Substrate Specificity of a Sulfhydryl Protease Purified from Germinating Corn

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Substrate specificity of a sulfhydryl protease (P-Ia) purified from germinating corn was investigated by using synthetic substrates and oxidized insulin B-chain. P-Ia showed a potent activity for *p*-nitrophenyl esters of various amino acid derivatives, except for those of carbobenzoxy-L-proline and carbobenzoxy-L-valine. Benzoylarginine- β -naphthylamide, a good substrate for papain and cathepsin B1, was not hydrolyzed by P-Ia. An investigation with acyl dipeptides showed that P-Ia hydrolyzed preferably the peptide bond adjacent to the carboxyl group of the aromatic amino acid. Oxidized insulin B-chain was hydrolyzed at the peptide bonds Gln⁴-His⁶, Glu¹³-Ala¹⁴, Ala¹⁴-Leu¹⁵, Leu¹⁵-Tyr¹⁶, Tyr¹⁶-Leu¹⁷ and Tyr²⁶-Thr²⁷. P-Ia, in spite of a sulfhydryl protease, seems to be characterized by its similarity to pepsin rather than papain, as far as the substrate specificity studied in the present work is concerned.

In our study with the degradation of zein during germination of corn, we have isolated a protease, designated P-Ia, which is considered to be a main enzyme responsible for the degradation of zein.^{1,2)} This protease, though active at a relatively low pH, is not an acid protease but a sulfhydryl one. The optimum of P-Ia lies at pH 3.0 when determined with hemoglobin as substrate. This optimum value of pH seems to be the lowest of all the sulfhydryl proteases known. Several proteases have been found in cereals,³⁻⁵⁾ but their characterizations are still insufficient. These proteases are considered to degrade reserve proteins during germination. Therefore, it will be necessary to clarify their substrate specificity for the elucidation of the action of these proteases on the reserve protein. In this paper we describe the substrate specificity of P-Ia elucidated by using synthetic substrates and oxidized insulin B-chain.

MATERIALS AND METHODS

Preparation of P-Ia. P-Ia was isolated from endosperm of germinating corn by the method described in the previous paper.²⁾

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Reagents for the measurement of esterase activity. Cbz**-Tyr-ONp (Protein Research Foundation), Cbz-Phe-ONp (Nakarai Chemicals), Cbz-Val-ONp (Fluka AG.) were used. Other *N*-acyl amino acid *p*-nitrophenyl esters were synthesized by condensing *N*-acyl amino acids with *p*-nitrophenol (Tokyo Kasei Kogyō) using dicyclohexylcarbodiimide (Tokyo Kasei Kogyō).⁶⁾ The following *N*-acyl amino acids were used for the synthesis: Ac-Gly (Nakarai Chemicals), Bz-Gly (Tokyo Kasei Kogyō), Cbz-Gly (Tokyo Kasei Kogyō), Cbz-Gln (Tokyo Kasei Kogyō), Cbz-Ala (Tokyo Kasei Kogyō), Cbz-Pro (Tokyo Kasei Kogyō), Cbz-Leu (Fluka AG.).

Measurement of esterase activity. To 0.2 ml of the enzyme solution was added 0.6 ml of 0.05 M citrate buffer (pH 3.5) containing 10 mM 2-ME. The reaction was started by adding 0.2 ml of a substrate solution in acetone and stopped by adding 5 ml of ethanol containing 2% HCl. The incubation was carried out at 37°C all the time. After incubation the solution was measured for its optical density at 340 nm and the amount of liberated *p*-nitrophenol was determined according to the standard calibration curve. The solution similarly incubated without enzyme was used as a control.

Measurement of the activity against benzoylarginine- β -naphthylamide (BANA). Benzoyl-DL-arginine- β -naphthylamide (Sigma) was dissolved in water so as to be 2 mM. To 0.5 ml of this solution was added

** Cbz-, carbobenzoxy-; -ONp, *p*-nitrophenyl ester; Ac-, acetyl; Bz-, benzoyl; 2-ME, 2-mercaptoethanol; BANA, benzoylarginine- β -naphthylamide.

0.5 ml of the enzyme solution in 0.1 M citrate buffer (pH 3.5) containing 10 mM 2-ME. After incubation 1 ml of 2% HCl-ethanol and 1 ml of 0.06% *p*-dimethylaminocinnamaldehyde in ethanol were added. The mixture was allowed to stand for 30 min and then measured for optical density at 540 nm. Papain (Sigma) and pepsin (Sigma) were used for comparison with P-Ia.

Measurement of the activity against N-acyl dipeptides. Cbz-Gly-Gly (Sigma), Cbz-Gly-Leu (Protein Research Foundation), Cbz-Gly-Phe (Protein Research Foundation), Cbz-Ala-Gly (Fluka AG.) and Cbz-Phe-Gly (Fluka AG.) were used as substrates. A mixture of 0.5 ml of the enzyme solution and 0.35 ml of 0.05 M citrate buffer (pH 3.5) containing 10 mM 2-ME was maintained at 37°C for 10 min. Therein was added each substrate which had been dissolved in 0.15 ml acetone. After incubation at 37°C an aliquot of the reaction mixture was subjected to the ninhydrin treatment according to the method of Yemm and Cocking.⁷⁾ A solution similarly incubated without enzyme was used as a control. Every measurement was expressed as leucine equivalent.

