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Staphylococcal Protease: A Proteolytic Enzyme Specific for Glutamoyl Bonds

(digestion of proteins/aminoacid composition/end-group analysis)

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ABSTRACT **An extracellular protease of *Staphylococcus aureus*, strain V8, previously shown to cleave specifically the peptide bonds on the carboxyl-terminal side of either aspartate or glutamate residues in phosphate buffer (pH 7.8) hydrolyzes only glutamoyl bonds in either ammonium bicarbonate (pH 7.8) or ammonium acetate (pH 4.0). Of all aspartoyl bonds tested, only the Asp-Gly linkage is cleaved at a detectable rate. The staphylococcal protease hydrolyzes all of the seventeen different glutamoyl bonds studied, although those involving hydrophobic aminoacid residues with bulky side chains are cleaved at a lower rate.**

The purification of a proteolytic enzyme from the culture filtrates of *Staphylococcus aureus*, strain V8, has recently been reported (1). Partial characterization of this protease indicated that it had a molecular weight of 12,000, was inhibited by diisopropyl fluorophosphate, and was active over the pH range of 3.5–9.5, but exhibited maximal activity at pH 4.0 and 7.8 with hemoglobin as substrate. Digestion of oxidized ribonuclease by the protease, followed by identification of the amino- and carboxyl-terminal residues of the liberated peptides, revealed that the protease cleaved specifically peptide bonds on the carboxyl-terminal side of either aspartic acid or glutamic acid. The specificity of the protease for bonds involving only dicarboxylic amino acids was also demonstrated by the inability of the enzyme to degrade casein in which all of its carboxyl groups had been coupled with glycine ethyl ester in amide linkage, whereas unmodified casein was readily digested.

At pH 7.8, in sodium or potassium phosphate buffer, both aspartoyl and glutamoyl peptide bonds were cleaved (1). However, the results of subsequent studies suggested that the protease could be rendered even more restrictive depending on the type of buffer used during the digestion of the protein substrates. In the present study, evidence is presented that indicates that only glutamoyl bonds are hydrolyzed when digestion occurs in ammonium bicarbonate buffer at pH 7.8, or in ammonium acetate buffer at pH 4.0.

MATERIALS AND METHODS

Reduced, carboxymethylated A and B chains of insulin (porcine) were purchased from Schwartz/Mann. Bovine pancreatic ribonuclease, egg-white lysozyme, and poly(L-glutamic acid) were obtained from Sigma. Horse-heart myoglobin was purchased from Pentex. The α subunit of *Escherichia coli* tryptophan synthase was donated by Dr. Charles Yanofsky. Staphylococcal protease was purified (1) by precipitation of the enzyme from culture filtrates with ammonium sulfate, followed by precipitation with acetone, and finally by chromatography on a DEAE-cellulose column. Proteins used for substrates were either oxidized with performic acid (2) or reduced and carboxymethylated (3). The prosthetic group of myoglobin was removed (4). Proteins were digested at 37° for 18 hr in 50 mM ammonium bicarbonate (pH 7.8) or ammonium acetate buffer (pH 4.0). The enzyme to substrate ratio varied from 1/30 to 1/40. The amino-terminal residues were identified as dansyl derivatives by chromatography on silica-gel plates (5) or on polyamide sheets (6). In one instance, the amino-terminal residues were determined quantitatively by the method of Stark and Smyth (7). Carboxyl-terminal residues were determined by the Akabori method (8), and were identified on an aminoacid analyzer. Peptides in the digests were separated by high-voltage electrophoresis at pH 3.7 or 6.4. The peptides derived from ribonuclease were fractionated by chromatography on a Dowex 50-X2 column. Aminoacid analysis was performed on a Beckman 120C analyzer. Samples were hydrolyzed with 6 N HCl under reduced pressure at 105° for 24 hr.

