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PURIFICATION OF AN ACID PROTEINASE FROM *ASPERGILLUS SAITOI* AND DETERMINATION OF PEPTIDE BOND SPECIFICITY

NORIO TANAKA, MICHIO TAKEUCHI and EIJI ICHISHIMA *

Laboratory of Enzymology and Microbial Chemistry, Tokyo Noko University, Fuchu, Tokyo 183 (Japan)

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Summary

The specificity and mode of action of an acid proteinase ** (EC 3.4.23.6) from *Aspergillus saitoi* were investigated with oxidized B-chain of insulin, angiotensin II and bradykinin. Further purification of acid proteinase was performed with *N,O*-dibenzyloxycarbonyl-tyrosine hexamethylene-diamino-Sepharose 4B affinity chromatography and isoelectric focusing. The purified enzyme was free of any other proteolytic activity demonstrated in *Asp. saitoi*.

Acid proteinase from *Asp. saitoi* hydrolyzed primarily two peptide bonds in the oxidized B-chain of insulin, the Leu(15)-Tyr(16) bond and the Phe(24)-Phe(25) bond. Additional cleavages of the bonds His(10)-Leu(11), Ala(14)-Leu(15) and Tyr(16)-Leu(17) were also noted. Primary splitting sites at Leu(15)-Tyr(16) and Phe(24)-Phe(25) with acid proteinase from *Asp. saitoi* were identical with those reported in the work of cathepsin D (EC 3.4.23.5) from human erythrocyte. Hydrolysis of angiotensin II was observed at the Tyr(4)-Ile(5) bond.

In conclusion, peptide bonds which have a hydrophobic amino acid such as phenylalanine, tyrosine, lecuine and isoleucine in the P'_1 position (as defined by Berger and Schechter, [29]) are preferentially cleaved by the trypsinogen-activating acid proteinase from *Asp. saitoi*.

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* To whom inquiries about this paper should be addressed.

** Acid proteinase activity is expressed in the unit, katal (symbol kat) [12] (1 kat = $6 \cdot 10^7$ U). One katal of the acid proteinase is defined as the amount of enzyme which yields the color equivalent of 1 mol of tyrosine $\cdot$ s^{-1} with the Folin-Ciocalteau phenol reagent, using casein as substrate at pH 2.7 and 30°C (Enzyme Nomenclature (1972) IUPAC and IUB recommendations, pp. 26—27, Elsevier, Amsterdam).

Introduction

The extracellular proteinase produced by *Aspergillus saitoi* (EC 3.4.23.6) shows optimal activity at pH 2.5—3.0 [1] and it digests a wide range of proteins [2,3,4]. However, like cathepsin D, it does not hydrolyse synthetic substrates (amides, esters) or small synthetic peptides [1]. *Asp. saitoi* also produces an acid carboxypeptidase that shows high activity with the substrate Z-Tyr-Leu (Z = benzyloxycarbonyl) [5,6,7]. However, in a previous paper from this laboratory a method was described for the separation of the two enzymes on a column of hexamethylene-diamino-Sepharose 4B covalently linked to *N,O*-dibenzyloxycarbonyltyrosine [8]. In the present studies we have obtained further purification of the acid proteinase by isoelectric focussing. We have also measured quantitatively the relative rates of hydrolysis by the proteinase of the peptide bonds of the oxidised B-chain of insulin. The results are discussed in relation to the bond specificities of pepsin and other acid proteinases.

Materials and Methods

Materials

Crystalline bovine insulin (I.U. 23-25/mg, Lot 169277) was purchased from Fluka AG, Buchs SG, Switzerland. Human angiotensin II (Lot. 72128-1) and Z-Tyr-Leu were from the Protein Foundation, Osaka. *N,O*-dibenzyloxycarbonyl-tyrosine was synthesized according to the method described in the previous paper [9]. Swine pepsin (3 times crystallised, Lot. 59356) was purchased from General Biochemicals Inc., U.S.A.

