Dye-sensitized Photooxidation of Neutral Protease from *Bacillus subtilis* var. *amylosacchariticus*: Assignment of Histidine Residue Oxidized

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Received February 14, 1991

The neutral protease of *Bacillus subtilis* var. *amylosacchariticus* was photooxidized in the presence of methylene blue, by which treatment the enzyme was rapidly inactivated. The inactive enzyme was digested with endoproteinase Asp-N, the resultant peptides were separated by HPLC, and their amino acid sequences were compared with those obtained from the unmodified enzyme. Of four peptides that contained histidine residues, only the recovery of one peptide was found to be decreased by the photooxidation with the appearance of a new peptide. Comparisons of amino acid compositions and sequences between these two peptides showed that the latter peptide lacked His²²⁸ of the former one, indicating that His²²⁸ was photooxidized. This result suggests that His²²⁸ is involved in the catalytic reaction of the neutral protease or interaction with substrates.

Bacillus subtilis var. *amylosacchariticus* (*B. amylosacchariticus*) produces a Zn-containing neutral endopeptidase of high specific activity.^{1–4)} Recently, its amino acid sequence⁵⁾ and the nucleotide sequence of the enzyme gene⁶⁾ were analyzed in our laboratory. Comparison of the amino acid sequence with those of other *Bacillus* neutral proteases showed that the *B. amylosacchariticus* enzyme is 89% homologous to that from *B. amyloliquefaciens* and 48% homologous to thermolysin, a thermostable Zn-containing endopeptidase from *B. thermoproteolyticus*. The putative active site amino acid residues, Zn-binding ligands, and two Ca-binding sites were well conserved among these proteases, as judged by comparison with those of thermolysin.^{7–11)}

In a previous paper, we reported that *B. amylosacchariticus* neutral protease was quite sensitive to photooxidation catalyzed by methylene blue (MB).¹²⁾ Amino acid analysis indicated that one histidine residue was oxidized with the concomitant loss of the enzyme activity. Thus, we attempted to identify

the histidine residue photooxidized. This paper deals with the separation of the peptide containing the modified residue and its assignment in the primary structure of *B. amylosacchariticus* neutral protease.

Materials and Methods

Materials. Methylene blue and other sequencing grade chemicals were purchased from Nacalai Tesque, Kyoto, and endoproteinase Asp-N of a *Pseudomonas fragi* mutant was from Boehringer Mannheim, Germany. Phenylisothiocyanate was from Pierce, U.S.A. N-blocked synthetic peptides were obtained from the Peptide Institute, Minoh. HPLC grade acetonitrile and 2-propanol were from Kishida Chemicals, Osaka. The neutral protease was purified as described previously¹⁾ with some modifications.⁵⁾

Assays of proteolytic activity and protein concentration. The caseinolytic activity was assayed by the method described previously,¹⁾ and protein concentration was estimated spectrophotometrically by assuming that $E_{1\%,1\text{cm}}$ at 280 nm was 13.8.¹⁾ The hydrolytic activity toward synthetic peptides was monitored by measuring the digestion products by HPLC using a Vydac C18 column (5 μm , 300 $\text{\AA}$, 4.6 $\times$ 250 mm).¹³⁾ A linear gradient system

of 0.075% TFA in water vs. 0.06% TFA in acetonitrile–2-propanol (3:1) was used at room temperature and at a flow rate of 1 ml/min.

Photooxidation of neutral protease. The enzyme, 2 mg, was dissolved in 2 ml of 0.1 M Tris–HCl buffer (pH 7.5) containing 2 mM calcium acetate and 0.01% MB in a quartz cell of 1-cm path length. The reaction mixture was placed at distance of 20 cm from the front lens of a 300-W slide projector and illuminated for 25 to 120 min, stirred slowly to supply oxygen.^{12,14} The reaction mixtures were periodically withdrawn and their proteolytic activities were checked. The remainders were mixed with 1/10 volume of 99% formic acid to inactivate the remaining activity and dialyzed against 0.1 M ammonium bicarbonate buffer (pH 8.0) to remove MB.

