity associated with either the metalloenzyme or staphylococcal protease was detectable. However, on addition of a sample of purified metalloenzyme after a suitable growth period, staphylococcal protease activity, as measured specifically with ZGPE, was detectable. Identical results were obtained by the addition of thermolysin but not of trypsin or chymotrypsin, suggesting that activation of staphylococcal protease is dependent on a protease of a defined specificity.

Purification of the precursor of staphylococcal protease. Accumulation of the precursor of staphylococcal protease by the parent organism was obtained when the growth medium contained EDTA. Under these conditions, the precursor was not activated, probably be-

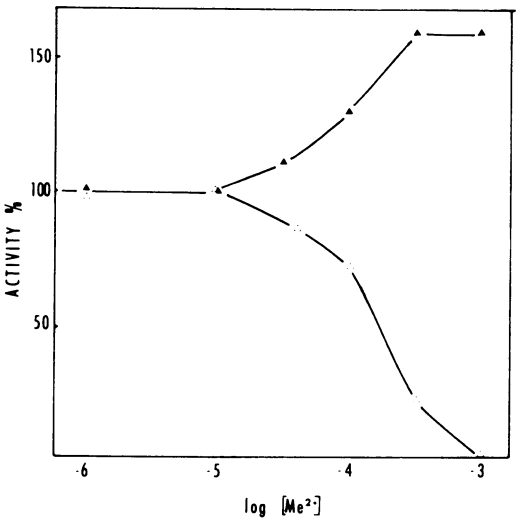

FIG. 4. *Enhancement of staphylococcal metalloprotease activity by Co^{2+} and inhibition by Zn^{2+}. Assays were performed by adding enzyme to a cuvette containing HEPES buffer and substrate, FAGLA, plus either Co^{2+} or Zn^{2+} at the concentrations indicated. Metal: Co^{2+} (▲); Zn^{2+} (△).*

TABLE 3. *Determination of N-terminal amino acid residue of peptides obtained from casein digests*

Amino acid	Proteases	
	S. aureus metallo-protease	Thermolysin
Alanine	0.29[a]	0.47
Valine	0.59	0.70
Isoleucine	0.37	0.50
Leucine	0.91	1.10
Phenylalanine	0.26	0.27
Tyrosine	0.33	0.34

[a] Number of μmoles of amino acid per μmole of norleucine added as an internal standard.

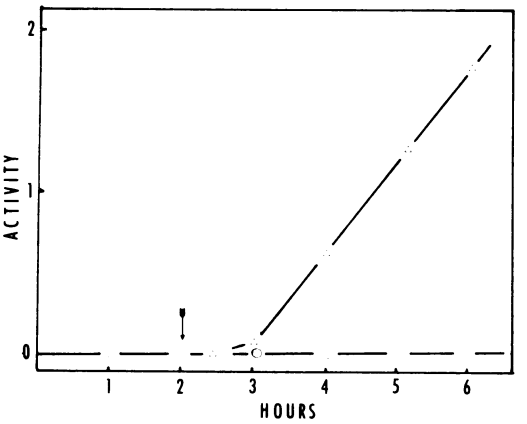

FIG. 5. *Time course study of staphylococcal protease activity in the culture medium of a mutant (V8-L2) deficient in metalloprotease activity. A 50-ml amount of the casein medium (9) was inoculated with 10 ml of cells of an overnight culture and incubated at 37°C. At the time indicated by the arrow, 1 mg of metalloprotease was added. Aliquots of 1 ml were withdrawn, centrifuged, and assayed for staphylococcal protease activity with ZGPE as substrate. A control containing no metalloprotease was run in parallel. Activity measured after addition of metalloprotease (△) and in control culture (○).*