Acid proteinase from Asp. saitoi

The proteinase was isolated from culture filtrates of *Asp. saitoi* ATCC-14332 and purified, until free from the acid carboxypeptidase, by published methods [1,8—10]. The specific activity of this step of the partially purified enzyme obtained from these steps was about 0.099 kat/kg of enzyme. Further purification of the acid proteinase was performed in isoelectric focusing [11]. Ampholines, pH 3.5—5.0, were incorporated into a sucrose gradient to 1%. The LKB electrofocusing column (110 ml volume) was used. The focusing time was generally 60 h with the column at 4°C. Fractions of 1.6 ml were collected. Absorbance at 280 nm in each fraction was estimated using microcuvets. Immediately after the pH had been determined using a Radiometer titrater TTT2 type pH meter, the purified enzyme was dialysed against 0.05 M acetate buffer (pH 2.7) to remove the Ampholines and then stored at 4°C.

Portions of purified fractions eluted from the column were incubated for 24 h with the sensitive substrate Z-Tyr-Leu at pH 3.5 and 30°C to detect acid carboxypeptidase activity. Hydrolysis of Z-Tyr-Leu was detected by paper-chromatography, using a solvent system of *n*-butanol/acetic acid/water, 4 : 1 : 2. The purified fractions did not contain any acid carboxypeptidase activity.

Oxidation of insulin

Oxidation of insulin was performed as described by Craig [13]. Oxidized B-cahin of insulin was prepared as described by Griffin [14]. Oxidized B-chain

preparation was sufficiently pure as judged by the N-terminal analysis with 1-fluoro-2,4-dinitrobenzene [15] and amino acid analysis.

Determination of time course for the hydrolysis of oxidized B-chain of insulin

Oxidized B-chain of insulin (7 mg, 2 μmol) was dissolved in 10 ml of 0.1 M acetate buffer, pH 2.7, and incubated for 20 min with 3 nkatal of acid proteinase from *Asp. saitoi* at 30°C. Aliquots (500 μl) were withdrawn at various time intervals and mixed 500 μl 0.6 M NaOH. The ninhydrin assay [5] was then carried on these samples. Total free amino groups of oxidized B-chain were determined with the hydrolyzate of 6 M HCl at 110°C for 22 h.

Separation and identification of peptides from the digest of B-chain

Oxidized B-chain of insulin (7 mg, 2 μmol) was dissolved in 10 ml dilute HCl, 3 nkat of acid proteinase from *Asp. saitoi* were added to the solution and then the pH was adjusted to 2.7 with dilute HCl. The mixture was incubated at 30°C for 20 min. One drop of conc. NH_4OH was added to inactivate the enzyme. The samples of hydrolyzate were stored at −20°C, and then the frozen digest was lyophilized.

Next the freeze-dried digest was dissolved in 50 μl 1 M NH_4OH, and was separated on Toyo filter paper in the first dimension by high-voltage paper electrophoresis in pyridine/acetic acid/water, 10 : 0.4 : 90, at pH 6.5 for 150 min at 35 V/cm. After drying, room temperature ascending chromatography was carried out in the solvent system *n*-butanol/acetic acid/water, 4 : 1 : 2 for 15—16 h. The peptide maps were stained with 0.02% ninhydrin-acetone reagent. Ninhydrin-positive zones were cut out from the remainder and peptides were washed with acetone, and eluted with water. The eluates were freeze-dried in vacuo.

Hydrolysis of angiotensin II and bradykinin

Angiotensin II (2 mg) was dissolved in 10 ml of dilute HCl at pH 2.7, and incubated at 30°C with 15 nkat acid proteinase from *Asp. saitoi* for 6 h.

Bradykinin (3 mg) was dissolved in 10 ml of dilute HCl at pH 2.7, and incubated at 30°C with 22.5 nkat acid proteinase from *Asp. saitoi* for 6 h. For digestion with pepsin, 2 mg of pepsin were used in 0.2 M HCl/KCl buffer (pH 1.8).

Amino acid analysis

Freeze-dried peptides were dissolved in 2 ml 6 M HCl and were hydrolyzed at 110°C for 24 h. Hydrolyzates of peptides were analyzed with Hitachi amino acid analyzer, model KLA-3B.

Determination of N-terminal amino acid of peptides

The N-terminal amino acids of the purified peptides (B-1, N-2, N-3, A-2) were determined by the DNP method of Sanger [15]. The DNP-amino acids were identified by paper chromatography with the solvent system, 1 M NaH_2PO_4/0.5 M Na_2HPO_4 buffer, pH 6.0.

