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PURIFICATION AND CHARACTERIZATION OF THE EXTRACELLULAR PROTEINASE OF *SERRATIA MARCESCENS*

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Summary

The extracellular proteinase produced by a derepressed strain of *Serratia marcescens* ATCC 25419 was purified and characterized. This strain produces more than 10-times the amount of extracellular proteinase produced by other strains of *Serratia* tested. The purified enzyme was prepared from the culture supernatant by $(NH_4)_2SO_4$ fractionation and DEAE-cellulose chromatography.

The purified enzyme has an $s^0_{20,w}$ of 3.95 and is a monomer of molecular weight 51 900. The proteinase has a broad pH optimum in the alkaline range with a maximum at pH 9.5. The enzyme will utilize a number of proteins as substrate. The products of digestion are primarily in the size range of tripeptides to hexapeptides. Peptides containing a sensitive bond and a minimum size of six amino acids are hydrolyzed. The proteinase is inhibited by chelating agents but unaffected by sulfhydryl or serine reagents and is devoid of esterase activity.

Introduction

Serratia marcescens ATCC 25419 is a pathogen for a variety of insects [1] and the causative agent for pathogenicity in the boll weevil has been shown to be an extracellular proteinase [2]. A proteinase from a *Serratia piscatorum* strain designated E-15 was purified and partially characterized by Miyata et al. [3—5]. This paper reports the purification of the extracellular proteinase produced by *Serratia marcescens* ATCC 25419 and compares its characteristics

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with those reported for *Serratia piscatorum*. The comparison of these two enzymes is of particular interest because the strain of *Serratia piscatorum* was isolated from the intestinal tract of larval silkworms.

Material and Methods

Bacteria. The strain of *Serratia marcescens* used in this study is maintained in the American Type Culture Collection (No. 25419).

Culture conditions. The organism was routinely maintained on brain heart infusion (Difco) agar slants at room temperature. Survival of the organisms appears to be greater when slants are stored in the dark. Stocks were routinely transferred at 6-week intervals.

The organism was cultured for maximal proteinase production by growth in 1-l cultures on BHI broth at room temperature on a rotary shaker for 36 h. A comparison of growth and proteinase production was obtained using a minimal medium which contained (per l): 10.5 g K_2HPO_4, 4.5 g KH_2PO_4, 0.397 g sodium citrate dihydrate, 0.107 g $MgSO_4 \cdot 7H_2O$, 1 g $(NH_4)SO_4$, 10 ml glycerol. This minimal medium was supplemented with 1% (w/v) gelatin or vitamin free casein for proteinase production.

Chemicals. *p*-Chloromercuribenzoate was purchased from Nutritional Biochemical Corporation; ethylenediamintetraacetic acid and 1.10-phenanthroline were purchased from Mallinckrodt Chemicals Works; iodoacetate was purchased from Eastman Chemical Company; diisopropylfluorophosphate, C-terminal octapeptide of glucagon, N-terminal hexapeptide of glucagon, and reduced carboxymethylated β-insulin and di- and tripeptides were purchased from Mann Research Laboratories; glycylvaline and glutathione were from Calbiochem, lysozyme, ribonuclease, *N*-benzoyl-L-arginine ethyl ester and *N*-benzoyl-L-tyrosine ethyl ester were purchased from Worthington Biochemical Corporation. The heptapeptide was isolated and purified from a tryptic digest of insulin. All other chemicals were reagent grade.

Enzymatic assay. One ml of 1% vitamin free casein in 0.1 M sodium phosphate (pH 7.5) was incubated at 30° C for 20 min with 1 ml of appropriately diluted enzyme solution. The reaction was stopped by the addition of three ml of 50% trichloroacetic acid. The reaction mixture was centrifuged at 23 000 $\times g$ for 15 min to remove precipitated protein and absorbance of the supernatant fluid was measured at 280 nm. One unit is defined as the amount of enzyme necessary to increase the 280 nm absorbance of the supernatant fluid 0.1 *A* unit above the appropriate blank under the conditions of the assay.

