Inhibition of *Scytalidium lignicolum* Acid Protease B by 1-Diazo-3-phenyl-2-propanone

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The acid protease B from *Scytalidium lignicolum*, a pepstatin-insensitive acid protease, was modified with 1-diazo-3-phenyl-2-propanone (DPP) in the presence of cupric ions. About 1 mol of DPP per mol of enzyme was incorporated with a concomitant loss of the activity. The modified enzyme was digested with thermolysin and DPP-labeled peptides were separated. One peptide, called S1, was purified and its amino acid sequence was found to be Ala-Thr-Ser-Asp-Thr-Ser-Gly-Ser, which corresponds to the sequence of residue Nos. 95~102 of the enzyme. Alkali treatment of the modified peptide resulted in complete removal of the modifier, suggesting that DPP is esterlinked to Asp-98 of the enzyme. The amino acid sequence around Asp-98 of the enzyme was significantly homologous to those around the active site aspartic acid residues of penicillopepsin and porcine pepsin. These results suggest that Asp 98 is one of the active site amino acids of the enzyme.

Proteolytic enzymes active at acidic pH, acid proteases, are now called aspartic proteases, since two aspartic acid residues have been shown to be involved in their catalytic reaction.¹⁾ These enzymes are commonly inhibited by *Streptomyces* pepsin inhibitor (S-PI: acetyl-pepstatin),²⁾ diazoacetyl-DL-norleucine methylester (DAN),³⁾ and 1,2-epoxy-3-(*p*-nitrophenoxy)propane (EPNP).⁴⁾ EPNP was reported to inhibit pepsin by ester-linking to Asp-32 and Asp-215,⁵⁾ while DAN was inhibitory to pepsin in the presence of cupric ions through the reaction with Asp-215.³⁾ Similar results were also reported in calf chymosin,⁶⁾ penicillopepsin,⁷⁾ and *Rhizopus* pepsin.⁸⁾

In 1972, Murao *et al.*⁹⁾ found that three new types of acid proteases produced by a strain of *Scytalidium lignicolum* are insensitive to S-PI (Ac-pepstatin). These enzymes, designated A1, A2, and B, were highly purified, and their enzymatic and physicochemical properties

were characterized by Murao and Oda.¹⁰⁾ The former two enzymes were exceptionally insensitive to acid protease inhibitors mentioned above, while the last one (B enzyme) was inhibited by EPNP but insensitive to DAN and pepstatin.^{10,11)} We¹²⁾ established the complete amino acid sequence of *S. lignicolum* acid protease B (SAB) and showed that there is only a little sequence homology between SAB and other acid proteases such as pepsin and penicillopepsin. This result and the discrepancy observed in the sensitivity to inhibitors seem to indicate that the active site structure of SAB is distinct from those of aspartic proteases hitherto reported.

Recently, we concluded that Glu-53 of SAB is one of the active site amino acid residues because Glu-53 was modified with EPNP with the concomitant loss of the activity and the amino acid sequence of the EPNP-labeled peptide was highly homologous to those around the active site Asp-215 of calf chymosin

Abbreviations: DPP, 1-diazo-3-phenyl-2-propanone; SAB, *Scytalidium lignicolum* acid protease B; S-PI, *Streptomyces* pepsin inhibitor (Ac-pepstatin); DAN, diazoacetyl-DL-norleucine methylester; EPNP, 1,2-epoxy-3-(*p*-nitrophenoxy)propane; *Rhizopus* pepsin, acid protease from *Rhizopus chinensis*.

and pepsin.^{13,14)}

To investigate whether this Glu-53 is the sole acidic amino acid participating in the catalytic reaction of SAB, we synthesized several diazo-compounds and surveyed their inhibitory activity on SAB with and without the addition of cupric ions.

This paper deals with the inhibition of SAB with 1-diazo-3-phenyl-2-propanone (DPP). DPP was found to inhibit SAB and Rhizopuspepsin as well as porcine pepsin in the presence of cupric ions. Analyses of DPP-labeled peptides indicated that Asp-98 of SAB and Asp-215 of pepsin were modified by DPP.