Estimation of the extent of hydrolysis of the various peptide bonds

The hydrolysis rates of the individual peptide bonds which were split were

then calculated by summation of the yields of the peptides located on either side of the bond being opened.

Results

Further purification of Asp. saitoi acid proteinase

Isoelectric focusing was used as the final purification step. The partially purified acid proteinase by published method [1,8,9,10] containing 85 nkat of activity in 10 ml was applied to the column. The specific activity of the partially purified preparation was 0.099 kat/kg of enzyme. Fig. 1 shows the eluent profile from an isoelectric focusing column. The specific activities of fractions Nos. 28 and 29 at pH 2.7 were 0.111 and 0.113 kat/kg of enzyme. The yield of purified enzyme from fractions Nos. 28 and 29 was about 70% in this step.

The preparation seemed to be homogeneous by free boundary electrophoresis, and the sedimentation pattern appeared monodisperse as described in the previous paper [10]. The purified fractions were incubated for 24 h with the sensitive substrate Z-Tyr-Leu at pH 3.0 and 30°C to detect acid carboxypeptidase activity. The purified fractions did not contain any acid carboxypeptidase activity.

In the previous paper [10], it was reported that the isoelectric point of the acid proteinase was at pH 3.65 on free-boundary electrophoresis. Isoelectric focusing in the present work showed that the isoelectric point of the enzyme was at pH 4.08.

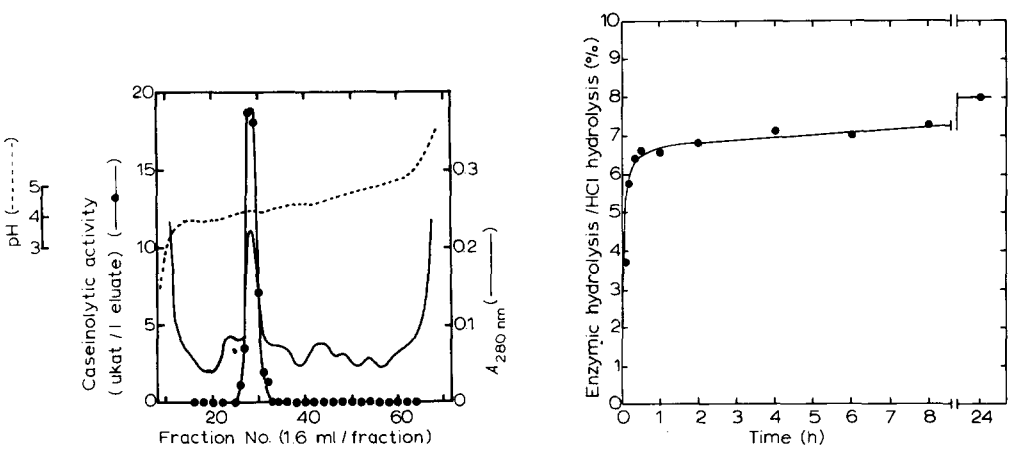

Fig. 1. Isoelectric focusing profile of acid proteinase from *Asp. saitoi* from Z-Tyr(Z)-hexamethylenediamino-Sepharose 4B run. Ampholines were incorporated into a sucrose gradient to 1%. The LKB electrofocusing column (110 ml volume) was used for the analysis of enzyme preparation. The focusing time was 60 h with the column to 4°C. Fractions of 1.6 ml were collected from the small column. The specific activities of Fractions Nos. 28 and 29 at pH 2.7 were 0.111 and 0.113 kat/kg of enzyme. The purified fraction did not contain any acid carboxypeptidase activity.

Fig. 2. Time course for the hydrolysis of oxidized B-chain of insulin by acid proteinase from *Asp. saitoi*. Increase in amino groups was determined by the ninhydrin method as described in the text. The reaction mixture contained 7 mg of B-chain and 3 nkat of the enzyme in 10 ml of 0.1 M acetate buffer (pH 2.7) at 30°C. Aliquots (500 µl) were withdrawn at various time intervals. Total free amino groups were determined with the hydrolyzates of 6 M HCl at 110°C for 22 h.