The assay for substrate specificity were as follows: substrates were dissolved at 5 mg/ml in 1 M Tris-HCl (pH 7.5). Proteinase was added to a final concentration of 30 μg/ml and the reaction incubated for 1 h at 30° C and stopped by the addition of an equal volume of 50% trichloroacetic acid. Hydrolysis of hemoglobin and casein was measured by increase in absorbance of the trichloroacetic acid-soluble fraction at 280 nm. Hydrolysis of lysozyme, ribonuclease and β-insulin as well as the aminoethylated derivatives of lysozyme and ribonuclease and peptides was measured by fingerprint analysis on paper chromatography in butan-2-ol/HCOOH/H_2O (7 : 1 : 2, v/v), and electrophoresis at pH 3.5 in pyridine/acetic acid/water (1 : 10 : 300, v/v) at 2400 V and 140 mA. Peptides

were located with ninhydrin spray. Hydrolysis of small peptides was determined by thin layer chromatography on silica gel in *n*-butanol/acetic acid/water (9 : 2 : 9, v/v).

The rates of hydrolysis of glucagon, carboxymethyl-β chain of insulin and carboxymethyl-α chain of insulin were measured by release of ninhydrin positive material.

Esterase activity was determined as previously determined [6].

Gel electrophoresis. Polyacrylamide gel electrophoresis was performed as described by Davis [7] except that the samples were applied in 40% sucrose without using sample gel. Gels were stained with 1% Amido Schwartz in 7% acetic acid and destained with 7% acetic acid.

Subunit molecular weight was determined on gel which contained 7.5% acrylamide, 0.1% sodium dodecylsulfate (SDS), 5.76 M urea and 0.1 M sodium phosphate (pH 7.2). Protein samples were denatured by boiling for 2 min in 1% SDS, 0.1% β-mercaptoethanol. A current of 5 mA/gel (15 cm) was applied for 18 h. The gels were stained for 1 h with 0.29% Coomassie Brilliant Blue in $CH_3OH/CH_3COOH/H_2O$, (5 : 1 : 5, v/v) and destained in 5% methanol/7.5% acetic acid at 37° C.

Sedimentation analysis. Sedimentation velocity was determined in a Beckman Model E analytical ultracentrifuge. A double sector synthetic boundary cell with quartz windows was employed. Sedimentation was at 56 000 rev./min in the An-D rotor; schlieren optics were used and photographs were taken at 8-min intervals beginning when a speed of 37 500 rev./min had been attained. Temperature was maintained at 20° C.

Protein was dissolved in 0.05 M sodium phosphate (pH 7.0), 0.1 M NaCl and subsequently dialyzed against three changes of the same buffer. Photographic plates were measured on a Nikon Model 6C profile projector. The value for v of 0.716 was computed from the amino acid composition [8].

Sedimentation equilibrium centrifugation. The molecular weight of the protein at pH 6.7 was determined by the sedimentation equilibrium method of Yphantis [9] using interference optics. The fluorochemical FC-43 was used as a base for a solution column 3 mm in height. The solvent was 0.05 M sodium phosphate buffer (pH 6.7) containing 0.1 M NaCl. Photographs were taken on Eastman Spectroscopic plates, type II G. Equilibrium was attained employing a rotor speed of 28 000 rev./min for 24 h at 20° C.

Amino acid analysis. Amino acid analysis was performed on a Beckman Model 120 C amino acid analyzer using the technique of Spackman et al. [10].

Protein measurement. Protein was measured by the technique of Warburg and Christian [11] and by the method of Lowry et al. [12].

Hydrolysis sites. The peptides produced after the indicated digestion time were separated by paper chromatography and electrophoresis. The solvents used for chromatography were butan-2-ol/$HCOOH/H_2O$, (7 : 1 : 2, v/v) or butan-1-ol/CH_3COOH/H_2O, (4 : 1 : 5, v/v). Electrophoresis was the same as described under enzymatic assay. The pure peptides were then analyzed for amino acid composition. Those peptides that demonstrated integral values of the component amino acids were then assigned a sequence that was consistent with the sequence of the substrate peptide.