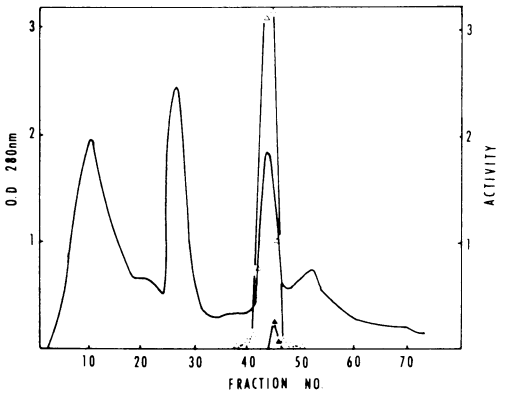

FIG. 6. *Chromatography on DEAE-cellulose. The proteins in culture supernatant of S. aureus V8 grown in the presence of 0.4 mM EDTA were obtained by ammonium sulfate precipitation. After dialysis, the enzyme was applied on a column (2 by 17 cm). The gradient bottles contained 0 and 0.6 M KCl, respectively, in 500 ml of Tris buffer, 10 mM, pH 7.5, and containing EDTA 2 mM. The flow rate was 36 ml/h. Activity, before (▲) and after (△) activation by metalloprotease. OD_{280nm}, Optical density at 280 nm.*

cause of the inhibition of the activating enzyme, the metalloprotease, by the chelating agent. Figure 6 shows the protein distribution profile obtained by chromatography on DEAE-cellulose with the growth medium concentrated by precipitation with ammonium sulfate. Fractions 41 to 47 contained the precursor which could be activated by the metalloprotease. The precursor eluted slightly ahead of the active enzyme which was present in very small quantities in fractions 45 and 46. When fractions 41 to 44 were pooled and rechromatographed on DEAE-cellulose under the same conditions, a single peak containing only the activable precursor was obtained. This precursor appeared to be pure as estimated by gel electrophoresis (Fig. 2B). Approximately 8 mg of pure precursor were obtained per liter of medium.

DISCUSSION

The procedure developed for the purification of the metalloenzyme from *S. aureus* gave a homogenous preparation with a good recovery. The purification of the enzyme was facilitated by using a mutant which secreted less than 5% of staphylococcal protease produced by the parent organism. Although the two enzymes eluted in two separate peaks from the DEAE-cellulose column, these peaks were proximate, and considerable overlapping occurred if the staphylococcal protease was present in large quantities. Another interesting and practical feature of the enzyme of the mutant is its higher stability in the growth medium as compared with that of the parent organism. It is not yet clear, however, whether or not the mutation responsible for these altered properties has occurred in the structural gene for the metalloenzyme or staphylococcal protease.

A comparison of the chemical and physical properties of the metalloprotease from *S. aureus* and other metalloproteases, such as thermolysin, revealed no striking differences. Their molecular weights are similar, and they have the same pH for maximum activity. The staphylococcal enzyme has a specific requirement for Zn^{2+} which can be replaced by Co^{2+} and has a specificity for peptide bonds on the N-terminal side of large hydrophobic residues, properties which are all shared with other metalloenzymes (17). On the other hand, there are significant differences in their amino acid compositions. Recently, it has been reported that the metalloenzyme purified from the parent strain *S. aureus* V8 has a requirement for Ca^{2+} rather than Zn^{2+} (21). This observation was not supported by the present data because the enzyme was completely inactive in the presence of *o*-phenanthroline, even in the presence of Ca^{2+}, and the addition of Zn^{2+} completely restored the activity of the inhibited enzyme.

In the present investigation, the role of the metalloprotease in the activation of staphylococcal protease from a precursor form was identified. The precursor, which is detected in the growth medium of metalloprotease-deficient mutants or of the parent organism grown under conditions inhibiting the activating enzyme, is specifically activated by this metalloprotease or by thermolysin. However, it is not inferred here that this precursor is the primary gene product of the staphylococcal protease gene. Indeed, although the activating enzyme must be absent in the growth medium to allow the accumulation of the precursor molecule, another proteolytic enzyme, the sulfhydryl protease, remains present. Therefore, the possibility that some processing of a primary precursor which would not generate an active enzyme cannot presently be excluded. Alternatively, processing might also occur during the transmembrane passage of the precursor by another protease located in the cell envelope as postulated for other extracellular enzymes (24). However, whether or not prior removal of a peptide segment occurs, it is clear that the final step in the processing of the precursor of staphylococcal protease requires the action of an enzyme with the substrate specificity of the metalloprotease.

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