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SUBSTRATE SPECIFICITY OF THREE DIFFERENT EXTRACELLULAR PROTEOLYTIC ENZYMES FROM *STAPHYLOCOCCUS AUREUS*

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SUMMARY

The proteolytic specificities of three different extracellular proteinases from *Staphylococcus aureus* have been determined Carboxymethylated derivatives of yeast and horse liver alcohol dehydrogenases were used as substrates Protease I had the most selective specificity, cleaving mainly peptide bonds at the carboxy-terminal side of a single type of residue, glutamic acid Protease II had the broadest specificity with no clear preferential cleavages of particular peptide bonds, but with extensive proteolytic activity Protease III had a rather broad specificity, cleaving at the amino-terminal side of hydrophobic residues The enzymes resemble different types of other known proteinases and their combined effect is great and probably of importance for growth and virulence of *S aureus*

INTRODUCTION

Proteolytic activity in *Staphylococcus aureus* has been known for a long time [1] According to the classification of Baird-Parker [2, 3], 91% of *S aureus* strains have proteolytic activity Drapeau and co-workers [4, 5] isolated a proteolytic enzyme specific for glutamoyl bonds from *S aureus* strain V8 Rydén et al [6] also isolated a protease with the same specificity from a mutant of *S aureus* strain 8325 N Three different extracellular proteolytic enzymes from *S aureus* strain V8 were purified and characterized by Arvidson and coworkers [7, 8] These enzymes were called Protease I, II and III [7, 8], and this nomenclature will be followed throughout this study The purpose of this work was to determine the substrate specificities of Protease I, II and III Carboxymethylated derivatives of alcohol dehydrogenase (EC 1 1 1 1) from yeast and horse liver were used as substrates

MATERIALS AND METHODS

Protease I, II and III were purified as previously described [7, 8] The pure enzymes were obtained in 0 05 M ammonium acetate buffer (pH 7 5) in a concentration of 0 5 mg/ml and had an activity of 150 units/ml (Protease I), 750 units/ml (Protease II) and 60 units/ml (Protease III) Proteolytic activity was determined ac-

cording to Kunitz [9] but with minor modifications as introduced by Arvidson et al [10]

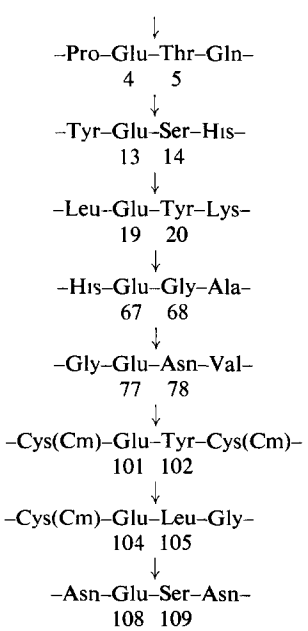
Yeast and horse liver alcohol dehydrogenases (EC 1 1 1 1) were obtained from Boehringer Mannheim, Germany, and [^{14}C]carboxymethylated in 6 M guanidine HCl after reduction, as previously described [11, 12] After dialysis the proteins were recovered by lyophilization Digestion with Protease I was performed in 0 06 M NH_4HCO_3 (pH 7 8) The same buffer containing 0 1 mM dithioerythritol was used for Protease II Digestion with Protease III was performed in 0 05 M ammonium acetate buffer (pH 7 5) containing 1 0 mM $CaCl_2$ All digestions were performed at 37 °C for 18 h The concentration of substrate was 3 mg/ml, the total amount of substrate was 25–100 mg and the enzyme substrate ratio was 1 100

Digestions were terminated by freezing After lyophilization, peptide mixtures were purified by different steps of paper chromatography and high-voltage paper electrophoresis at pH 1 9, 3 5 and 6,5, as previously described [11] Peptides were detected by autoradiography and by staining guide strips with a cadmium–ninhydrin reagent [13]

The compositions of peptides were determined after hydrolysis with 6 M HCl at 110 °C for 24 h Liberated amino acids were estimated by paper electrophoresis at pH 1 9 or quantitatively determined on a Beckman 120 B amino acid analyzer

N-terminal analysis was performed by dansylation and amino acid sequences were determined by the dansyl-Edman procedure [14–17]

C-terminal residues were determined by hydrazinolysis of the peptides at 110 °C for 6 h These analyses permitted identification of the peptides in the known amino acid sequences of the yeast [18] and horse liver [19] enzymes