Results and Discussion

Serratia marcescences ATCC 25419 produces an extracellular proteinase in response to exogenously supplied proteins or peptides. Addition of 1% casein to minimal medium resulted in an increase from 0.05 u/ml to 2.28 u/ml, while addition of 1% casein hydrolysate resulted in 1.3 u/ml. A mixture of equal weights of the 20 common amino acids to give a total weight of 1 g/100 ml of culture medium resulted in 0 u/ml of activity. Due to the fact that the mixture of amino acids resulted in no proteinase production it seems likely that the induction observed upon the addition of casein hydrolysate is due to residual peptides which are present in the commercial preparation used for this study. However, Loria et al. [13] reported that leucine is the inducer for the enzyme.

Cell-free extracts, prepared by sonication at 4° C, were devoid of proteolytic activity when grown with or without an exogenous supply of protein. Maximum proteinase production is obtained by cells growing on minimal medium supplemented with 1% casein or 1% gelatin as the protein source or on brain heart infusion after 36 h incubation at room temperature. Regardless of the medium used proteinase activity was greater when cultures were shaken on a rotary shaker rather than aerated by sparging with compressed air.

Proteinase purification. Eighteen one liter shaken cultures of *S. marcescens* ATCC 25419 grown on brain heart infusuion broth or minimal medium supplemented with 1% protein (vitamin-free casein or U.S.P. gelatin) were pooled and cleared of cells by centrifugation in a Sharples Super Centrifuge. The culture fluid was collected and immediately used for proteinase purification. The supernatant fluid was rapidly cooled to 4° C by immersing in a −10° C cooling bath and the precipitate forming at 80% satn. $(NH_4)_2SO_4$ was collected by centrifugation in a Sharples Centrifuge and redissolved in a minimal volume of 0.05 M sodium phosphate buffer pH 6.7 (buffer 1). The redissolved precipitate was dialyzed against three changes of buffer 1 for 12 h to remove the salt. After dialysis the protein concentration was adjusted to 10—20 mg/ml with buffer 1, the precipitate forming at 30—50% satn. $(NH_4)_2SO_4$ was collected by centrifugation, redissolved in a minimal volume of 0.5 M phosphate buffer pH 8.0 (buffer 2). This material was added to a 30 × 4.5 cm DEAE cellulose column previously equilibrated with buffer 2. The column was washed with 2 l of buffer 2 and the enzyme was eluted with a linear gradient consisting of 1 l buffer 2 in the mixing vessel and 1 l of 0.6 M phosphate buffer (pH 8.0) in the reservoir (10-ml fractions). The active fractions (Fig. 1), which appeared in the first quarter of the gradient were pooled, dialyzed against deionized water and lyophilized. The dried material can be stored at −70° C for a period of several months without loss of activity. The results of a typical purification are presented in Table I.

SDS-gel electrophoresis. Although the purification procedure described above is simple, the results of electrophoresis on denaturing polyacrylamide gels indicate a high degree of homogeneity of the proteinase preparation. By this criterion we judge the proteinase preparation to be more than 95% pure. Electrophoresis in nondenaturing gels resulted in one major band and faint traces of three to four minor bands.