RESULTS

Aminoacid composition of peptides

Digestion of either the A or the B chain of insulin by staphylococcal protease in ammonium bicarbonate buffer yielded only three peptides on electrophoresis. The aminoacid composition of these peptides is presented in Table 1. Knowing the aminoacid sequence of the insulin chains (9), it is evident from Table 1 that the peptides derived from the A chain corresponded to sequences 1–4, 5–17, and 18–21, and those derived from the B chain to sequences 1–13, 14–21, and 22–30. These results, therefore, indicate that only the two glutamoyl bonds present at positions 4,5 and 17,18 in the A chain and 13,14 and 21,22 in the B chain were cleaved by the protease. Digestion of ribonuclease under the same conditions yielded six peptides, the aminoacid composition of which (Table 2) coincided exactly with those expected for the peptides cleaved at the five glutamoyl bonds at positions 2,3; 9,10; 49,50; 86,87; and 111,112. Apparently, none of the five aspartoyl bonds also present in ribonuclease had been hydrolyzed. Table 2 also shows the aminoacid composition of the three peptides derived from lysozyme. Again, as deduced from the composition of these peptides, only the two glutamoyl bonds of lysozyme (positions: 7,8; 35,36) could have been split; none of the aspartoyl bonds could have been cleaved at a rate comparable to that of the glutamoyl bonds.

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TABLE 1. *Aminoacid composition of peptides derived from insulin chains by the action of staphylococcal protease*

	A Chain			B Chain		
	Sequences			Sequences		
	1–4	5–17	18–21	1–13	14–21	22–30
Lys	—	—	—	—	—	0.98 (1)
His	—	—	—	1.85 (2)	—	—
Arg	—	—	—	—	—	0.97 (1)
Cys	—	2.68 (3)	0.95 (1)	0.95 (1)	0.92 (1)	—
Asp	—	—	2.18 (2)	0.99 (1)	—	—
Thr	—	0.88 (1)	—	—	—	1.00 (1)
Ser	—	1.97 (2)	—	0.95 (1)	—	—
Glu*	1.10 (1)	3.41 (3)	—	2.12 (2)	1.11 (1)	—
Pro	—	—	—	—	—	1.10 (1)
Gly	1.22 (1)	—	—	1.35 (1)	1.01 (1)	1.12 (1)
Ala	—	—	—	—	0.85 (1)	1.04 (1)
Val	0.86 (1)	—	—	1.90 (2)	1.06 (1)	—
Ile	0.80 (1)	0.85 (1)	—	—	—	—
Leu	—	2.23 (2)	—	1.98 (2)	2.16 (2)	—
Tyr	—	0.95 (1)	0.86 (1)	—	0.88 (1)	0.89 (1)
Phe	—	—	—	0.82 (1)	—	1.89 (2)

Values in parentheses represent the aminoacid composition of the peptides expected if only glutamoyl bonds in the insulin chains were cleaved.

* Also includes glutamine.

Dansylation

The possibility of slow hydrolysis of the aspartoyl bonds present in lysozyme and ribonuclease could not be completely ruled out. This possibility was tested by subjecting the various digests to dansylation and subsequently by identifying the dansyl derivatives. This highly sensitive method should reveal the presence of trace amounts of amino-terminal residues, if present. From ribonuclease, only the derivatives of serine, threonine, glycine, and arginine were detected as new amino-terminal residues. Since these residues are adjacent to glutamic acids in the aminoacid sequence, none of the aspartoyl bonds could have been hydrolyzed. Dansylation of the peptides obtained after digestion of lysozyme yielded the derivatives of threonine, leucine, and a trace of glycine. Since the release of glycine as an amino-terminal residue is not to be expected, its presence could have arisen from partial cleavage of one or more of the four Asp-Gly bonds of lysozyme. Confirmation was obtained by hydrazinolysis, which revealed traces of aspartic acid, in addition to glutamic acid, as new carboxyl-terminal residues, whereas in the case of ribonuclease only glutamic acid was found.