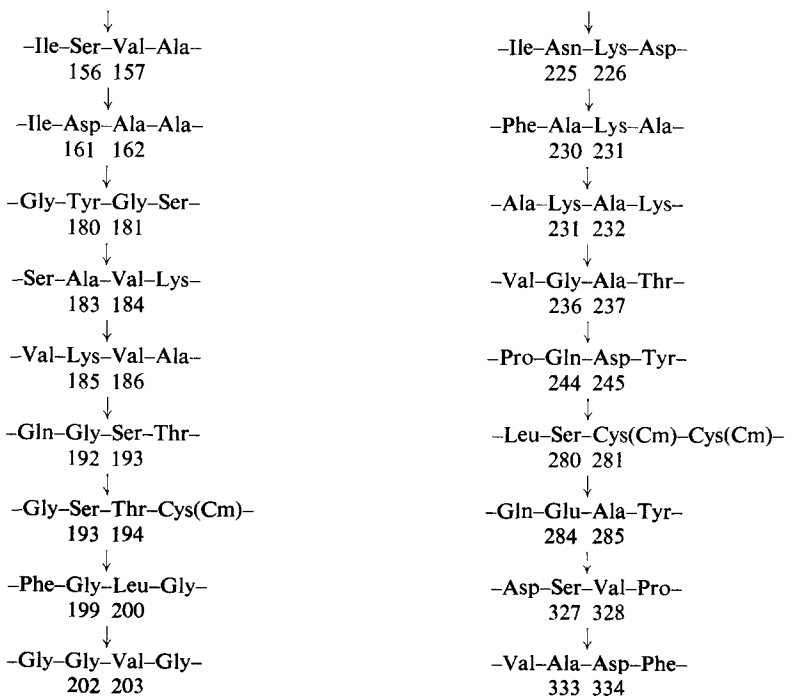
Scheme 1 Peptide bond cleavages by Protease I Carboxymethylated yeast alcohol dehydrogenase was used as substrate in 0 06 M NH_4HCO_3 (pH 7 8) and identified peptide bond cleavages were positioned in the primary structure of the protein Cm, Carboxymethyl-

Protease I

Using carboxymethylated yeast alcohol dehydrogenase as substrate a limited proteolysis was obtained as judged by the presence of large insoluble peptides From the supernatant after centrifugation, five different peptides were identified They consisted of peptides containing Residues 5–13, 14–19, 68–77, 102–104 and 105–108 in the polypeptide chain [18] The peptide bonds cleaved are given in Scheme I Remaining soluble peptides originate from regions of the protein not yet definitely established All these peptide bond cleavages are therefore not clear, but hydrolysis of several glutamoyl bonds were proven, although single cleavages of other bonds could not be excluded From these results and those in Scheme I it is obvious that Protease I is highly specific for peptide bonds at the carboxy-terminal side of glutamic acid No clear influences by the nature of the following residue or adjacent residues are noticed (Scheme I)

Protease II

This proteinase yielded a completely soluble digest containing a large number of small peptides when carboxymethylated horse liver alcohol dehydrogenase was used as substrate Peptide bond cleavages listed in Scheme II are based on the following peptides 157–161, 181–183, 186–192, 186–193, 200–202, 226–230, 231–236, 232–244, 281–284 and 328–333 Apart from these ten peptides several di- and tripeptides

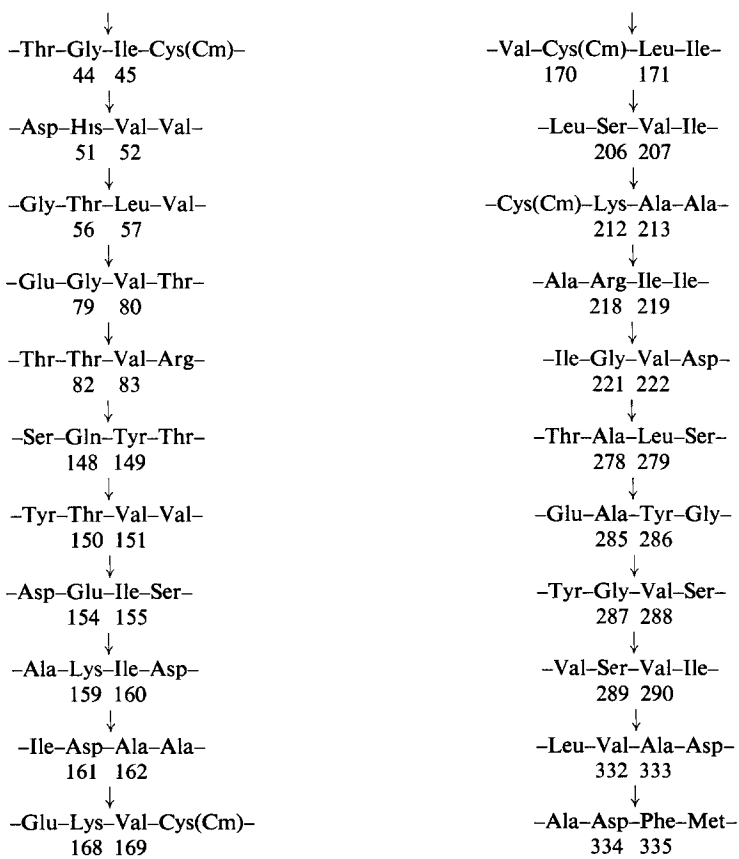


Scheme II Peptide bond cleavages by Protease II Carboxymethylated horse liver alcohol dehydrogenase was used as substrate in 0 06 M NH_4HCO_3 (pH 7 8) with 0 1 mM dithioerythritol Identified peptide bond cleavages were positioned in the primary structure of the protein Cm, carboxymethyl-