Comparison of the relative electrophoretic mobility of the proteinase in

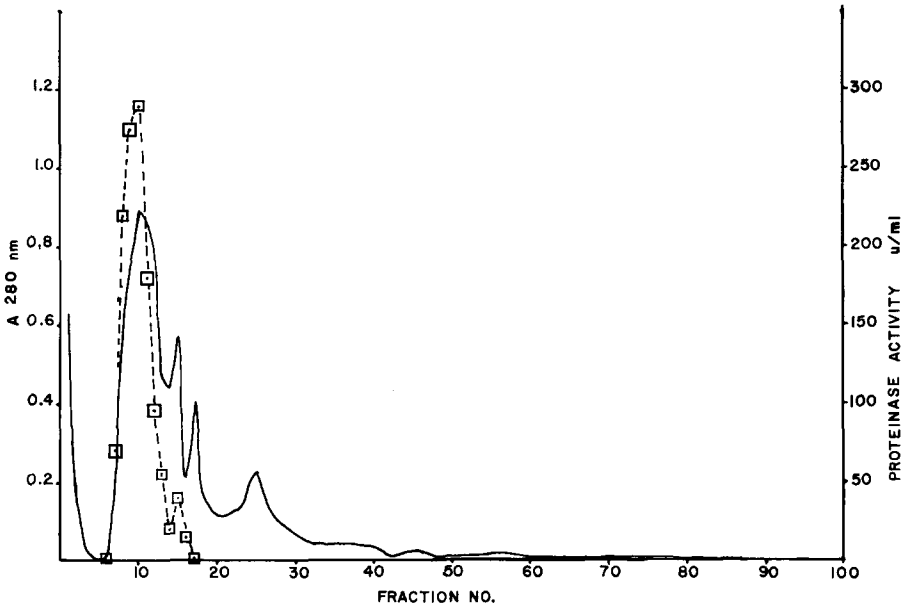


Fig. 1. Elution of *S. marcescens* proteinase from DEAE-cellulose chromatography. The elution was carried out with a linear gradient of phosphate buffer. ———, A_{280nm}; ⊡- - - - -⊡, proteinase activity (u/ml).

SDS-gels with a set of proteins of known molecular weight indicated a subunit molecular weight of 47 500. Standards used for molecular weight determination were the major polypeptides of reovirus (kindly supplied by Dr. K. Payne).

Sedimentation analysis. The sedimentation coefficient for the *Serratia* proteinase was determined in a Beckman Model E analytical ultracentrifuge, and a single symmetrical peak was observed. The sedimentation coefficient was determined at four concentrations of protein and extrapolated to zero: the value of $s^0_{20,w}$ was 3.95.

Sedimentation equilibrium centrifugation. The plot of the natural log of the fringe displacement as a function of comparator X coordinate yielded a straight line, and the molecular weight calculated for the enzyme was 51 900.

Amino acid composition. The amino acid composition of *S. marcescens* proteinase is shown in Table II. Very few basic amino acid residues were detected in the protein.

TABLE I

PURIFICATION SCHEME FOR *SERRATIA MARCESCENS* PROTEINASE

Fraction	Volume (ml)	Activity (units X 10^{-5})	Protein (mg)	Specific activity	Recovery (%)
Culture fluid	18 000	11.67	432 000	2.7	100
Dissolved 80% $(NH_4)_2SO_4$ precipitate	920	8.30	19 300	43	71
Dissolved 30—50% $(NH_4)_2SO_4$ precipitate	136	7.76	4 650	167	67
DEAE-cellulose active fractions	260	6.07	1 450	420	52

TABLE II

AMINO ACID COMPOSITION OF *SERRATIA MARCESCENS* PROTEINASE

Amino acid	Residues/mol *	Amino acid	Residues/mol *
Lysine	15	Alanine	47
Histidine	10	Half cystine	0
Arginine	9	Valine	27
Aspartic acid	76	Methionine	1
Threonine **	36	Isoleucine	20
Serine **	38	Leucine	27
Glutamic acid	42	Tyrosine	21
Proline	11	Phenylalanine	31
Glycine	59	Tryptophan ***	8

* Based on a molecular weight of 51 900 for the protein.
** Extrapolated to zero hydrolysis time.
*** Goodwin and Morton [14].

