Inactivation of *Scytalidium lignicolum* Acid Protease B with 1,2-Epoxy-3-(4'-azido-2'-nitrophenoxy)propane[†]

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The acid protease B of *Scytalidium lignicolum* (SAB) was modified with 1,2-epoxy-3-(4'-azido-2'-nitrophenoxy)propane [EANP] under acidic conditions. EANP was incorporated stoichiometrically into SAB, and the enzyme was almost completely inactivated. The modified enzyme was digested with thermolysin and EANP-labeled peptides were separated by high performance liquid chromatography (HPLC). The amino acid sequence of the major peptide labeled was Ile-Leu-Glu-Thr-Gly, which corresponds to the sequence of residue Nos. 51 ~ 55 of the enzyme. Treatment of the peptide with 0.5 N hydroxylamine (pH 10.5) resulted in the complete removal of the modifier from the labeled peptide, suggesting that EANP is ester-linked to the Glu-53 residue of the enzyme. The amino acid sequence (-Cys-Gln-Thr-Ala-Ile-Leu-Glu-Thr-Gly-) around Glu-53 of SAB shows high homology with those around the active site aspartic acids of calf chymosin and porcine pepsin. The results indicate the involvement of Glu-53 in the enzyme reaction of SAB.

Acid proteases, now called aspartic proteases, are known to be commonly inhibited by pepstatin, S-PI, diazoacetyl-DL-norleucine methylester (DAN), and 1,2-epoxy-3-(*p*-nitrophenoxy)propane (EPNP). Recently, however, some of acid proteases produced by bacteria,^{1~4)} fungi,^{5~7)} and *Basidiomycetes*^{8~10)} were shown to be insensitive to pepstatin and DAN. Among them, the acid protease B from *Scytalidium lignicolum* (SAB) has been reported to be inhibited by EPNP⁶⁾ and we have established the complete primary structure of the enzyme¹¹⁾ and found that glutamic acid-53 (Glu-53) of the enzyme is modified by EPNP with a concomitant loss of the ac-

tivity.^{12, 13)}

To confirm this result and to survey whether or not one more acidic amino acid residue is also related to the enzyme activity, we synthesized several diazo- and azido- compounds and examined their inhibitory effect on SAB. One of the diazo compounds tested, 1-diazo-3-phenyl-2-propanone (DPP), was found to inhibit the enzyme through the modification of Asp-98 of SAB¹⁴⁾ and also one of the azido compounds, 1,2-epoxy-3-(4'-azido-2'-nitrophenoxy)propane [EANP], to react with the Glu-53, resulting in the inactivation of the enzyme activity. This report deals with the inactivation of *S. lignicolum* acid protease B by

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Abbreviations: EANP, 1,2-epoxy-3-(4'-azido-2'-nitrophenoxy)propane; EANP-OH, 1,2-dihydroxy-3-(4'-azido-2'-nitrophenoxy)propane; S-PI, *Streptomyces* pepsin inhibitor (acetyl-pepstatin); DAN, diazoacetyl-DL-norleucine methylester; EPNP, 1,2-epoxy-3-(*p*-nitrophenoxy)propane; DPP, 1-diazo-3-phenyl-2-propanone; HPLC, high performance liquid chromatography; TFA, trifluoroacetic acid; PTH-, phenylthiohydantoin.

EANP and the identification of the EANP-reactive residue of the enzyme.

Materials and Methods

Materials. The acid protease B (SAB) was purified by the method of Murao *et al.*⁶⁾ with some modifications. 4-Amino-2-nitrophenol and epichlorohydrin were purchased from Nakarai Kagaku Co., Kyoto. EANP was prepared by the method of Hixson *et al.*¹⁵⁾ with a slight modification; mp 97.5~98.5°C; Elemental analysis, Calcd for $C_9H_8O_4N_4$: C 45.76, H 3.41, N 23.72; Found: C 45.75, H 3.46, N 23.32. Purity was confirmed by NMR data. 1,2-Dihydroxy-3-(4'-azido-2'-nitrophenoxy) propane [EANP-OH], a hydrolysate of EANP, was prepared by treating EANP with 5 mM citric acid at 45°C for 12 hr.

