ACTION OF STAPHYLOCOCCAL PROTEINASE ON PEPTIDES OF VARYING CHAIN LENGTH AND COMPOSITION

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Received July 12,1976

SUMMARY

Staphylococcal proteinase was found to hydrolyze - Glu-X-bond in 29 peptides where X is polar, hydrophobic or basic. No hydrolysis was observed between adjacent glutamyl residues or at a Glu-Pro bond. When Glu is within two residues of the NH_2-terminus or two or three residues from the COOH-terminus, hydrolysis was limited or undetectable. This suggests that the peptide must be bound to the enzyme over a region extending several residues on either side of the potentially susceptible bond. The peptides studied were all derived from the NAD-specific glutamate dehydrogenase of Neurospora crassa.

Purification and properties of a protease from the culture filtrate of Staphylococcus aureus, strain V8, have been reported (1,2). The specificity of this proteolytic enzyme for peptide bonds involving the carboxyl groups of only dicarboxylic amino acid residues makes the protease a useful additional tool for studies of amino acid sequences. Earlier reports (2) have suggested that the specificity of the protease may depend upon the type of buffer used during the digestion. It appeared that COOH-linked bonds of glutamyl residues are hydrolyzed in ammonium bicarbonate buffer at pH 7.8, or in ammonium acetate buffer at pH 4.0, whereas peptide bonds on the COOH-terminal side of either aspartyl or glutamyl residues are cleaved in sodium or potassium phosphate buffer at pH 7.8.

We have employed this protease for sequence determinations of peptides from the NAD-specific glutamate dehydrogenase of Neurospora crassa (3,4). The protease was used to hydrolyze fragments between 5 and 134 residues in

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length produced by cyanogen bromide or tryptic cleavage of the protein. In view of the importance of the protease for sequence determinations, we report here on the effect of peptide chain length and neighboring residues on potentially susceptible bonds.

MATERIALS AND METHODS: The protease from Staphylococcal aureus, V8 strain, was a generous gift from Dr. G. R. Drapeau. Another sample of the protease, which appeared identical in its specificity and activity towards a number of substrates, was also purchased from Miles-Yeda. Peptides were obtained from the NAD-specific glutamate dehydrogenase from Neurospora crassa and will be described elsewhere. Peptides were hydrolyzed in 0.1 M ammonium bicarbonate (pH 8.0) or 0.05 M potassium phosphate (pH 7.8) at 40° at an enzyme to substrate ratio of 1/20 for 6h, or longer where indicated. Products were isolated by chromatography on columns (1.8 x 150 cm) of Sephadex G-25, preparative electrophoresis on paper at pH 1.9, 2.6 or 6.5 or preparative chromatography on paper with 1-butanol-pyridine-acetic acid-water (15:10:3:12 v/v). Edman degradation was performed as described earlier (5). In some cases, the extent of hydrolysis was monitored by the intensity of color produced by the substrate or products with ninhydrin after electrophoresis on paper at pH 1.9 or 3.6. In cases of incomplete hydrolysis, relative intensities of spots were measured by extracting into methanol (3 ml), filtering, and measuring the absorbance at 570 nm of the filtrate. Yields of products of a peptide containing ^{14}C carboxymethylcysteine were measured by cutting out the spots obtained by ninhydrin staining, and measuring counts by scintillation. In each case, evidence of the acidic nature of the glutamyl or aspartyl residues at or near the point of cleavage was obtained by sequence determination of an overlapping peptide, or by hydrolysis with carboxypeptidase A, occasionally at pH 5.5 (6) or with aminopeptidase M.

RESULTS AND DISCUSSION: The amino acid sequences in the neighborhood of peptide bonds which were hydrolyzed by the protease are listed in Table I. Evidence for the purity and sequence of peptides will be described elsewhere. In 0.1 M ammonium bicarbonate (pH 8.0) instances of cleavage at the COOH-terminal side of 29 unique glutamic acid residues were observed whereas only one aspartyl bond of 29 was hydrolyzed. The aspartyl residue occurred in the sequence: -Gln-Asp-Asn-Ala-.