Hydrolysis of oxidized B-chain of insulin

The rate of hydrolysis of oxidized insulin B-chain was examined (Fig. 2). The reaction for B-chain was initiated with rapid hydrolysis. During the rapid hydrolysis of oxidized B-chain, two peptide bonds per oxidized insulin B-chain molecule might be hydrolyzed.

The peptides were isolated and purified as described: a total of 9 peptides were obtained. The strong spots shown in the fingerprint map of the digest of oxidized insulin B-chain were A-2, N-2 and B-1. The weak spots gave only small amounts of amino acids after hydrolysis and were therefore considered to have arisen from insignificant cleavage.

Table I lists the peptides, and their amino acid compositions and final uncorrected recoveries. All of the peptides were readily identifiable from the known sequence of oxidized B-chain of insulin. Identified N-terminal amino acids of the B-1, N-2, N-3 and A-2 peptides are also shown in the Table I. The N-terminals of four weak spots (N-1, A-1, A-3 and B-2) were not determined. One weak diffuse spot (A-4) was not isolated.

The results of Table I can be summarized to give the cleavage points shown in Fig. 3. The results in the Table I show that the acid proteinase from *Asp. saitoi* hydrolyzed primarily two peptide bonds in the oxidized B-chain of

TABLE I

AMINO ACID COMPOSITIONS OF PEPTIDES OBTAINED FROM THE DIGEST OF OXIDIZED INSULIN B-CHAIN BY ACID PROTEINASE FROM *ASPERGILLUS SAITOI*

The results of single analyses of 24-h hydrolyzates (6 M HCl, 110°C) are given. The values in parentheses denote the theoretical number of residues of a given amino acid in the peptide.

Amino acid	Peptides (residues/molecule)							
	B-1	B-2	N-1	N-2	N-3	A-1	A-2	A-3
Lys	1.02 (1)							
His		1.08 * (2)		0.77 (2)	0.60 (2)			
Arg						0.99 (1)	1.00 (1)	0.56 (1)
$CySO_3H$		0.60 (1)		1.25 (1)	1.00 (1)	1.00 (1)	1.54 (1)	1.43 (1)
Asp		1.00 (1)		1.41 (1)	1.00 (1)			
Thr	0.91 (1)							
Ser		0.79 (1)		0.84 (1)	0.59 (1)			
Glu		1.41 * (1)	0.42 (1)	1.87 (2)	1.35 (2)	1.08 (1)	0.94 (1)	0.83 (1)
Pro	0.87 (1)							
Gly		0.79 (1)		1.33 (1)	1.00 (1)	2.36 (2)	1.96 (2)	1.86 (2)
Ala	1.00 (1)		1.00 (1)	1.00 (1)	0.38 (1)			
Val		0.79 (1)	0.68 (1)	1.75 (2)	1.44 (2)	1.09 (1)	1.17 (1)	1.00 (1)
Leu		2.00 * (1)	1.48 (2)	2.45 (3)	1.22 (2)	1.58 (2)	0.66 (1)	0.32 (1)
Tyr	0.33 (1)		0.71 (1)			0.87 (1)	0.18 (1)	
Phe	0.30 (1)	0.25 (1)		0.41 (1)	0.10 (1)	0.95 (1)	0.49 (1)	0.43 (1)
Recovery μmoles (uncorrected)	0.222	0.029	0.020	0.106	0.070	0.061	0.227	0.058
N-Terminal amino acid	Phe	—	—	Phe	Phe	—	Tyr	—
Suggested segment	25—30	1—10	11—16	1—15	1—14	15—24	16—24	17—24

* Uncertain because of the relatively high background.