Materials and Methods

Materials. *Scytalidium lignicolum* ATCC24568 was a generous gift from Professor S. Murao of the Kumamoto Institute of Technology. The strain was cultured aerobically in a medium containing 5% glucose, 1.5% meat extract, 0.2% KH_2PO_4 , 0.05% $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, and 0.1% yeast extract (pH 3.5) at 25°C for 90 hr and the acid protease B (SAB) was purified from the culture filtrate by the method of Murao *et al.*,^{9,10)} with a slight modification. Rhizopuspepsin was purified as described previously.¹⁵⁾ Porcine pepsin and thermolysin were purchased from Sigma Chemical Co., U.S.A. *N*^α-Benzyloxycarbonyl-L-phenylalanyl diazomethane (ZPDM) was prepared by the method of Ong and Perlmann,¹⁶⁾ yield: 41%, mp: 84~85°C. Elemental analysis, Calcd: C, 66.86; H, 5.30; N, 13.00%. Found: C, 66.99; H, 5.49; N, 11.88%. 1-Diazo-3-phenyl-2-propanone (DPP) was synthesized from phenylacetyl-chloride and diazomethane as described by Plattner and Heusser.¹⁷⁾ DPP was obtained as yellow crystals in a 90% yield, mp 46°C. The IR spectra showed a diazo band at $2,110\text{ cm}^{-1}$ and thin-layer chromatography on silica gel plates (Merck, F254) in ethyl acetate gave a single spot with a *R_f* value of 0.73, differing from that (0.90) of the starting material, in ultraviolet irradiation. Elemental analysis, Calcd. for C, 67.49; H, 5.03; N, 17.49%; Found: C, 67.68; H, 5.09; N, 16.58%. All other chemicals were of reagent grade.

Assay of proteolytic activity and inhibition studies. The proteolytic activity was assayed by the method mentioned previously¹⁵⁾ and inhibition studies were done as follows: enzymes (0.025%) dissolved in 4 ml of 0.1 M sodium acetate buffer at appropriate pHs were incubated with 0.1 ml of cupric acetate solution (a 50-fold molar excess over enzyme) at 14°C for 10 min. Then, the reaction was started by the addition of ZPDM or DPP (the same ratio as cupric ion) dissolved in 0.1 ml of methanol at 14°C under stirring

in the dark. Samples of the reaction mixture were periodically withdrawn and the residual activities were assayed.

Preparation of modified enzymes. Pepsin (4 mg) or SAB (8 mg) dissolved in 1 ml of 0.1 M sodium acetate buffer (pH 4.8) containing a 50-fold molar excess of cupric acetate was incubated with DPP for 12 hr at 14°C as above and the reaction mixture was dialyzed against three changes of 1 l each of 10 mM acetic acid (pH 3.4) at 4°C for every 4 hr to remove excess reagents. The dialyzed solution containing the DPP-modified enzyme was gel filtered on a column (7.5 × 600 mm) of Toso, TSK gel G3000SW equilibrated with 10 mM acetic acid at a flow rate of 0.7 ml/min to remove traces of contaminants. Elution of DPP-modified enzyme was monitored by following absorbances at 280 nm and 315 nm.

The DPP incorporated into the enzyme was spectrophotometrically measured by using the molar extinction coefficients of DPP and enzymes. The values used for DPP were 5,610 at 315 nm and 230 at 280 nm. The molar extinction coefficients of SAB and pepsin at 280 nm were assumed to be 34,700¹⁰⁾ and 46,000,⁴⁾ respectively.

Proteolytic digestion of modified enzyme and the separation of peptides. DPP-modified SAB (3.4 mg) was dialyzed against 3 l of 0.1 M sodium acetate buffer, pH 6.5, containing 5 mM CaCl_2 at 4°C and digested with thermolysin (molar ratio of enzyme/substrate: 1/100) at 37°C for 2 hr in a total volume of 0.8 ml. Samples (100 μl) of the digest were put on a reverse phase HPLC column (4.6 × 250 mm, 5 μm) of Toso, ODS-120T equilibrated with 0.05% trifluoroacetic acid (TFA) at 25°C and eluted with an increasing gradient of acetonitrile containing 0.05% TFA (0~15% for 30 min starting at 10 min after injection, and then 15~70% for 40 min). The flow rate was 1.0 ml/min and the elution of peptides was monitored by following absorbances at 214 nm and 315 nm, respectively.

Removal of modifier from the labeled peptides. The DPP-modified peptides were incubated with 0.5 N hydroxylamine (pH 10.5) at 37°C for 24 hr and the reaction mixtures were analyzed by HPLC. Elution profiles of peptides and modifier were spectrophotometrically monitored at 214 nm and 315 nm.

Identification of amino acid sequence of the modified peptides. The amino acid sequences of DPP-labeled peptide fragments were identified by manual Edmann degradation by the method of Kobayashi and Tarr.¹⁸⁾

Results

Effects of DPP and ZPDM on activities of SAB, pepsin, and Rhizopuspepsin

Three acid proteases were incubated with DPP or ZPDM at pH 4.8 in the presence or

absence of cupric ions. The results are shown in Table I. DPP inhibited all of these acid proteases in the presence of Cu^{2+} , while ZPDM was inhibitory only to Rhizopuspepsin and pepsin.