Assay of proteolytic activity. The protease activity was assayed by the casein-Folin method. To 3 ml of 1.3% Hammarsten casein (Merck) solution in 20 mM lactate buffer (pH 2.3), 1 ml of enzyme solution was added at 37°C. After incubation for 10 min, the reaction was stopped by the addition of 4 ml of 0.44 M trichloroacetic acid (TCA). The mixture was kept at 37°C for 20 min and then filtered. One ml of the filtrate was mixed with 5 ml of 0.44 M Na_2CO_3 and 1 ml of 3-fold diluted Folin solution. After incubation at 37°C for 20 min, the absorbance at 660 nm was measured. One unit was defined as the enzyme activity which liberates TCA-soluble products equivalent to 1 μ g tyrosine per min under these conditions. The protein concentration was estimated by assuming that $E_{1\%, 1\text{ cm}}$ at 280 nm of SAB is 15.8.⁶⁾

Inhibition studies. Enzyme (0.17%) dissolved in 1 ml of 0.1 M sodium acetate buffer of various pHs was mixed with 1 ml of EANP (a 100-fold molar excess over enzyme) suspended in the same buffer under gentle stirring at 30°C in the dark or light. Aliquots of the reaction mixture were periodically removed and the residual activities were assayed.

Modification of SAB with EANP. SAB (7 mg) dissolved in 4 ml of 10 mM sodium acetate buffer (pH 3.4) was mixed with a suspension of EANP as above. After 48 hr, the reaction mixture was dialyzed against three changes of 1 l each of 10 mM sodium acetate buffer, pH 3.4, and two changes of 1 l of 0.1 M sodium acetate (pH 6.5) containing 10 mM $CaCl_2$ at 4°C every 3 hr to remove excess reagents.

The content of the modifier incorporated into the enzyme was calculated from molar absorption coefficients of EANP-OH and SAB. EANP-OH has maximum absorptions at 251 nm and 365 nm, and the molar absorption coefficients were experimentally estimated to be 19,500 M^{-1}, cm^{-1} at 251 nm, 4,890 M^{-1}, cm^{-1} at 280 nm, and 2,110 M^{-1}, cm^{-1} at 365 nm. The molar absorption coefficient of SAB at 280 nm was reported to be

34,700 M^{-1}, cm^{-1} and that at 251 nm was experimentally found to be 16,330 M^{-1}, cm^{-1} .

Proteolytic digestion of EANP-modified SAB and the separation of peptides. EANP-modified SAB (6 mg) was digested with thermolysin (50 μ g) at 37°C for 3.5 hr in 1 ml of 0.1 M sodium acetate buffer, pH 6.5, containing 10 mM $CaCl_2$. Samples of the digest were put on a reverse phase HPLC column (Vydac C18: 4.6 $\times$ 250 mm, 5 μ m) equilibrated with 0.075% trifluoroacetic acid (TFA) in water and eluted with an increasing gradient of organic solvent (acetonitrile-2-propanol: 3:1, v/v) containing 0.06% TFA (0~40% for 60 min, then 40~70% for 10 min) at a flow rate of 1.0 ml/min and 25°C. The elution of peptides was monitored by following absorbances at 214 nm and 365 nm.

Removal of modifier from EANP-labeled peptide. The peptide labeled with EANP was incubated with 0.5 N hydroxylamine (pH 10.5) at 37°C for 24 hr and the reaction mixture was used for HPLC.

Sequencing of amino acids of EANP-labeled peptide. The peptide was purified by HPLC to homogeneity and its amino acid sequence was found by manual Edman degradation by the method of Kobayashi and Tarr.¹⁶⁾ The amino acid composition was determined by the phenylthiocarbamyl-amino acid method.^{17, 18)}

Results and Discussion

Inactivation of SAB by EANP

Figure 1 shows activity changes of the enzyme caused by incubation with EANP at various pHs, and Fig. 2 illustrates analysis of

Fig. 1. Effects of pH on the Inhibitory Activity of EANP for the Enzyme.