Effect of pH and inhibitors. The enzyme is active over a pH range of 7.5 to 10.6 with a broad pH optimum of 9.5. The sulfhydryl reagents, *p*-chloromercuribenzoate and iodoacetic acid were not inhibitory at 1 mM. Diisopropylphosphofluoridate was not inhibitory precluding involvement of serine in the active site. Ethylene diaminetetracetic acid (EDTA) and 1,10-phenanthroline (at 10 mM) inhibited the enzyme 85—100%. Neither Ca^{2+}, Zn^{2+} nor Fe^{2+} could restore the activity of enzyme treated with chelating agents.

Specificity of Serratia proteinase. Various proteins and peptides were used as substrates to determine the specificity of the *Serratia* extracellular proteinase. Enzymic hydrolysis was measured by following the increase in trichloroacetic acid soluble material by absorbance at 280 nM, fingerprint methodology or by the release of ninhydrin reacting α-amino groups. Table III indicates that the

TABLE III

SUBSTRATE SPECIFICITY OF *SERRATIA* PROTEINASE

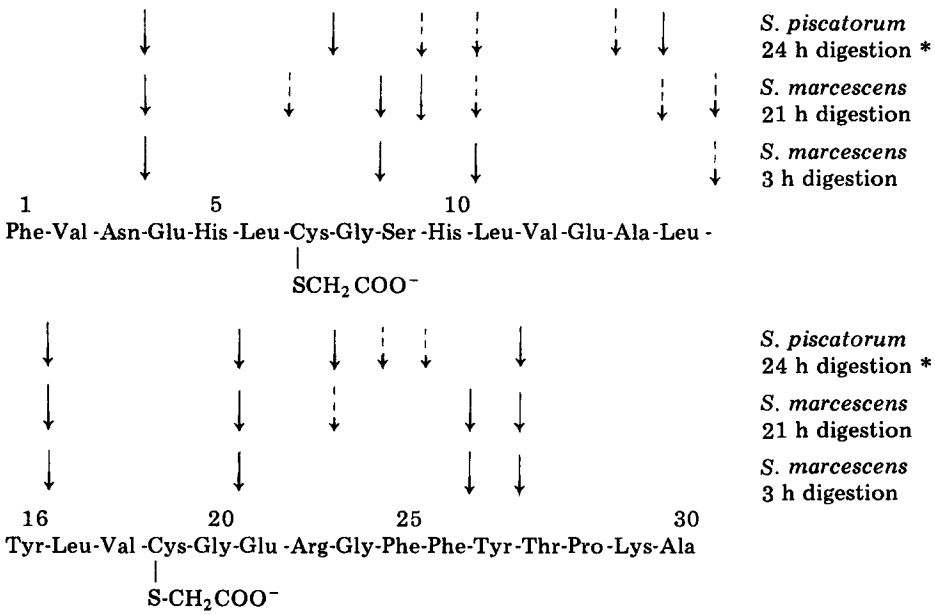
Substrate	Hydrolysis [a]	
	ΔA_{280}	Peptides in Fingerprint
Hemoglobin	0.41	N.D.
Casein	0.91	N.D.
Lysozyme	—	[b]
Aminoethylated lysozyme	—	56
Ribonuclease	—	64
Aminoethylated ribonuclease	—	69
Cm-β-insulin	—	22
Glucagon	—	10
C-terminal octapeptide of glucagon	—	4
N-terminal hexapeptide of glucagon	—	2
N-benzoyl-L-arginine ethyl ester	0.00 [c]	—
N-benzoyl-L-tyrosine ethyl ester	0.00 [d]	—

[a] 30 μg/ml proteinase for 60 min at 30°C.
[b] Substrate remained at origin. Spots were weakly stained.
[c] A_{253}/min.
[d] ΔA_{256}/min.

enzyme is effective against several proteins. The extensive hydrolysis of carboxymethyl-β-insulin suggests a very broad specificity in terms of susceptible bonds. Since the β-chain of insulin contains only 30 residues many of the peptides found after digestion with *Serratia* proteinase have partial homology. This table also indicates that the enzyme is capable of hydrolyzing peptides as small as six amino acids, if they contain a sensitive bond that is not a terminal peptide bond. The rate of hydrolysis of a substrate is directly dependent upon the positive charge of the substrate.

