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SERINE-CONTAINING ACTIVE CENTER OF ALKALINE PROTEINASE OF *ASPERGILLUS FLAVUS**

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SUMMARY

1. From the soluble portion of the tryptic digest of oxidized diisopropyl [^{32}P]-phosphoryl-derivative of alkaline proteinase of *Aspergillus flavus* a radioactive fragment was isolated and its amino acid composition determined to be Asp_7, Met_1, Thr_5, Ser_8, Glu_4, Pro_3, Gly_7, Ala_9, Val_6, Ile_5, Leu_6, Tyr_2 and Phe_2.

2. Four radioactive peptides were isolated from a partial acid hydrolysate of the slightly soluble portion of the tryptic digest. These peptides provide evidence of the amino acid sequence Gly–Thr–Ser–Met–Ala around the reactive serine residue of alkaline proteinase. This amino acid sequence represents a part of the large tryptic fragment.

3. Similarities in the amino acid sequences of serine-containing active centers of microbial proteinases are discussed.

INTRODUCTION

It is known that the so-called serine proteolytic and esterolytic enzymes can be inactivated by the reaction with diisopropylphosphorofluoridate. Studies on these diisopropylphosphoryl-(DIP-)derivatives have established the amino acid sequence around the reactive serine residue in a number of enzymes[1–3]. On the other hand only a few reports have been published concerning the active centers of microbial proteinases[3–8]. In the previous paper we reported the isolation and characterization of an alkaline extracellular proteinase from *Aspergillus flavus*[9]. We provided evidence showing that this proteinase is a serine enzyme containing one reactive serine residue which can be inhibited by diisopropylphosphorofluoridate[9]. The aim of this paper is to report on the determination of the amino acid sequence around this serine residue. The results obtained are compared with data known on active centers of other microbial proteinases.

Abbreviations: DIP-, diisopropylphosphoryl-, DNS-, dimethylaminonaphtalenesulfonyl-.

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METHODS

Tryptic digestion of alkaline proteinase

[^{32}P]DIP-proteinase was prepared and oxidized by the method described in the preceding paper[9]. The lyophilized protein (150 mg) was dissolved in 50 ml of water, heated at 37°, and its pH adjusted to 8.5 by 1 M NaOH in an autotitrator. After the addition of 8 mg of 1-tosylamido-2-(phenyl)-ethylchloromethyl ketone-treated trypsin[10], the digestion was allowed to proceed for 75 min. The addition of 4 mg of trypsin after this period did not lead to additional hydrolysis. Therefore, 30 min later the solution was acidified with 10% formic acid to pH 3, when a precipitate was formed. The latter was washed twice with 5 ml of 0.2 M pyridine formate at pH 3. The pooled supernatants contained 40% of radioactivity, while 60% of the total radioactivity was present in the insoluble portion.

The soluble portion was not concentrated by evaporation but directly applied at room temperature to a column of Dowex 50-X2 in pyridinium form which was previously equilibrated with the first buffer. The details are given in the legend to Fig. 1. The effluents were continuously monitored with the aid of the apparatus of TYKVA AND GRÜNBERGER[11] equipped with an activity recorder utilizing the scintillation of anthracene. From each fraction two aliquots were withdrawn as follows: 0.2 ml was subjected to radioactivity measurement and 0.5 ml to paper chromatography in System S_1 (*n*-butanol–acetic acid–pyridine–water, 30:6:20:24, by vol.)[12]. According to the results obtained, identical fractions were pooled (*cf.* Fig. 1) and rotary evaporated.