Residues on the carboxyl-terminal side of the hydrolyzed bonds include those with polar, charged and non-polar side-chains. However, although cleavage at the Glu-Asp bond was observed in the sequence: -Asp-Glu-Asp-Leu-, no instance of cleavage between two adjacent glutamyl residues was noted. Three -Glu-Glu- sequences were found intact in peptides isolated from Staphylococcal protease hydrolyzates (Table II).

Hydrolysis at a Glu-Pro bond was not observed. Treatment of an octa-

TABLE I

Bonds Hydrolyzed by Staphylococcal Protease in 0.1 M Ammonium Bicarbonate (pH 8) at 40°).

-Trp-Asp-Asn-GLU-Lys-Ile-Arg-Arg-	-Arg-His-Arg-GLU-Ala-Phe-COOH-
-Pro-Asn-Gly-GLU-His-Val-Asn-Gly-	NH_2-Ser-Lys-GLU-Ala-Tyr-Gln-Ile-
-Val-Ser-Asn-GLU-His-Glu-Glu-Hse-COOH	-Leu-Asp-Met-GLU-Ala-Ser-Asp-His-
-Ile-Trp-Arg-GLU-His-Glu-Gln-Thr-	-Phe-Leu-Pro-GLU-Val-Glu-Tyr-Pro-
-Pro-Arg-Tyr-GLU-His-Arg-Leu-Glu	-Pro-His-Ile-GLU-Val-Phe-Asp-Ile-
-Thr-Ile-Ile-GLU-Arg-Val-Pro-Asp-	-Thr-Arg-Leu-GLU-Val-Ile-Ser-Asp-
-Ser-Leu-Asp-GLU-Asp-Leu-Gln-Arg-	-Tyr-Val-Lys-GLU-Val-Gln-Asn-Lys-
NH_2 -Leu-Ser-Asn-GLU-Thr-Tyr-COOH	-Gly-Ser-Glu-GLU-Met-Arg-COOH
-Arg-Phe-Val-GLU-Thr-Arg-Arg-	-Ser-His-Val-GLU-Ile-Leu-Ser-Lys-
-His-Arg-Leu-GLU-Ser-Lys-Tyr-Leu-	-Asp-Lys-Asp-GLU-Leu-Arg-Arg-Leu-
NH_2-Leu-GLU-Ser-Lys-Tyr-Leu-	-Gln-Arg-Ser-GLU-Leu-Trp-Asp-Asn-
NH_2-Glu-His-GLU-Gln-Thr-Gly-Leu-	-Asn-Ala-Pro-GLU-Phe-Tyr-Gln-Ala-
-Phe-Asp-Ile-GLU-Gly-Ser-Glu-Glu-	-Ala-Arg-Leu-GLU-Phe-Glu-Ala-Ile-
-Asp-Ile-Pro-GLU-Gly-Gly-Ser-Lys-	NH_2-Leu-Gly-Thr-GLU-Tyr-Thr-Ser-Leu-
-Leu-Glu-Phe-GLU-Ala-Ile-Trp-Arg-	-Lys-Ile-Gln-ASP-Asn-Ala-Arg-Leu-

TABLE II

Peptides Containing Glutamyl Residues Not Hydrolyzed.

-Asp-Lys-Arg-Glu-Glu-Ile-Arg-Leu-
-Asn-Glu-His-Glu-Glu-Hse-COOH
-Glu-Gly-Ser-Glu-Glu-Met-Arg-COOH
-Asp-Thr-Ser-Glu-Pro-Gly-Met-Thr-

Conditions:- 0.1 M ammonium bicarbonate (pH 8) for 6h at 40°.

peptide, NH_2-Ile-Asp-Thr-Ser-Glu-Pro-Gly-Hse-COOH (R_{Asp} at pH 1.9, 0.80) with the protease yielded no other spots staining with ninhydrin after electrophoresis at pH 1.9, and only PTH-isoleucine was obtained by one step

of Edman degradation. The length of peptide in the neighborhood of the glutamyl residue was not a factor since no hydrolysis was observed in an overlapping peptide of 36 residues (Table III). Although cleavage of -Glu-Pro- bonds has been reported (2), it would appear that some bonds of this type have a limited susceptibility towards the protease. Behrens and Brown (7) have also reported that Glu-Pro bonds are resistant to Staphylococcal proteinase.