The enzyme was incubated at 30°C for 72 hr with (—●—) or without EANP (—○—) in the dark. The other conditions are described in the text.

the course of enzyme inactivation by EANP in the presence and absence of casein. The maximum inactivation was observed at pH 3.4 and the presence of substrate effectively prevented the inactivation. This result suggests that EANP reacts at or near the substrate-binding site of the enzyme.

Incubation of SAB with EANP at 30°C for

Fig. 2. Activity Changes of the Enzyme upon Incubation with EANP in the Presence and Absence of Casein.

The enzyme was incubated with EANP at 30°C and pH 3.4. The concentration of casein was 0.5%. See the text for experimental details.

—○—, without EANP; —●—, with EANP; —□—, with EANP and casein.

Fig. 3. Correlation of Activity Change with Binding Ratio of EANP to the Enzyme.

The enzyme was incubated with a 100-fold molar excess of EANP at pH 3.4 and 30°C. To analyze the activity change and the extent of EANP-incorporation into the enzyme, aliquots of the reaction mixture were periodically withdrawn, and after removal of excess reagents, the remaining activities and the binding ratio of EANP to the enzyme were assayed.

48 hr resulted in 90% loss of the original activity, and the numbers of EANP bound per mole of SAB were calculated to be 0.95. As shown in Fig. 3, there was observed a linear correlation between the numbers of EANP bound per mole of SAB and the degree of the enzyme inactivation and stoichiometric binding of EANP to the enzyme.

Identification of EANP-reactive amino acid residue in SAB

The EANP-modified SAB was digested with thermolysin and the resultant peptides were separated by HPLC. The elution profile of the thermolysin digest is shown in Fig. 4. Three peaks showing absorbance at 365 nm, S1, S2, and S3, were observed. They were pooled and

Fig. 4. HPLC Pattern of Thermolysin-digest of EANP-modified Enzyme.

See the text for experimental details.

Table I. AMINO ACID SEQUENCE OF EANP-LABELED PEPTIDES

Peptide	Amino acid sequence	Yield (%)
S2	Ile-Leu-Glu-Thr-Gly	30
S3	Ile-Leu-Glu-Thr-Gly	3

purified by HPLC to homogeneity.

The analytical data of the EANP-labeled peptides, S2 and S3, are shown in Table I. The amino acid sequence of peptide S2 was Ile-Leu-Glu-Thr-Gly, which corresponds to the sequence of residue Nos. 51~55 of SAB, and S3 peptide had the same sequence as that of S2, though the yield was quite low. The reason why the retention time is different between the two peptides is unknown. The amount of S1 was too small to establish its amino acid sequence. The glutamic acid residue in S2 will be No. 53 of SAB, which has previously been reported to be the EPNP-reactive site in SAB.^{12, 13)}

Removal of modifier from EANP-labeled peptide

If EANP is ester-linked to a carboxyl group of an acidic amino acid residue in SAB, the modifier is expected to be released from the peptide by alkali treatment. To examine this possibility, S2 peptide was incubated with 0.5N hydroxylamine (pH 10.5) at 37°C for 24 hr, and HPLC patterns before and after alkali treatment were compared. As shown in Fig. 5, peak S2 disappeared, as monitored by absorbance at 365 nm after alkali treatment while another peak, S2A, appeared. This fraction contained neither amino acid nor peptide,

and its retention time, 40 min, exactly coincided with that of EANP-OH, a hydrolysate of EANP.

Fig. 5. HPLC Profiles of EANP-labeled Peptide before and after Treatment with 0.5N Hydroxylamine.

About 23 μ g of peptide was chromatographed by HPLC under the same conditions as in Fig. 4. The other conditions are described in the text. A and B indicate the profiles after and before alkali treatment. X, hydroxylamine.