Some small peptides that were not hydrolyzed by the enzyme were $(Gly)_{2-6}$, Gly-Glu, Tyr-Leu, Gly-Ser, His-Leu, Gly-Thr, Gly-Leu-Tyr, Leu-Trp-Met, and Leu-Tyr-Leu; a number of these contain bonds that are sensitive to hydrolysis when present as part of a larger polypeptide. The peptides bonds that are hydrolyzed by the proteinase in carboxymethylated insulin β-chain and in glucagon are shown below:

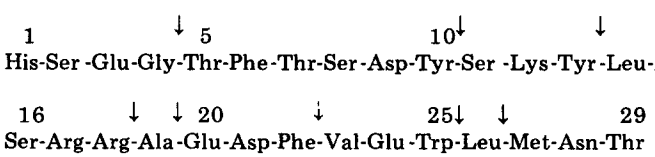
Hydrolysis sites in carboxymethylated insulin β-chain by *Serratia* proteases



Solid arrows indicate the major sites of peptide bond cleavage and dashed arrows less readily hydrolyzed bonds.

* The substrate used in this study (Miyata et al. [4]) was the performic oxidized β-chain.

Hydrolysis sites in glucagon by *S. marcescens* protease



The peptide bonds hydrolyzed in these two substrates indicate that glycine, alanine, tyrosine, arginine, asparagine, histidine, leucine and phenylalanine are

the amino acids contributing the carboxyl function. The hydrolysis of peptide bonds in hexa-, hepta- and octapeptides is shown below:

Hydrolysis sites in three peptides found in glucagon and insulin

C-terminal octapeptide of Glucagon Phe-Val-Glu-Trp↓-Leu↓-Met-Asn-Thr

N-terminal hexapeptide of Glucagon His-Ser↓-Glu-Gly-Thr-Phe

Tryptic Insulin heptapeptide Gly-Phe-Phe↓-Tyr-Thr-Pro-Lys

The C-terminal octapeptide of glucagon is hydrolyzed at the exact two bonds hydrolyzed in the intact glucagon. That is not the case with the tryptic heptapeptide from insulin. It contains two sensitive bonds (Phe-Tye; Tyr-Thr) in the intact insulin β-chain, however the heptapeptide is hydrolyzed only between phenylalanine and tyrosine. The N-terminal hexapeptide from glucagon contains a sensitive bond between glycine and threonine when it is part of the intact glucagon molecule. However, when the hexapeptide is digested with the proteinase that bond is not hydrolyzed but instead the serine-glutamine bond is hydrolyzed. This seems to indicate a need for at least three amino acids on the carboxyl side of a sensitive bond in order for the enzyme to attack the bond. This is also supported by the finding that in both digests of carboxymethylated-β-chain of insulin and the glucagon digest the smallest isolated peptide in the digest was a tripeptide. There were no free amino acids or dipeptides released.

This proteinase seems to be very similar to the enzyme purified from *Serratia piscatorum* [3]. The sedimentation values are close, 3.95 S compared to 3.95 S for the enzyme from *S. piscatorum*. The molecular weight for the enzyme from *S. marcescens* is 51 900 compared to 60 000 for the other enzyme. The hydrolysis sites on the insulin β-chain are similar although not identical. The pH optima and sensitivities to inhibitory agents for the two enzymes are similar. However the enzyme from *S. marcescens* was not reactivated by Zn^{2+} after EDTA treatments and the enzyme from *S. piscatorum* was not affected by *O*-phenanthroline while the other enzyme was inhibited.

It seems likely that the proteinase produced by *Serratia* strains that are pathogenic for insects are all going to be very similar. This is consistent with the idea that these enzymes are the toxic agent, and hence for pathogenicity to be present the enzyme activity would be preserved in the various microorganisms.

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