Kinetic measurement. Initial rates were measured and the two parameters, K_m and k_{cat} , were calculated by using an equation⁸⁾ induced from the Michaelis-Menten equation.

Hydrolysis of oxidized insulin B-chain. About 15 mg of oxidized insulin B-chain (Boehringer Mannheim) was dissolved in 3 ml of 0.1 N HCl and the pH was adjusted to 4.0 with 0.05 N sodium acetate. After 0.4 ml of P-Ia solution was added, the pH was readjusted exactly to 4.0 with 0.01 N HCl and the incubation carried out at 37°C.

Identification of peptides in the hydrolysate of oxidized insulin B-chain. The hydrolysate solution was neutralized with dil. NaOH and, after heating at 100°C for 5 min, spotted on a 0.25 mm pre-coated TLC plate (20 cm × 20 cm) of silica gel (Merck). Butanol-acetic acid-water (4:1:2) was used as a development solvent. After dried completely, the plate was developed again with the same solvent. A small strip was cut off from the twice-developed plate and treated with ninhydrin reagent for color development. The silica gel powder corresponding to the same R_f position as in the strip was collected and treated with dil. HCl (pH 2) to extract a single peptide. The peptide was hydrolyzed with 6 N HCl at 110°C for 24 hr to determine its constituent amino acids with a Hitachi amino acid analyzer (KLA-5).

RESULTS AND DISCUSSION

A comparative study with Cbz-Tyr-ONp

TABLE I. ACTION OF P-Ia, COMPARED TO PAPAIN AND PEPSIN, ON CARBOBENZOXITYROSINE *p*-NITROPHENOL AND BENZOYLARGININE- β -NAPHTHYLAMIDE (BANA)^a

Substrate	Protease		
	P-Ia	Papain	Pepsin
Hemoglobin ^b	100	100	100
Cbz-Tyr-ONp ^c	22	100	0
BANA ^c	0	100	0

^a Every activity was measured at pH 3.5 using 0.05 M citrate buffer. 2-Mercaptoethanol was added to make a 10 mM concentration in the case of P-Ia and papain. Proteases having the same activity for hemoglobin were used, and the activity of papain was expressed as 100 in the case of each substrate.

^b Activity measured by the method described before.²⁾

^c Activity was measured by the method described in the experimental part of this paper.

and BANA as substrates showed that papain had a relatively high activity for Cbz-Tyr-ONp and BANA whereas pepsin having the same activity against hemoglobin as papain had no activity for any of the substrates (Table I). P-Ia showed a relatively weak activity for Cbz-Tyr-ONp, not for BANA. In this regard P-Ia seems to be similar to pepsin rather than papain. It should be noted that P-Ia shows no activity for BANA. Several endo-type intracellular proteases of animal origin are known which have optimum pH values in an acidic region. Cathepsin D⁹⁾ and cathepsin B¹⁰⁾ are the most popular among them. Some endo-type proteases in cereals are also active under an acidic condition. In certain respects these had been thought to be similar to cathepsin D or cathepsin B1. In the previous report²⁾ we concluded that P-Ia was a sulfhydryl protease. In this respect, P-Ia is not related to cathepsin D. It appears, therefore, that P-Ia is close to cathepsin B1 which is known to be a sulfhydryl protease and active under an acidic condition. However, P-Ia did not actually act on BANA which is a very good substrate for cathepsin B1.¹¹⁾ From these studies it is clear that P-Ia is neither cathepsin D type nor cathepsin B1 type enzyme.

Table II shows the kinetic parameters for

TABLE II. KINETIC PARAMETERS FOR P-Ia
ACTING ON *p*-NITROPHENYL ESTERS^a

Substrate	<i>K_m</i> (μ M)	<i>k_{cat}</i> (sec ⁻¹)	<i>k_{cat}/K_m</i> (sec ⁻¹ μ M ⁻¹)
Ac-Gly-ONp	14.2	0.990	0.0697
Bz-Gly-ONp	25.3	0.739	0.0293
Cbz-Gly-ONp	32.2	0.535	0.0166
Cbz-Ala-ONp	7.81	0.509	0.0652
Cbz-Val-ONp	275.0	0.112	0.0004
Cbz-Leu-ONp	7.55	0.563	0.0746
Cbz-Gln-ONp	49.0	0.835	0.0170
Cbz-Pro-ONp	1000.0	0.054	0.00005
Cbz-Tyr-ONp	9.53	0.589	0.0618
Cbz-Phe-ONp	7.80	0.383	0.0491

^a At pH 3.5 and 37°C in the presence of 20% acetone.