In addition to a Glu-Pro and Glu-Glu sequence, the 36 residue peptide (Table III) had an NH_2-terminal glutamyl residue that was not removed by the protease. Another example of this lack of exopeptidase activity (a common feature of other proteases such as trypsin and chymotrypsin) was obtained in the study of Glu-His-Glu-Gln-Thr-Gly-Leu-Pro-Arg (Table I). The NH_2-terminal tripeptide Glu-His-Glu was isolated in good yield and no free glutamic acid was detected. The same Glu-His bond was cleaved in a digest of 134-residue peptide that contained the nonapeptide sequence.

TABLE III

Action of Staphylococcal Proteinase on a 36-residue Peptide [a]

Glu-Asp-Lys-Arg-Glu-Glu-Ile-Arg-Leu-Asp-Met-GLU-Ala-Ser-Asp-His-Ala-Ile-Tyr-Ile-Asp-Thr-Ser-Glu-Pro-Gly-Met-Thr-Ser-Asn-Thr-Phe-Asp-Gly-Pro-Arg

[a] Hydrolysis occurred only at the underlined bond.

In certain sequences, given in Table IV, the protease did not liberate an NH_2-terminal dipeptide. The peptide Leu-Glu-Val-Ile-Ser-Asp-Arg (Sequence No. 1, Table IV) gave only the ninhydrin-staining spot exhibited by the starting material after 6h in presence of the protease. One step of Edman degradation released only the original NH_2-terminal amino acid as PTH-Leu. In an overlapping peptide containing the same sequence, but extended at the NH_2-

TABLE IV

Observations on Hydrolysis of Glutamyl Residues near the NH_2-or COOH-terminus

No.	Sequence of peptide	Extent of cleavage (%)[a]	Method [b]
1.	NH_2-Leu-Glu-Val-Ile-Ser-Asp-	0	A + C
2.	NH_2-Ala-Glu-Thr-Pro-Gly-Ile-	0	A + C
3.	NH_2-Ser-Glu-Val-Leu-Asp-Ala-	0	A
4.	NH_2-Val-Glu-Tyr-Pro-Lys-Pro-	0	A + B
5.	NH_2-Ser-Glu-Leu-Trp-Asp-Asn-	0	A
6.	NH_2-Leu-Glu-Ser-Lys-Tyr-Leu-	100	A + B
7.	NH_2-Lys-Ala-Glu-Thr-Pro-Gly-Ile-	0	A
8.	NH_2-Pro-Lys-Glu-Thr-Arg-Leu-Glu-	0	A + B
9.	NH_2-Ser-Lys-Glu-Ala-Tyr-Gln-Ile-	100	A
10.	-Thr-Ile-Ile-Glu-Arg-COOH	0	A
11.	-Arg-His-Arg-Glu-Ala-Phe-COOH	67	A + C
12.	NH_2-Leu-Ser-Asn-Glu-Thr-Tyr-COOH	10	A
13.	Ser-Asn-Glu-Thr-Tyr-COOH	0	A
	Ser-Asn-Glu-Thr-Tyr-COOH	80 [c]	A
14.	-Trp-Asp-Asn-Glu-Lys-Ile-Arg-COOH	0	A
15.	-Trp-Asp-Asn-Glu-Lys-Ile-Arg-Arg-COOH	100	A

[a] NH_4HCO_3 was the buffer used except where marked c; in this case K_2HPO_4 was used. Time of hydrolysis was 6 hours except for Peptide 4 which was hydrolyzed for 4 hours.