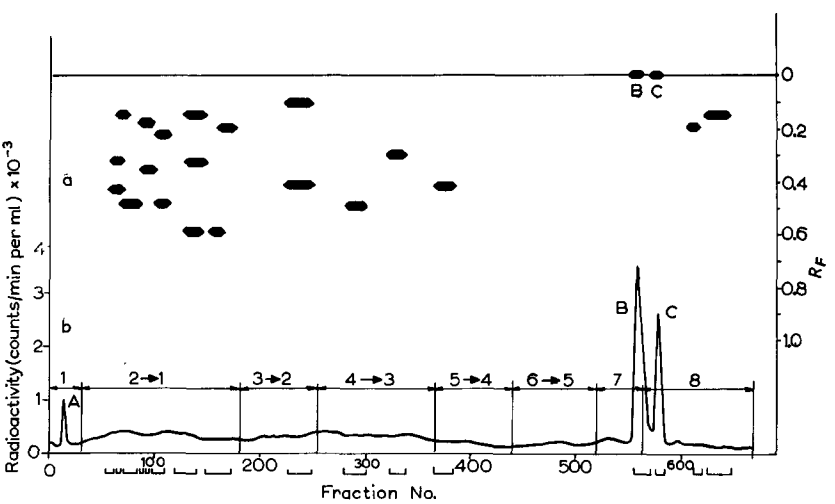

Fig. 1. Ion-exchange chromatography of the soluble portion of the tryptic digest and its evaluation by paper chromatography and radioactivity measurement. 10-ml fractions emerging from a 2 cm × 65 cm column of Dowex 50-X2, 200–400 mesh, were collected at 10-min intervals. The following buffers were used as eluents: 1, pyridine formate (pH 3.0; 0.2 M); 2, pyridine formate (pH 3.5; 0.4 M); 3, pyridine acetate (pH 4.5; 0.8 M); 5, pyridine acetate (pH 5.0; 1 M); 6, pyridine acetate, (pH 6.0; 2 M); 7, 2 M pyridine; 8, a mixture of 2 M pyridine and 2 M ammonia. Linear elution gradients were applied between fractions No. 30 and 552. a, paper chromatography of aliquots of fractions in system S_1 (*n*-butanol–acetic acid–pyridine–water, 30:6:20:24, by vol.) (ref. 12); b, results of radioactivity measurement.

Radioactive material of Peaks B (fraction No. 555–575; 10.9 mg) and C (fractions No. 576–584; 7.8 mg) was purified by paper chromatography on Whatman No. 1 paper in System S_2 (phenol–collidine–water, 8:2:1, by vol.). The R_F-value of this material was 0.9. The radioactivity on paper chromatograms and also electropheretograms was scanned automatically with a Frieseke–Hoepfer methane through-flow counter. A part (1/40) of this material was hydrolyzed in 6 M HCl at 100° for 1 h. The presence of radioactive phosphoserine was revealed with the aid of a standard after descending paper electrophoresis[13] (E_1) in acetic acid (32 ml/1 l, pH 2.3). An analogous method was employed for the proof of phosphoroserine in the insoluble portion of the tryptic digest.

11 mg of the insoluble portion of the tryptic digest was subjected to partial acid hydrolyses in 1 ml of 6 M HCl (40 min at 100°). The dry residue of the hydrolysate was fractionated by repeated electrophoresis (E_1) on Whatman No. 1 paper. Ninhydrin-positive zones were purified further by descending paper electrophoresis (E_2) in pyridine acetate buffer at pH 3.5 (1 ml of pyridine + 10 ml of acetic acid + 989 ml of water). The isolated radioactive peptides were designated P_1, P_2, P_3 and P_4.

The peptides obtained from different hydrolysates were characterized by the dimethylaminonaphtalenesulfonyl-(DNS-)method according to GRAY AND HARTLEY[14] and by quantitative amino acid analysis according to the procedure of SPACKMAN, STEIN AND MOOR[16]. The amino acid composition of peptides obtained in only small amounts was determined either by paper chromatography of their total acid hydrolysates in System S_3 (ref. 13) (*n*-butanol–acetic acid–water, 144:13:43, by vol.) or by conversion of the amino acids present in the hydrolysates into their DNS-derivatives[14] and identification of these derivatives by thin-layer chromatography[15].

RESULTS AND DISCUSSION

The starting material of [^{32}P]DIP-proteinase was homogeneous with respect to its end groups (N-terminal glycine, C-terminal alanine), and its amino acid composition was identical with the published data[9]. The enzyme molecule contains one residue of methionine which is labile and undergoes spontaneous oxidation. We were aware of the possibility of uncontrollable losses during the fractionation of peptides by paper chromatography and/or electrophoresis. Therefore we used the oxidized form of [^{32}P]DIP-labeled proteinase, containing the stable methionine sulfone instead of methionine. In spite of the fact that the digestion with trypsin was complete and did not continue further after addition of new portions of enzyme, it was not specific enough. The radioactive fragments had different solubilities. Peptides which were very slightly soluble and precipitated after acidification of the hydrolysate contained 60% of the radioactivity, these were recovered by centrifugation and submitted to partial acid hydrolysis. The supernatant containing 40% of radioactivity was directly applied to the Dowex 50-X2 column and chromatographed. The radioactive material emerged in three peaks (*cf.* Fig. 1). The first peak (A), which emerged with the front, did not contain peptides and was therefore not treated further. The material of the remaining two peaks (B and C) showed identical R_F-values when chromatographed in systems S_1 ($R_F = 0$) and S_2 ($R_F = 0.9$). The complete acid hydrolysates of Fractions B and C did not contain basic amino acids. The amino acid composition of these

two fractions was identical, and they both contained radioactive phosphoserine and methionine sulphone. In our opinion these two fractions are derived from the same site of the molecule and differ only slightly, *e.g.*, in their amide content. Peak B contained a larger amount of material than Peak C. The total acid hydrolysate of peptide isolated from Fraction B was analyzed on the 150-cm column of the amino acid analyzer[16] (Table I). Fractions B and C were also subjected to enzymic digestion, but we did not isolate sufficient amounts of homogeneous peptides. In addition to these radioactive peptides we obtained a number of other, nonradioactive fragments by chromatography of the tryptic digest. The description of the isolation and characterization of these peptides will not be reported here.