In Fig. 1, the effects of pH on enzyme inhibition by DPP for SAB and pepsin were

Table I. EFFECTS OF SOME DIAZO-COMPOUNDS ON ACTIVITIES OF THREE ACID PROTEASES

The enzymes were incubated with the chemicals in the presence and absence of cupric ions at 14°C and pH 4.8 for 10 hr, and the residual activities were assayed. The other conditions are described in the text.

Chemicals	Cu^{2+}	Residual activity (relative value)		
		SAB	Rhizopuspepsin	Pepsin
None	—	100	100	100
None	+	96	93	98
DAN	—	94	92	92
DAN	+	91	0	0
ZPDM	—	92	94	95
ZPDM	+	91	0	0
DPP	—	93	95	96
DPP	+	2	0	0

DAN, diazoacetyl-DL-norleucine methylester; ZPDM, *N*-benzyloxycarbonyl phenylalanyl diazomethane; DPP, 1-diazo-3-phenyl-2-propanone.

shown. Inhibition by DPP for SAB was pH-dependent, more potent at higher pH, but pepsin was inhibited by DPP equally between pH 4 and 5.5. Course analyses of enzyme inhibitions by DPP are illustrated in Fig. 2. Pepsin was more sensitive to DPP, and more than 10 hr's incubation at pH 4.8 and 14°C was necessary for complete inhibition of SAB. The presence of 0.5% casein completely prevented the inactivation of both enzymes by DPP.

Enzymatic digestion of inactive modified enzymes and isolation of DPP-labeled peptides

The DPP-modified enzymes were separated from the excess reagents by gel filtration (Fig. 3). By measuring absorbances of the modified enzymes at 280 nm and 315 nm, the numbers of DPP molecules bound per mole of pepsin and SAB were calculated to be 1.1 and 1.3 residues, respectively.

The DPP-modified enzymes were digested with thermolysin as described in **Materials and Methods**, and the resultant peptides were separated by HPLC. The elution profiles of thermolysin-digest of the modified pepsin are shown in Fig. 4. One major peak (P1) showing

Fig. 1. Effects of pH on Inactivation of Pepsin and SAB by DPP.

The enzymes were incubated with DPP in the presence (○) or absence (●) of cupric ions at 14°C for 1 hr (A: pepsin) and 4 hr (B: SAB) at pHs indicated. The concentrations of Cu^{2+} and DPP were 50-fold molar excess of the enzymes.

Fig. 2. Changes of Enzyme Activities upon Incubation with DPP.

Pepsin (A) and SAB (B) were incubated with DPP in the presence (○) or absence (●) of cupric ions at 14°C and pH 4.8. Samples of the reaction mixtures were periodically removed and the residual activities were assayed.

absorbance at 315 nm was observed. In case of the modified SAB, three peaks, one major (S1) and two minor (S2 and P3) peaks were detected (Fig. 5). These fractions were further purified and lyophilized.

Amino acid sequences of peptides derived from the DPP-labeled sites of enzymes

The analytical data on the DPP-labeled peptide obtained from the modified pepsin are shown in Table II-A. The amino acid sequence of P1 was Ile-Val-Asp-Thr-Gly, which coincides with the sequence of residue Nos. 213~217 of pepsin. The Asp-215 should be labeled, as it is known to be one of the active site amino acid residues of pepsin.

The DPP-labeled site of SAB was also analyzed. The amino acid sequence of the major peak, S1, was Ala-Thr-Ser-Asp-Thr-Ser-Gly-Ser. This sequence agrees well with that of Nos. 95~102 of SAB. Furthermore, this sequence shows significant homology to those of the active site aspartic acid residues of pepsin and other acid proteases hitherto reported. A minor peak, S2, was obtained as a mixture of two peptides and the yield was very low. In peak S3, neither amino acid nor peptide was detected. This peak was tentatively assumed to

Fig. 3. Gel Filtration Pattern of DPP-Modified SAB on a TSK Gel G3000SW Column (7.5 × 600 mm) in 10 mM Acetic Acid (pH 3.4).

About 100 µg of the modified enzyme was filtered on gel at a flow rate of 0.7 ml/min at 25°C.

be 3-phenyl-2-oxopropanol, a hydrolyzate of DPP, since alkali treatment of the DPP-labeled peptides causes the prominent increase in this peak, as shown later.

If DPP is ester-linked to the carboxyl group of acidic amino acid residues of SAB, the modifier is expected to be released from pep-

Fig. 4. HPLC Pattern of the Thermolysin-digest of DPP-Modified Pepsin.

The thermolysin-digest, 100 μ l (70 μ g), was put on a column of TSK gel ODS-120A. An increasing linear gradient system of organic solvent (acetonitrile-2-propanol, 3:1) in 0.05% TFA was used at a flow rate of 1.0/min and 25°C.