Enzymatic digestion of the intact and oxidized enzymes and separation of the resultant peptides. The intact and photooxidized enzymes (about 1.6 mg) were digested with endoproteinase Asp-N for 24 hr at 37°C and pH 8.0 in a total volume of 2 ml. The ratio of enzyme to substrate was 1/2000 (wt/wt). Urea, 250 mg, was added to

a part (500 μ l) of the reaction mixture to completely dissolve insoluble peptides, and then the peptides were separated by reversed-phase HPLC in a manner similar to that used for separation of digestion products from the synthetic substrates as above. Some fractions were further purified on a TSK-gel ODS 120T column (5 μ m, 120 Å, 4.6 $\times$ 250 mm) using the above solvent system.

Analyses of amino acid sequence and composition. Amino acids of peptides were sequenced by manual Edman degradation.¹⁵ Phenylthiohydantoin (PTH) amino acids were measured by HPLC as described by Kobayashi and Tarr.¹⁵ Amino acid composition of peptides was determined according to Spackmann *et al.*,¹⁶ after hydrolysis with 6 N HCl containing a trace of phenol *in vacuo* at 150°C for 1 hr.

Results and Discussion

Photooxidation of the neutral protease and reduction of the activity

By photooxidation of the enzyme for 25 min

Fig. 1. HPLC Profiles of Endoproteinase Asp-N Digests of the Neutral Protease Photooxidized for 25 min and the Native Enzyme.

Black peaks indicate histidine-containing peptides. See the text for experimental detail.

A, modified enzyme (about 400 μ g); B, intact enzyme (about 300 μ g).

Table I. AMINO ACID SEQUENCES OF AMINO TERMINAL REGIONS OF HIS-CONTAINING PEPTIDES OBTAINED BY ENDOPROTEINASE ASP-N DIGESTION OF THE NATIVE AND PHOTOOXIDIZED ENZYMES*

Peak	Amino acid sequence and residue number	Yield (%)
HC-1**	⁷³ Asp-Ala- His -Tyr-Asn-Leu-Gly-Lys-Val-Tyr--- ⁸²	32
HP-1	⁷³ Asp-Ala- His -Tyr-Asn-Leu-Gly-Lys-Val-Tyr--- ⁸²	28
HC-2	⁹⁵ Asp-Asn-Lys-Gly-Ser-Lys-Ile-Val-Ser-Ser-Val- His -Tyr--- ¹⁰⁷	30
HP-2	⁹⁵ Asp-Asn-Lys-Gly-Ser-Lys-Ile-Val-Ser-Ser-Val- His -Tyr--- ¹⁰⁷	24
HC-3	¹³⁹ Asp-Val-Thr-Ala- His -Glu-Met-Thr- His -Gly--- ¹⁴⁸	18
HP-3	¹³⁹ Asp-Val-Thr-Ala- His -Glu-Met-Thr- His -Gly--- ¹⁴⁸	21
HC-4	²²³ Asp-Tyr-Gly-Gly-Val- His -Thr-Asn--- ²³⁰	28
HP-4a	²²³ Asp-Tyr-Gly-Gly-Val- His -Thr-Asn--- ²³⁰	17
HP-4b	²²³ Asp-Tyr-Gly-Gly-Val- ? -Thr-Asn--- ²³⁰	21

* 25 min illumination.

** See Figs. 1 and 2.

? Unknown product derived from histidine.