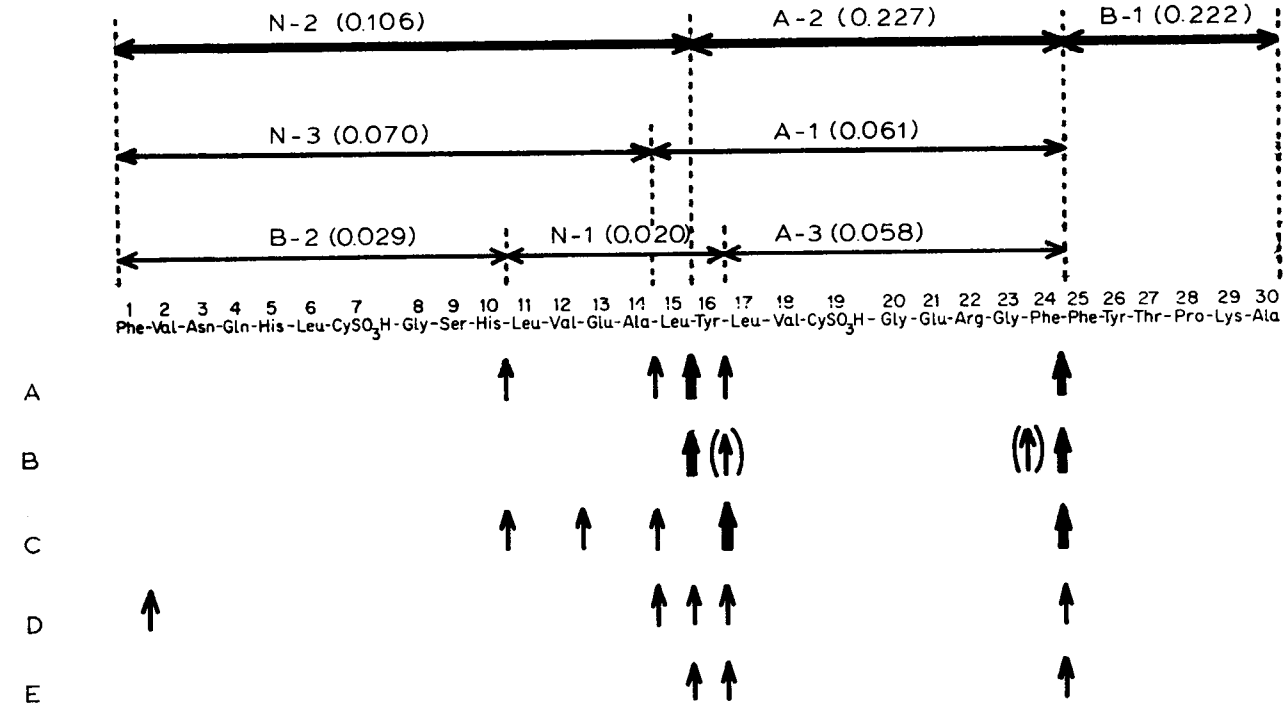

Fig. 3. Summary of the specificities of *Asp. saitoi* proteinase towards oxidized B-chain of insulin. A: *Asp. saitoi* acid proteinase, 20-min incubation at pH 2.7, *e/s*; 1 : 260, 30°C. B: Cathepsin D from human erythrocyte, 4-h incubation at pH 2.8 and 37°C [17]. C: Porcine pepsin C at pH 2.0 [18]. D: *Mucor pusillus* chymosin-like acid proteinase, 10-min incubation at pH 3.8, *e/s*; 1 : 1000, 38°C [19]. E: *Mucor miehei* chymosin-like acid proteinase, 30-min incubation at pH 3.8, *e/s*; 1 : 1000, 38°C [19].

TABLE II

THE HYDROPHOBICITIES OF SIDE CHAIN OF AMINO ACID RESIDUES ADJACENT TO THESE INVOLVED IN THE BOND TO BE SPLIT BY THE ACID PROTEINASE FROM *ASPERGILLUS SAITOI*

The arrow shows the position of the splitting site. The relative hydrolysis rate on oxidized B-chain of insulin was calculated by summation of the yields of the peptides located on either side of the bond being opened.