Table II. AMINO ACID SEQUENCES OF PEPTIDES CONTAINING DPP-REACTIVE RESIDUES OBTAINED BY THERMOLYSIN-DIGESTION OF THE MODIFIED PEPSIN (A) AND *Scytalidium* ACID PROTEASE B (B)

Peptide	Amino acid sequence	Yield (%)	Inhibitor bound (mol/mol)
A			
P1	Ile-Val- ²¹⁵ Asp-Thr-Gly	34	1.1
B			
S1	Ala-Thr-Ser- ⁹⁸ Asp-Thr-Ser-Gly-Ser	27	1.2
S2	Leu-Glu-Asn, Ile-Glu-Met-Ser	3	n.d.
S3	No amino acid residue	/	/

n.d., not determined.

tides by alkali treatment, resulting in the disappearance of absorbance at 315 nm. To examine this possibility, the S1 peak was incubated with 0.5N hydroxylamine (pH 10.5) at 37°C for 24hr, and HPLC patterns before and after

alkali treatment were compared. Peak S1 disappeared upon this treatment, while another peak corresponding to Peak S3 increased greatly. This indicates that the dissociation of the modifier from S1 took place by alkali treat-

Fig. 5. Elution Profile of the Thermolysin-digest of DPP-Modified SAB.

About 50 μ l (50 μ g) of the digest was put on the column.
The other conditions were described in the text.

ment and that the binding of the modifier to peptide is base-labile, presumably be an ester-linkage between DPP and carboxyl group of acidic amino acid in the peptide. Thus, the Asp-98 of S1 peptide seems to be the most plausible candidate for binding to DPP.

Discussion

It has been reported that sequences around the active site amino acids in acid proteases show high homology,^{1,6,19-21} regardless of their origins. As shown in Fig. 6, a significant homology was observed between sequences around Asp-98 of SAB and active sites of other acid proteases.

Active site Asp-215 of pepsin is reactive to DAN⁵) and DPP, whereas another active site Asp-32 is esterified with EPNP.^{4,5}) Interestingly, the amino acid sequence around

Asp-98 of SAB, which is reactive to DPP, is more homologous to that around Asp-32 than Asp-215 of pepsin. On the contrary, the sequence around Glu-53 of SAB, which is reactive to EPNP and is possibly one of the active site amino acids of SAB, is more homologous to that around Asp-215 than Asp-32 of pepsin.^{13,14}) These may be responsible for the differences in properties such as sensitivity to inhibitors and substrate specificity between SAB and pepsin.^{10,11})

Peak S2 was a mixture of peptides. We attempted to purify these peptides to homogeneity by repeating HPLC but such procedures led to the removal of the modifier from peptides as judged by absorbance at 315 nm, and the recovery was quite low. Thus, it was very hard to elucidate the meaning for the appearance of this peak.

Taking into account the present results and

	53
SAB	-Cys-Gln-Thr-Ala-Ile-Leu-Glu-Thr-Gly-Phe-
	*
Calf chymosin	-Cys-Gln-----Ala-Ile-Leu-Asp-Thr-Gly-Thr-
Porcine pepsin	-Cys-Gln-----Ala-Ile-Val-Asp-Thr-Gly-Thr-
Penicillopepsin	-----Ser-----Gly-Ile-Ala-Asp-Thr-Gly-Thr-
Rhizopuspepsin	-Ser-Phe-Asp-Gly-Ile-Leu-Asp-Thr-Gly-Thr-
	98
SAB	-Thr-Ser-Asp-Thr-Ser-Gly-Ser-Ala-Thr-Leu-
	*
Calf chymosin	-Leu-Phe-Asp-Thr-----Gly-Ser-Ser-Asp-Leu-
Porcine pepsin	-Ile-Phe-Asp-Thr-----Gly-Ser-Ser-Asn-Leu-
Penicillopepsin	-Asn-Phe-Asp-Thr-----Gly-Ser-Ser-Asp-Phe-
Rhizopuspepsin	-Asp-Phe-Asp-Thr-----Gly-Ser-Ala-Asp-Leu-

* Active site aspartic acid residues in acid proteases

Fig. 6. Comparison of Amino Acid Sequences Around Glu-53 and Asp-98 of *Scytalidium lignicolum* Acid Protease B (SAB) with Those Around Active Site Aspartic Acid Residues of Several Acid Proteases.

the previous ones,^{13,14)} we conclude that two acidic amino acids, Glu-53 and Asp-98, participate in the catalytic reaction of SAB, unlike pepsin and other acid proteases, in which two aspartic acids have been reported to be involved in the enzyme reaction. Involvement of two acidic amino acid residues in the catalytic reaction of *Scytalidium* acid proteases has previously been suggested by Oda and Murao from kinetic and chemical modification experiments.^{22,23)}

Site-directed mutagenesis is now in progress in our laboratory to obtain more detailed information on the active site structure of SAB.

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