Table II. AMINO ACID COMPOSITIONS OF PEPTIDES HC-4, HP-4a AND HP-4b

Amino acids	HC-4	Peptides HP-4a	HP-4b
Asx (4)	3.5	4.2	3.6
Glx (2)	2.3	2.6	2.5
Ser (5)	4.7	4.4	4.4
Gly (4)	4.0	4.3	4.2
His (1)	1.2	1.1	0.1
Arg (1)	0.9	1.2	0.9
Thr (7)	6.5	7.0	6.7
Ala (3)	3.4	3.5	3.3
Pro (2)	1.8	1.7	2.3
Tyr (5)	5.0	4.8	5.5
Val (2)	1.5	1.4	1.4
Met (0)			
Cys (0)			
Ile (3)	2.7	2.5	3.0
Leu (3)	2.7	3.2	3.3
Phe (1)	1.0	1.0	0.9
Lys (4)	3.5	4.3	4.4
Trp (0)			
Residue No.	223-269		

The number in parenthesis indicates the composition (mol/mol) expected from the amino acid sequence.

under the condition described in Materials and Methods, the caseinolytic activity was reduced to less than 30% of the initial activity. In the

90 min photooxidation, the enzyme activity was almost completely lost. The hydrolytic activity toward synthetic substrate, Z-Gly-Leu-NH₂, was also reduced parallelly to the caseinolytic activity. Amino acid analysis indicated that loss in the activity is closely related to the modification of one histidine residue.

Digestion of the modified enzyme with endoproteinase Asp-N and identification of the histidine residue photooxidized.

An HPLC elution profile of endoproteinase Asp-N digests of the neutral protease modified by 25 min-photooxidation is illustrated and compared with that from the intact protein in Fig. 1. Of 16 main peaks derived from the native enzyme, four peptides (black peaks in Fig. 1) contain histidine residues, as has been reported previously.⁵⁾ One of them, peak HC-4 (HP-4a in the modified enzyme) was decreased by the photooxidation, and a concomitant increase in a new peak (HP-4b) was observed.

Amino acid sequences of amino terminal regions of His-containing peptides are shown in Table I, and amino acid compositions of three peptides, HC-4, HP-4a, and HP-4b, are shown in Table II, Peak HC-4 obtained from

Fig. 2. HPLC Profile of Endoproteinase Asp-N Digests of the Neutral Protease Photooxidized for 90 min. Black peaks indicate histidine-containing peptides. See the text for experimental detail.

the native enzyme was found to correspond to the peptide of residue Nos. 223-269, and decreased by the photooxidation, accompanied by the activity loss of the enzyme. The amino acid sequence and composition of a new peptide, HP-4b, were identical to those of HC-4, except that the former peptide lacked a histidine residue. These results strongly suggest that His²²⁸ is closely related to the hydrolytic reaction of the enzyme. There is, however, some discrepancy between the degree of enzyme inactivation and a relative ratio of HP-4b to HP-4a; inactivation percentage of the enzyme is somewhat higher than the accumulation degree of HP-4b. This is presumably due to a further change of HP-4b to an unknown product during the photooxidation reaction, as shown later.

An HPLC elution profile of the enzymatic digests of the neutral protease modified by 90-min photooxidation is illustrated in Fig. 2. A further decrease in peak HP-4a and concomitant increase in peak HP-4b were observed, though the recovery of the latter peak was significantly lower than that we expected. Unfortunately, we could not identify the photooxidation-derived product from histidine. In addition, further elongation of illumination time caused both peptides, HC-4 (HP-4a) and HP-4b to vanish, suggesting that further destruction or conversion of HP-4b to another unknown product took place.

Taking into account a high homology in amino acid sequences between thermolysin and *B. amylosacchariticus* neutral protease and the three dimensional structure of the former enzyme,^{8-11,17} we conclude that His²²⁸ of this enzyme corresponds to His²³¹ of thermolysin and is involved in the catalytic reaction or substrate-binding of the enzyme.

To elucidate more detailed structure in active sites of the neutral protease, analysis by site-directed mutagenesis and a computer-modelling of the three dimensional structure are now in progress.

Acknowledgment. We are grateful to Dr. R. Kobayashi for his valuable advice in amino acid sequence analysis.

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