TABLE 2. *Aminoacid composition of peptides derived from ribonuclease and lysozyme*

	Ribonuclease						Lysozyme*		
	Sequences						Sequences		
	1–2	3–9	10–49	50–86	87–111	112–124	1–7	8–35	36–129
Lys	1.09 (1)	1.05 (1)	3.25 (3)	2.14 (2)	3.38 (3)	—	1.04 (1)	2.04 (2)	3.20 (3)
His	—	—	2.30 (2)	—	0.77 (1)	0.86 (1)	—	0.83 (1)	—
Arg	—	—	3.16 (3)	0.91 (1)	—	—	1.20 (1)	2.02 (2)	7.80 (8)
Cys	—	—	1.96 (2)	3.95 (4)	2.24 (2)	—	0.80 (1)	0.95 (1)	6.05 (6)
Asp	—	—	6.26 (6)	5.39 (5)	2.00 (2)	2.14 (2)	—	3.25 (3)	17.90 (18)
Met	—	—	3.00 (3)	0.81 (1)	—	—	—	0.83 (1)	1.03 (1)
Thr	—	0.95 (1)	3.00 (3)	2.92 (3)	3.00 (3)	—	—	—	6.80 (7)
Ser	—	—	6.65 (7)	4.70 (5)	1.93 (2)	1.10 (1)	—	0.77 (1)	8.50 (9)
Glu†	0.91 (1)	1.06 (1)	2.64 (3)	5.23 (5)	2.36 (2)	—	1.02 (1)	1.45 (1)	3.06 (3)
Pro	—	—	0.89 (1)	—	0.79 (1)	2.07 (2)	—	—	2.30 (2)
Gly	—	—	—	1.16 (1)	1.11 (1)	0.97 (1)	1.09 (1)	3.12 (3)	8.46 (8)
Ala	—	3.00 (3)	2.14 (2)	3.04 (3)	4.00 (3)	1.16 (1)	—	4.94 (5)	7.17 (7)
Val	—	—	1.68 (2)	2.96 (3)	0.91 (1)	2.88 (3)	1.07 (1)	1.19 (1)	4.20 (4)
Ile	—	—	—	0.96 (1)	1.75 (2)	—	—	—	5.70 (6)
Leu	—	—	1.11 (1)	1.06 (1)	—	—	—	3.05 (3)	5.10 (5)
Tyr	—	—	0.96 (1)	1.67 (2)	1.57 (2)	0.88 (1)	—	1.75 (2)	0.86 (1)
Phe	—	0.94 (1)	1.03 (1)	—	—	0.90 (1)	0.79 (1)	0.80 (1)	0.87 (1)

Values in parentheses represent the aminoacid composition of the peptide expected if only the glutamoyl of the proteins were cleaved.

* Try was not determined. † Also includes glutamine.

Cleavage of other glutamoyl bonds

Hydrolysis by the protease of all nine different glutamoyl bonds encountered in the insulin chains, ribonuclease, and lysozyme suggested that the nature of the residue contributing the amino group to the peptide linkage is not critical. Further support of this hypothesis came from studies of other glutamoyl bonds that can be found in myoglobin and the α subunit of *E. coli* tryptophan synthase (9). Digestion of these proteins by the protease and identification of the new amino-terminal residues released (which were the residues adjacent to glutamic acid in the sequence) indicated that all of the glutamoyl bonds were hydrolyzed in these two protein substrates. Hydrazinolysis demonstrated the presence of only glutamic acid in both cases, except for a trace of aspartic acid in digests of the tryptophan synthase subunit. Whether or not this aspartic acid residue that appears as a new carboxyl-terminal is the result of some cleavage of the Asp-Gly bond could not be established unambiguously in view of the presence of a Glu-Gly sequence in the protein. Table 3 summarizes these results. Apparently, all of the seventeen different glutamoyl bonds present in the protein substrates studied, including a Glu-Pro linkage, were cleaved by the protease.

Specificity of the protease in ammonium acetate buffer, pH 4.0

No significant differences in the specificity of the protease were noted when insulin chains, ribonuclease, lysozyme, or myoglobin were digested at pH 4.0. However, the release of glycine as the new amino-terminal, resulting from the partial cleavage of one or more of the Asp-Gly linkages of lysozyme that occurred at pH 7.8, was not detected. Moreover, there were no new aspartic acid residues at the carboxyl-terminal positions of the peptides produced from lysozyme.