were isolated, but could, due to small size, not be unambigously identified in the amino acid sequence of the protein, since similar sequences occur at several positions It is obvious from Scheme II that the side chains of the residues on either side of the susceptible bonds have no common properties In addition, N- and C-terminal analyses of the unpurified digest revealed that most amino acids were present in these two positions of the peptides

Protease III

This proteinase, too, yielded a completely soluble digest with carboxymethylated horse liver alcohol dehydrogenase as substrate The cleavages listed in Scheme III are based on the following peptides 45–51, 52–56, 80–82, 149–150, 151–154, 160–161, 169–170, 207–212, 219–221, 279–285, 286–287, 288–289 and 333–334 These thirteen peptides could all be identified in the primary structure of the protein Several other small peptides were also isolated, but their positions in the amino acid sequence of the protein could not be unambigously determined All of these peptides, however,



Scheme III Peptide bond cleavages by Protease III Carboxymethylated horse liver alcohol dehydrogenase was used as substrate in 0 05 ammonium acetate buffer (pH 7 5) with 1 0 mM $CaCl_2$ Identified peptide bond cleavages were positioned in the primary structure of the protein Cm, carboxymethyl-

had a hydrophobic N-terminus, which the identified peptides also had (Scheme III) Furthermore N-terminal analysis of the unresolved digest obtained after treatment with Protease III revealed that only hydrophobic amino-terminal residues were present It is therefore clear that this protease preferentially cleaves peptide bonds involving the amino-terminal side of hydrophobic residues, i e alanine, isoleucine, leucine, phenylalanine, tyrosine and valine (Scheme III)

DISCUSSION

It is established in this investigation that the three proteinases produced by *S aureus* strain V8 have different substrate specificities This was indicated already by the differences in the physical appearances of the digests and was confirmed after purification and characterization of the peptides

Protease I has the most selective specificity, cleaving mainly at the carboxy-terminal side of glutamic acid (Scheme I) Its specificity is therefore identical to that of the proteinases reported by Drapeau et al [5] and Rydén et al [6], but it may be noted that differences in molecular weight and sensitivity to diisopropyl fluorophosphate have been reported for these proteinases [4, 6, 8] *S aureus* is so far the only microorganism reported to have a glutamoyl-specific protease, although this has been reported from a plant, germinated *Sorghum* [20] It was not possible, in the present investigation, to demonstrate any influence of the amino acids in the vicinity of the susceptible bonds but such factors may not be excluded (cf [5])

Protease II has the broadest specificity and most amino acids were found both at the carboxy- and amino-terminal side of peptide bonds cleaved, as well as at adjacent positions (Scheme II) This type of specificity together with other characteristics of Protease II, such as activation by reducing agents [8], inactivation by heavy metals [8] and low molecular weight [8] are all properties reminiscent of a papain type of proteinase

Protease III has a proteolytic specificity for the amino-terminal side of hydrophobic residues (Scheme III) No clear influence by the residue contributing the carboxyl group of a susceptible bond could be demonstrated The substrate specificity of Protease III, as well as molecular weight [7] and sensitivity to EDTA [7], are all properties typical of a thermolysin type of proteinase

S aureus, therefore, has three proteinases with entirely different specificities One with a clear specificity for cleaving mainly on the carboxy-terminal side of a single type of residue, one for cleavage on the amino-terminal side of a group of related residues and a third with no obvious specificity but with extensive proteolytic activity The enzymes also differ in size and sensitivity to chemical reagents and resemble different types of known proteases

The exact function of these enzymes is not known *S aureus*, however, has a growth demand for amino acids [10, 21–25] especially in case of toxin production It is therefore probable that degradation of tissue proteins by extracellular proteolytic enzymes is of value in order to supply the *S aureus* cell with amino acids and small peptides that can be transported across the bacterial membrane The combined effect of three proteinases with different substrate specificities is therefore presumably of great physiological importance for this parasitically adapted bacterium It has even been reported that the presence of certain peptides stimulates growth and toxin

production in *S aureus* [26] The presence of proteinases can therefore have an effect on the bacterial virulence, which is presently under further investigation

ACKNOWLEDGMENTS

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