Substrate	Amino acid residues * adjacent to those involved in the bond to be split by the enzyme $S_3\ S_2\ S_1\ S'_1\ S'_2\ S'_3$ $-P_3-P_2-P_1-P'_1-P'_2-P'_3-$	Hydrophobicities of side chain of amino acid residues ** Δgt (kcal/mol)	Relative rate of hydrolysis
(A) Oxidized B-chain of insulin			
Major splitting site	—R —G —F $\overset{24}{\downarrow}$ $\overset{25}{}$F —Y —T —	—?—0—2.5$\overset{\downarrow}{}$2.5—2.3—0.4—	1.00
	—E —A —L $\overset{15}{\downarrow}$ $\overset{16}{}$Y —L —V —	—0.55—0.5—1.8$\overset{\downarrow}{}$2.3—1.8—1.5—	0.59
Minor splitting site	—V —E —A $\overset{14}{\downarrow}$ $\overset{15}{}$L —Y —L —	—1.5—0.55—0.5$\overset{\downarrow}{}$1.8—2.3—1.8—	0.23
	—A —L —Y $\overset{16}{\downarrow}$ $\overset{17}{}$L —V —C_{SO_3H}—	—0.5—1.8—2.3$\overset{\downarrow}{}$1.8—1.5—?—	0.14
	—G —S —H $\overset{10}{\downarrow}$ $\overset{11}{}$L —V —E —	—0—(—)0.3—0.5$\overset{\downarrow}{}$—1.8—1.5—0.55—	0.09
(B) Angiotensin II	—R —V —Y $\overset{4}{\downarrow}$ $\overset{5}{}$I —H —P —	—?—1.5—2.3$\overset{\downarrow}{}$2.97—0.5—2.6—	
(C) Bovine trypsinogen ***	—D —D —K $\overset{6}{\downarrow}$ $\overset{7}{}$I —V —G —	—0.54—0.54—1.5$\overset{\downarrow}{}$2.97—1.5—0—	K_m = 0.023 nm k_{cat} = 0.14 s^{-1} (pH 3.0)

* One-letter amino acid abbreviations were used.

** Data from the references [31,32].

*** Data from the reference [4].

insulin, the Leu(15)-Tyr(16) bond and the Phe(24)-Phe(25) bond. This conclusion is supported by the fact that the recoveries of peptides in B-1, N-2 and A-2 were 0.222, 0.106 and 0.227 μmol, respectively.

The bonds His(10)-Leu(11), Ala(14)-Leu(15) and Tyr(16)-Leu(17) in the oxidized B-chain of insulin appears to be secondary sites of hydrolysis by the acid proteinase from *Asp. saitoi.*

The hydrolysis rate of the individual peptide bonds which were split was calculated. The results are shown in Table II.

Hydrolysis of angiotensin II

When angiotensin II was digested with acid proteinase from *Asp. saitoi* for 6 h, the products could be resolved into two bands by high-voltage paper electrophoresis at pH 6.5. The digestion products of angiotensin II were subjected to amino acid analysis. The results are shown in Table III. Therefore, the peptide bond that had been hydrolyzed by acid proteinase from *Asp. saitoi* was the Tyr(4)-Ile(5) bond in angiotensin II.

Hydrolysis of bradykinin

When bradykinin was digested with acid proteinase from *Asp. saitoi* for 6 h, the products could be resolved into two bands by high-voltage paper electrophoresis at pH 6.5. Bradykinin was also digested by pepsin. Electrophoretic diagrams of the two were somehow different. The strong spots, BA-1 and BP-2, were identified as the undigested bradykinin. The other weak spots shown in the paper were not isolated. Therefore, bradykinin was a resistant peptide for the hydrolysis by *Asp. saitoi* acid proteinase and by pepsin.

Discussion

Determination of peptide bond specificity using oxidized B-chain of insulin and angiotensin II as substrates

Acid proteinases, which are most active at low pH and are generally inhibited by diazoacetyl-DL-norleucine methyl ester in the presence of cupric ions and by pepstatin, show specificity against aromatic or bulky amino acid residues at both sides of the splitting point ($-P_1-P_1'-$) in substrates, though this is not clearly reflected in the hydrolysis of the oxidized insulin B-chain, in which they all show considerably broad specificity [16].

Primary splitting sites at 15-16 and 24-25 in oxidized B-chain of insulin with *Asp. saitoi* acid proteinase were identical with those reported in the work of cathepsin D (EC 3.4.23.5) from human erythrocyte [17]. Splitting sites of cathepsin D from human erythrocyte [17], porcine pepsin C (EC 3.4.23.3) [18] and *Mucor pusillus* and *Mucor miehei* chymosin-like acid proteinases [19] in the oxidized B-chain of insulin are shown in the Fig. 3.