[b] A. Examination of digest by paper electrophoresis, B. isolation and analysis of the products, C. edman degradation of the digest.

terminal end by five residues, i.e., Pro-Lys-Glu-Thr-Arg-Leu-Glu-Val-Ile-Ser-Asp-Arg, quantitative cleavage was observed at the Glu-Val bond but not at the Glu-Thr bond. This observation indicates that the protease has an extended active site and requires interaction with the substrate at least one or two residues NH_2-terminal to the potentially scissile bond. A large active site has been demonstrated for other proteases (8-10).

Other examples of peptides containing glutamyl residues, two or three

residues from the NH_2-terminus, which did not undergo hydrolysis are included in Table IV. However, since these peptides differed from others in the types of residues in the neighborhood of the potential cleavage sites, factors other than the distance to the NH_2-terminus may be involved in reducing the rate of cleavage. A rapid rate of cleavage removed the dipeptide Leu-Glu from Peptide 6 (Table IV), and the enhanced rate compared to that observed with Peptide 1 may have been due to the increased polar character of the residues on the COOH-terminal of the glutamic acid residue in Peptide 6 compared to those of Peptide 1. A low rate of hydrolysis at bonds two or three residues from the COOH-terminus of peptides containing either the sequence -Glu-X-Y-COOH or -Glu-X-Y-Z-COOH (Peptides 10-14, Table IV) was also observed. The importance of a subsite equivalent to positions three or four residues to the COOH-terminal side of the cleavage point in the substrate is demonstrated by the marked increase in rate of hydrolysis of Peptide 15 (Table IV), which is only one residue longer than Peptide 14.

Hydrolysis in the presence of potassium phosphate rather than ammonium bicarbonate did not appear to change markedly the specificity of the enzyme, as reported earlier (2). No hydrolysis was observed on the COOH-terminal side of aspartyl residues in peptides listed in Table V after incubation with the protease in 0.05 $\underline{M}$ potassium phosphate (pH 7.8). Two instances of enhanced rates of cleavage at glutamyl residues in phosphate were observed. The ^{14}C-labelled Peptide 5 (Table V) was hydrolyzed at its Glu-His bond to an extent of 35% in ammonium bicarbonate, and 90% in 0.5 $\underline{M}$ potassium phosphate, in each case after 16h at 40°. A marked increase in rate at the Glu-Thr bond of Peptide 13 (Table IV) was also observed. Thus, it is reasonable to conclude that the protease is either more active or more stable in potassium phosphate, and the apparent change in specificity of the enzyme observed previously (2) may be due to scission at aspartyl residues that hydrolyze slowly in the presence of either buffer.

We recognize that many factors may be involved in the action of the

TABLE V

Peptides Containing Aspartyl Residues at Which no Hydrolysis Occurred [a]

1.	Val-Pro-Asp-Ser-Tyr-Arg
2.	Leu-Gly-Gly-Ile-Pro-His-Asp-Ser-Tyr-Gly-Met-Thr-Thr-COOH
3.	<Glu-Thr-Gly-Gly-Pro-Asp-Gly-Asp-Leu-Gly-Ser-Asn-Glu-Ile-Leu-Leu [b]
4.	Leu-Glu-Ser-Lys-Tyr-Leu-Asp-Gly-Asp-Asp-Thr-Ser-Lys-Arg
5.	Val-Leu-CMCys-Asp-Asp-Thr-Asn-Val-Thr-Leu-Pro-Asn-Gly-Glu-His-Val-Asn-Gly-Thr-Ala-Phe-Arg- [b]

[a] Experiments were performed in potassium phosphate buffer.

[b] Hydrolysis occurred only at the glutamyl residue.

proteinase other than those reported above; however, the present information should be useful for investigating peptide sequences employing Staphylococcal proteinase as a hydrolytic agent.

ACKNOWLEDGEMENT: This work was aided by NIH grant GM-11061

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