Enzyme	Amino acid sequence	References
53		
Acid protease B of <i>S. lignicolum</i>	-Cys-Gln-Thr-Ala-Ile-Leu-Glu-Thr-Gly-Phe-	
215		
Calf chymosin	-Cys-Gln-----Ala-Ile-Leu-Asp-Thr-Gly-Thr-	19
Porcine pepsin	-Cys-Gln-----Ala-Ile-Val-Asp-Thr-Gly-Thr-	19
Penicillopepsin	-----Ser-----Gly-Ile-Ala-Asp-Thr-Gly-Thr-	20
Rhizopuspepsin	-Ser-Phe-Asp-Gly-Ile-Leu-Asp-Thr-Gly-Thr-	21, 22
98		
Acid protease B of <i>S. lignicolum</i>	-Thr-Ser-Asp-Thr-Ser-Gly-Ser-Ala-Thr-Leu-	
32		
Calf chymosin	-Leu-Phe-Asp-Thr-----Gly-Ser-Ser-Asp-Leu-	19
Porcine pepsin	-Ile-Phe-Asp-Thr-----Gly-Ser-Ser-Asn-Leu-	19
Penicillopepsin	-Asn-Phe-Asp-Thr-----Gly-Ser-Ser-Asp-Phe-	20
Rhizopuspepsin	-Asp-Phe-Asp-Thr-----Gly-Ser-Ala-Asp-Leu-	21, 22

Fig. 6. Comparison of Amino Acid Sequences around Glu-53 and Asp-98 of *Scytalidium lignicolum* Acid Protease B with Those of Active Site Aspartic Acid Residues of Some Other Pepstatin-sensitive Acid Proteases.

Another peak, S2B, which appeared after alkali treatment, showed an amino acid composition of Ile, Leu, Glu, Thr, and Gly in an equimolar ratio, indicating that it was derived from Peak S2. These results show that the dissociation of the modifier from S2 takes place by alkali treatment and that the binding of the modifier to the peptide is base-labile, presumably being an ester linkage between EANP and carboxyl group of acidic amino acid in peptide. Thus, Glu-53 may be one of the active site amino acid residues of SAB, as we have suggested.^{12, 13)}

It has been reported that sequences around active site amino acids in acid proteases show high homology, regardless of their origins.^{19~22)} As shown in Fig. 6, a significant homology was observed between sequences around Glu-53 of SAB and the active site Asp-215 of other acid proteases, except that Asp is replaced with Glu in SAB.

In general, acid proteases have two aspartic acid residues participating in the catalytic reaction.^{19~22)} In the case of SAB, Asp-98 has been suggested to be another acidic amino acid involved in the enzyme activity, since a diazo compound, 1-diazo-3-phenyl-2-propanone (DPP), modifies this residue in the presence of cupric ions, accompanied by the complete loss in the activity of SAB,¹⁴⁾ and the amino acid sequence around Asp-98 of SAB is homologous to that of active site Asp-32 of other acid proteases (Fig. 6).

To confirm the participation of two acidic amino acids in the enzyme reaction of SAB, we attempted to synthesize epoxide derivatives containing diazo or azido groups, which were expected to react as bifunctional reagents, and examined their inhibitory activity under various conditions: under illumination, at different pHs and in the presence or absence of cupric ions. EANP was found to be most inhibitory toward the enzyme under the conditions shown in Figs. 1 and 2. Contrary to our expectation, however, EANP was reactive only with Glu-53.

In the case of pepsin, Asp-32 is supposed to be ionized and Asp-215 to be in a protonated

form for its activity.^{23, 24)} Hence, Asp-215 is reactive to DAN (diazo-reactive) and Asp-32 is reactive to EPNP (epoxide-reactive). In the case of SAB, however, the amino acid sequence around Glu-53, which is reactive to EANP as well as EPNP (epoxide-reactive), is more homologous to that around Asp-215 (diazo-reactive) than to the sequence around Asp-32 (epoxide-reactive) of pepsin. Furthermore, the amino acid sequence around Asp-98, which is reactive to DPP (diazo-reactive), is more homologous to that of epoxide-reactive Asp-32 of pepsin. It remains to be clarified by further experiments whether such diversity is responsible to the differences in properties such as sensitivity to inhibitors and substrate specificity between SAB and other pepstatin-sensitive acid proteases.

From these results and previous ones, we conclude that two acidic amino acids, Glu-53 and Asp-98, participate in the enzyme activity of SAB, unlike pepstatin-sensitive acid proteases, in which two aspartic acid residues are involved in their catalytic reaction.

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