Relative rate of cleavage of glutamoyl bonds

Since peptides containing more than one glutamic acid residue could not be found in digests obtained from the insulin chains and ribonuclease, quantitative cleavage of the glutamoyl bonds was suggested*. Digestion of myoglobin under identical conditions, however, appeared to be incomplete, as estimated by peptide mapping. Since the dansylation procedure revealed that all glutamoyl bonds in myoglobin were hydrolyzed by the protease, it would appear that some bonds were hydrolyzed at a lower rate than others. Information bearing on the rate of hydrolysis of the different glutamoyl bonds of myoglobin was obtained by quantitative measurement (by the cyanate procedure) of the amount of each amino-terminal residue released. As shown in Table 4, the residues lysine, aspartic acid, threonine, alanine, and methionine were released nearly quantitatively. However, the release of valine, leucine, and phenylalanine was only about 25%. These results suggest that glutamoyl bonds involving hydrophobic amino acids with bulky side chains are somewhat resistant to hydrolysis by the protease.

TABLE 3. *Cleavage of glutamoyl bonds in various proteins by staphylococcal protease*

Glutamoyl bonds tested	Substrates	Glutamoyl bonds tested	Substrates
Glu—Lys	5,6	Glu—Pro	6
Glu—His	N.D.‡	Glu—Gly	3,6
Glu—Arg	2,3,6	Glu—Ala	2,5,6
Glu—Cys	N.D.‡	Glu—Val	5
Glu—Asp	5	Glu—Met	5,6
Glu—Asn	1,6	Glu—Ile	N.D.‡
Glu—Thr	3,5	Glu—Leu	4,5,6
Glu—Ser	3,4,6	Glu—Tyr	6
Glu—Glu	—*	Glu—Phe	5,6
Glu—Gln	1,6	Glu—Try	5†

1, insulin A chain; *2*, insulin B chain; *3*, ribonuclease; *4*, lysozyme; *5*, myoglobin; *6*, α Subunit of *E. coli* tryptophan synthase.

* Oligopeptides were obtained from a digest of poly(L-glutamic acid).

† Identified as the phenylthiohydantoin derivative.

‡ N.D., not determined.

TABLE 4. *Yields of new amino-terminal residues liberated from myoglobin*

Amino-terminal residues	Recovery* (%)
Lysine	95.1
Aspartate	76.4
Threonine	76.6
Alanine	79.1
Valine	21.0
Methionine	104.4
Leucine	32.1
Phenylalanine	25.5
Tryptophan	N.D.†

* Calculated as percent of the amino-terminal glycine.

† N.D., not determined.

DISCUSSION

The results presented in this study strongly support the previous conclusion that the staphylococcal protease specifically hydrolyzes peptide bonds involving dicarboxylic amino-acid residues (1). Since none of the glutaminoyl bonds were cleaved, these results also indicate that the γ-carboxyl group of glutamic acid must be unsubstituted for hydrolysis to occur. While digestion of ribonuclease in phosphate buffer at pH 7.8 resulted in the cleavage of both aspartoyl and glutamoyl bonds (1), only glutamoyl bonds were cleaved when digestion was performed in ammonium bicarbonate or ammonium acetate buffer. The reason for this buffer-dependent specificity is not clear.

Of all the aspartoyl bonds present in the proteins tested, only cleavage of the Asp-Gly bonds was detectable. The fact that glutamoyl, but not aspartoyl, bonds are cleaved by the protease indicates that the length of the side chain is critical for the enzyme specificity. However, when a small aminoacid residue is adjacent to an aspartic acid in a polypeptide chain, the β-carboxyl group of aspartic acid may become accessible to the enzyme-binding site; this accessibility could explain the partial cleavage of the Asp-Gly bond.

Glutamic acid is a relatively rare amino acid in the sequence of proteins; it occurs on the average once for every 20 residues (9). Digestion of proteins by the staphylococcal protease,

* Lysozyme was not completely digested by the protease, due to its poor solubility after oxidation or carboxymethylation.

therefore, would liberate peptides twice as long as those generally obtained by tryptic digestion. These larger peptides produced by selective proteolysis could be digested further by the protease in phosphate buffer at pH 7.8, thus cleaving the aspartoyl bonds. In addition, this enzyme would also help in differentiating between aspartic acid or glutamic acid and their amide forms, asparagine or glutamine, since bonds involving the latter amino acids are not hydrolyzed. Although conditions for the quantitative cleavage of certain glutamoyl bonds need further investigation, we feel that the staphylococcal protease will be a valuable tool for studies of protein sequence.

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