TABLE I

AMINO ACID COMPOSITION OF ISOLATED RADIOACTIVE PEPTIDES

Amino acid	*Radioactive peptides*					
	B	*Nearest integer*	P_1	P_2	P_3	P_4
Asp	6.80	7				
Methionine sulfone	0.98	1	1	0.84	0.87	
Thr	4.97	5	1	1.13	1.00	1
Ser	7.60	8	1	1.02	1.10	1
Glu	4.11	4				
Pro	2.88	3				
Gly	7.23	7		0.14	0.23	1
Ala	9.20	9	1	0.17		
Val	5.52	6				
Ile	4.66	5				
Leu	6.31	6				
Tyr	1.53	2				
Phe	2.20	2				
Total		65	4	3	3	3
N-terminal amino acid	Gly		Thr	Thr		Gly

The portion of the radioactive material which precipitated on acidification of the tryptic digest was not homogeneous from the viewpoint of end-group analysis. The total acid hydrolysate of this material contained radioactive phosphoserine and methionine sulphone. Since [^{32}P]DIP-proteinase contains only one residue of methionine and only one reactive serine residue (*cf.* ref. 9), it is obvious that this almost insoluble portion of radioactive material is derived from the same site of the molecule as Fractions B and C. Therefore this material was not fractionated but directly submitted to partial acid hydrolysis and thus solubilized. Four homogeneous radioactive peptides, P_1, P_2, P_3 and P_4 (Table I) were isolated from the hydrolysate. From a combination of the results of the amino acid analyses of these peptides the amino acid sequence around the reactive serine residue in the active center of oxidized [^{32}P]DIP-proteinase can be deduced as follows (Sêr = [^{32}P]phosphoryl serine or monoisopropyl[^{32}P]phosphoryl serine):

P_1 Thr (Sẽr, $MetSO_2$, Ala)
P_2 Thr (Sẽr, $MetSO_2$)
P_3 (Thr, Sẽr, $MetSO_2$)
P_4 Gly(Thr, Sẽr)

Gly–Thr–Sẽr–$MetSO_2$–Ala

$MetSO_2$ = methionine sulphone.

The difference in mobility of Peptides P_2 and P_3 may be explained by partial deisopropylation of DIP-serine. It is likely that this sequence in the alkaline proteinase of *A. flavus* is preceded by an asparagine residue and that at the carboxyl side of the sequence is glutamine. The corresponding peptides, however, have not been obtained in a pure state, and therefore we consider the extended sequence Asn–Gly–Thr–Sẽr–Met–Ala–Gln merely as a working hypothesis which should be verified by future sequence studies. From the above discussion it becomes obvious that this active center constitutes a part of the large fragment isolated from the soluble portion of the tryptic digest. If this fragment (which does not contain basic amino acids) were a specific tryptic peptide, it should be derived from the C-terminus of the molecule.

The finding that methionine, a very reactive amino acid, is adjacent to the reactive serine in the active center is worthy of special note, since it is likely that experiments with the modification of this methionine residue could throw more light on the functioning of the active center. The amino acid sequence in the active center as proposed by us is in accordance with the amino acid sequence Thr–Sẽr–Met–Ala proposed by SHAW to exist in the proteinase of *A. oryzae*. (The unpublished work of D. C. SHAW was mentioned by SANGER[3].) This identity of active centers together with the similarity of amino acid composition seems to suggest a similarity if not identity of aspergillopeptidase B and alkaline proteinase of *A. flavus* (see DISCUSSION in paper[9]). The amino acid sequence reported by us is identical with the amino acid sequence of the active center of subtilisin (Table II) even though *Bacillus subtilis* and *A. flavus* are genetically far apart. We must mention here the paper of SHAW AND WELLS[17]. These authors found that caseinase from French beans has the same

TABLE II

COMPARISON OF ACTIVE CENTER OF ALKALINE PROTEINASE OF *A. flavus* WITH KNOWN SERINE-CONTAINING ACTIVE CENTERS OF MICROBIAL PROTEINASES

The reactive serine residue is marked by an asterisk.

Proteinase	*Origin*	*Ref. No.*	*Active center*
Alkaline proteinase	*A. flavus*	This paper	Gly–Thr–Sẽr–Met–Ala
'Mold protease'	*A. oryzae*	3	Thr–Sẽr–Met–Ala
Subtilisin	*B. subtilis*	4	Asn–Gly–Thr–Sẽr–Met–Ala
Bacteriolytic Protease	Sorangium sp.	5, 6	Asp–Sẽr–Gly–Gly
Pronase	*Streptomyces griseus*	7	Asp–Sẽr–Gly
Protease	Arthrobacter	8	Ser–Sẽr–Gly

active center, Thr–Ser–Met–Ala. The remaining three microbial proteinases listed in Table II show other amino acid sequences of their active centers. The proteinase of Sorangium[5,6] and pronase[7] are obviously of the trypsin–chymotrypsin type, while the proteinase from Arthrobacter[8] differs from all the others. Very interesting information can be expected to result from a comparison of the active center of alkaline proteinase of *A. flavus* with those of the other proteinases from Aspergilli when the necessary data become available.

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