SHORT COMMUNICATIONS

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Specificity of alkaline proteinase of *Aspergillus flavus*

In preceding papers we reported on the preparation of an extracellular proteinase from *Aspergillus flavus*[1] and on the determination of the serine active center[2], and discussed the similarity between proteinases of various kinds of aspergilli[3–5]. In this paper, the specificity of the proteinase isolated by us (*cf.* ref. 6) will be described and compared with the known specificities of the proteinases mentioned above.

Cleavage of the B-chain of oxidized insulin. The B-chain (5 mg) of oxidized insulin[7,8] was digested by the alkaline proteinase of *A. flavus*[1] 1 h at 37° and pH 8.5. The molar enzyme to substrate ratio was 1:100. Individual peptides were isolated by preparative paper chromatography in System S_1 (*n*-butanol–acetic acid–pyridine–water, 30:6:20:24, by vol.)[9] and by high-voltage paper electrophoresis[10] at pH 1.9. The amino acid composition was determined by the method of SPACKMAN *et al.*[11]. The peptides obtained are represented by horizontal lines in Fig. 1. We also obtained a complex mixture of peptides in yields lower than 0.04 μmole, peptides which could not be purified further owing to their small amounts.

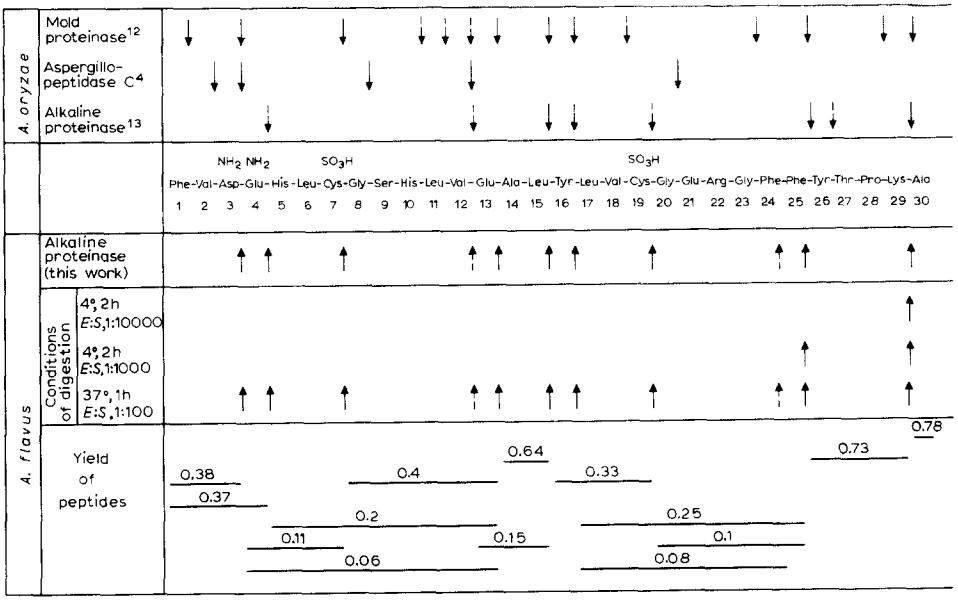

Fig. 1. Sites of cleavage of oxidized B-chain of insulin by alkaline proteinases from Aspergilli. The yield in μmoles of each individual peptide calculated from the data of amino acid analysis is given above the bar. The peptide containing Residues 5–13 was assigned its location after determination of its N-terminal group by the dimethylaminonaphthalenesulfonation technique[16,17].

* Abbreviations used: Ac–, acetyl–; Boc–, *tert.*-butyloxycarbonyl–; –*p*Na, *p*-nitroanilide; Suc–, succinyl–; Z, benzyloxycarbonyl–; –OEt, ethoxy; –OMe, methoxy.

We investigated the cleavage under various conditions and thus found the sites of primary attack which correspond to the isolated peptides in order of their yield (*cf.* Fig. 1).

Cleavage of synthetic substrates. A volume of 200 μl of 0.05 M trimethylamine buffer, which had been adjusted to pH 4, 5, 6, 7, 8 or 9 by acetic acid, was mixed with 20 μl of a solution containing 0.05 μmole of substrate in water (or in 50% ethanol) and with 10 μl of the enzyme solution. The resulting molar enzyme to substrate ratio was always 1:20. The reaction mixture was incubated 16 h at 37°. The cleavage was studied by paper chromatography in System S_1. The sites of cleavage of substrates containing a higher number of amino acid residues were established in analogous experiments carried out on a preparative scale (1 μmole of substrate at pH 8). The isolated digestion products were characterized by amino acid analysis[11]. In the pH range 7–9 the following substrates* were cleaved completely:

Gly–Phe–OMe
Gly–Phe–Leu
Leu–Leu–*p*Na

Gly–Phe–*p*Na
Leu–Gly–*p*Na
Gly–Tyr–OEt

Asn–Ala–Pro–Leu–Gly–NH_2
Boc–β–Ala–Trp–Met–Asp–Phe–NH_2
Z–Gly–Pro–Gly–Gly–Pro–Ala

There was no cleavage of the following substrates:

Glu–Asn
Glu–Glu–Ala
Suc–Phe–*p*Na
Pro–Leu–Gly–NH_2
Ac–Phe–Tyr
Gly–Pro–Ala

Our results obtained concerning the digestion of synthetic peptides show that the proteinase cleaves peptide bonds at the carboxyl side of phenylalanine, leucine, tyrosine, aspartic acid, and methionine. A fact deserving interest is that the proteinase also splits the bond at the carboxyl side of glycine, if the carboxyl of the adjacent phenylalanine residues is bound by a peptidic or at least pseudopeptidic residue, not, however, if the phenylalanine carboxyl is esterified. It is likely that in some cases the substitution of the N-terminal amino acid prevents the cleavage. By contrast, the elongation of peptide Pro–Leu–Gly–NH_2 at its N-terminus rendered the peptide susceptible to cleavage.

The specificity of the alkaline proteinase of *A. flavus*, as observed with the digestion of the B-chain of oxidized insulin, strongly resembles the specificity of the mold proteinase of *A. oryzae* described by SANGER *et al.*[12] (*cf.* Fig. 1); it differs, however, from the specificity of aspergillopeptidase C of *A. oryzae* as reported by NORDWIG AND JAHN[4]. The specificity of our proteinase corresponds to the specificity of the alkaline proteinase isolated from *A. oryzae* by MORIHARA AND TSUZUKI[13]. These authors, however, report for their preparation a molecular weight of 52 000, which is different from the value of 18 000–19 000 reported by other authors[3,4] and by us[1]. Our results provide additional evidence of the great similarity existing between the proteinase of *A. flavus* and the alkaline proteinase of *A. oryzae* (*cf.* refs. 1, 2).

The data presented above show that the specificity of the alkaline proteinase of *A. flavus* is rather broad. In their study on the binding site of papain, SCHECHTER AND BERGER[14] reported a seven-residue binding site of aspergillopeptidase B (*cf.* ref. 3). For this reason, we arranged all the peptides which are cleaved by our proteinase in such a manner that the amino acids equally distant from the site of cleavage were below each other. When comparing the neighborhood of the cleaved bond, we did not find any obvious regularity. The low specificity of the proteinase was in good agreement with the assumed multiplicity of the binding site and obviously plays a functional role in the proteinases of lower organisms.

The results of our investigation of the cleavage of both the B-chain of oxidized insulin and also the synthetic peptides lead us to conclude that the proteinase isolated by us falls into the group of serine proteinases of broad specificity[13], a group to which also belongs, among others, subtilisin with the same serine active center[2,15].

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