Splitting sites at 14-15, 15-16, 16-17 and 24-25 with pepsin were identical with those reported in the present study, while we did not find the six splitting sites at 1-2, 4-5, 11-12, 13-14, 23-24 and 25-26 reported by Sanger and Tuppy [20]. Like pepsin, chymosin (EC 3.4.23.4) [21] and many acid proteinases, *Rhyzopus* acid proteinase [22], *Penicillium janthinellum* acid proteinase [23] and *Acrocylindrium* acid proteinase [24] are enzymes of low specificity.

A characteristic of the acid proteinases that have just been discussed is that they are inhibited by diazoacetyl-DL-norleucine methyl ester and by S-PI, the *Streptomyces* pepsin inhibitor [25]. A different class of acid proteinase represented by acid proteinase A from *Aspergillus niger* var. magnasporus [26]. It is insensitive to this inhibitor and the pattern of its hydrolysis of the B-chain of insulin is quite different: bonds 3-4, 13-14, 26-27 are major sites and 20-21 is a minor site.

Angiotensin II has been used to be a very limited extent as a proteinase substrate. However, while the *Asp. saitoi* acid proteinase cleaves the 4-5 bond, pepsin cleaves the 3-4 bond [27].

Pepsin C (EC 3.4.23.3) shows a slightly more restricted specificity than pepsin. It should be noted that porcine pepsin C fails to cleave Ac-Phe-Tyr(I_2), which is known as a good synthetic-substrate for porcine pepsin, at measurable rate. Huang and Tang [28] reported that digests of oxidized ribonuclease A and glucagon with human gastricsin (EC 3.4.23.3) and pepsin were compared by IR-120 B column chromatography and two dimentional high voltage paper electrophoresis to separate the resulting peptides. They indicate that the specificities of both enzymes are broad and similar. However, from the studies with proteins as well as synthetic substrates, definite differences have been shown.

Structure of the substrate

The size of the active site of several proteases has been shown to extended over many subsites. For examples, the active site of papain (EC 3.4.22.2) and elastase (EC 3.4.21.11) have been shown to extended over 6—7 subsites (21—25 Å) [29] and that of carboxypeptidase A (EC 3.4.12.2) over about 5 subsites (approx. 18 Å) [30]. Extended activities are important both for increasing enzyme-substrate affinity and turnover rates. The results of Morihara also indicate that the microbial acid proteinases require oligopeptides larger than

TABLE III

AMINO ACID COMPOSITIONS OBTAINED FROM THE DIGEST OF ANGIOTENSIN II * BY ACID PROTEINASE FROM *ASPERGILLUS SAITOI*

Amino acid	Peptides (residues/molecule)	
	I	II
His	1.00 (1)	
Arg		0.94 (1)
Asp		1.00 (1)
Pro	1.18 (1)	
Val		0.98 (1)
Ile	0.80 (1)	
Tyr		0.84 (1)
Phe	0.86 (1)	
Recovery (μmol) (uncorrected)	0.343	0.827
Suggested segment	5—8	1—4

* Asp—Arg—Val—Tyr—Ile—His—Pro—Phe.

hexapeptides as the sensitive substrates, the backborn chain as well as the side chain at the respective positions being significant for hydrolysis [16].

The effect on the hydrolysis rate of side chains in the amino acid residue of the substrates was elucidated in the present experiments. The hydrophobicities [31,32] of side chain of amino acid residues adjacent to those involved in the bonds to be split by the acid proteinase from *Asp. saitoi* are indicated in Table II. The peptide bonds which have a hydrophobic amino acid such as phenylalanine, tyrosine, leucine and isoleucine in the P'_1 position are preferentially cleaved by the acid proteinase from *Asp. saitoi*. These results show that the S'_1-P'_1 interactions might be important in increasing enzyme-substrate affinity and turnover rate.

The present results show that the acid proteinase from *Asp. saitoi* exhibited a lower preference for a hydrophobic amino acid in the P'_2 position than in the P'_1 position. While, the hydrophobicity in the P'_3 position might not be important for the activity of the acid proteinase from *Asp. saitoi*. Furthermore, it can be assumed that the acid proteinase from *Asp. saitoi* does not require the hydrophobic amino acid in the P_1, P_2 and P_3 positions of the splitting point.

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