P-Ia to act on *N*-acyl amino acid *p*-nitrophenyl esters. From the data obtained with Cbz-Val-ONp it is estimated that the amino acid moiety having the β -branched side-chain can fit with difficulty to the active site of P-Ia as well as to those of most proteases. This is the case with the proline residue. The substitution of Gly in Cbz-Gly-ONp with any of Ala, Leu, Tyr and Phe was effective in decreasing *K_m* to any extent. But this extent is rather small probably because of a greater contribution of *p*-nitrophenyl residue to the decrease of *K_m*.

The primary specificity of P-Ia was shown more clearly from the experiment with *N*-acyl dipeptides (Table III). It is obvious that a peptide bond involving the α -carboxyl group of hydrophobic amino acid, especially of aromatic amino acid, is split more readily. A possible effect of amino acid residue giving amino group to the peptide bond to be hydrolyzed remains to be studied. Interestingly enough, the *K_m* value for Cbz-Gly-Phe is

TABLE III. KINETIC PARAMETERS FOR P-Ia
ACTING ON CARBOBENZOXY DIPEPTIDES^a

	<i>K_m</i> (mM)	<i>k_{cat}</i> (sec ⁻¹)	<i>k_{cat}/K_m</i> (sec ⁻¹ mM ⁻¹)
Cbz-Gly-Gly	40	0.089	0.0022
Cbz-Gly-Leu	13.3	0.062	0.0047
Cbz-Gly-Phe	0.551	0.014	0.0254
Cbz-Ala-Gly	2.45	0.100	0.0408
Cbz-Phe-Gly	1.05	0.234	0.2229

^a At pH 3.5 and 37°C in the presence of 15% acetone.

FIG. 1. Specificity of P-Ia in Relation to Its Active Site.

as small as that for Cbz-Phe-Gly whereas *k_{cat}* for the former is much smaller than that for the latter. To explain this phenomenon we could present a schematic diagram (Fig. 1), in which it is postulated that *S₁* has a high affinity to the aromatic amino acid residue and peptide bond is easily split at the C-terminal of the amino acid residue. Therefore, when Cbz-Gly-Phe fits to the active site of P-Ia, the phenylalanine residue is expected to prefer *S₁* to *S₁'*. In our experimental condition at pH 3.5, the α -carboxyl group is not fully ionized and, consequently, the phenylalanine residue can fit more easily to *S₁* and presumably act as a competitive inhibitor. In the case of Cbz-Gly-Leu also, an almost similar effect may occur.

To clarify the substrate specificity of a protease the action on protein should be investigated. Oxidized insulin B-chain has been used most often for this purpose. The splitting points are known from the past studies with many common proteases.¹²⁻¹⁴ Therefore, the action of P-Ia against oxidized insulin B-chain was investigated. During the treatment of the substrate with P-Ia, new spots E, F and I appeared on the TLC plate after 2 hr, with the increase of the spot number up to about ten after 46 hr (Fig. 2). Among those six main spots named A, C, E, F, G and I were isolated. Amino acid compositions of the peptide fragments corresponding to the six spots are shown

FIG. 2. Thin-layer Chromatograms of Oxidized Insulin B-Chain Hydrolyzed with P-Ia for Various Period of Hydrolysis.

Butanol:acetic acid:water (4:1:2) was used for the development. The spots were detected by the ninhydrin method.

in Table IV. Free amino acids in the P-Ia hydrolysate were also determined. From these results six peptides were considered to be derived from the positions shown in Fig. 3 and therefore the cleavage points were esti-

TABLE IV. AMINO ACID COMPOSITIONS OF THE PEPTIDE FRAGMENTS ISOLATED FROM THE HYDROLYSATE OF OXIDIZED INSULIN B-CHAIN^a

Constituent amino acid	Peptide fragment						Free amino acid
	A	C	E	F	G	I	
Lys	1.2	—	—	0.9	—	—	—
His	—	2.0	—	—	—	—	—
Arg	—	—	—	0.9	—	—	—
CySO ₃ H	—	0.8	—	1.1	—	—	—
Asp	—	0.9	1.0	—	—	—	—
Thr	1.0	—	—	1.1	—	—	—
Ser	—	1.0	—	—	—	—	—
Glu	—	2.0	0.7	1.0	—	—	—
Pro	1.2	—	—	1.0	—	—	—
Gly	—	1.7	—	2.3	—	—	—
Ala	1.0	0.8	—	1.2	0.9	0.9	1.0
Val	—	2.1	1.0	1.2	—	—	—
Leu	—	2.1	—	1.1	1.1	1.2	1.3
Tyr	—	—	—	1.0	1.1	—	1.3
Phe	—	1.2	1.2	1.8	—	—	—

^a Data given as approximate integrals.

mated as shown in Fig. 4. Among these cleavage points the peptide bond between Tyr¹⁶

FIG. 3. Most Probable Sequences and Positions of the Isolated Peptides.

FIG. 4. Estimated Points of Cleavage of Oxidized Insulin B-Chain. Arrows of dotted line show the bonds hydrolyzed to a small extent.

and Leu¹⁷ is considered to be hydrolyzed most easily, because peptide F appeared first of all. Figure 4 includes the cleavage points already studied with papain¹³⁾ and pepsin.¹⁴⁾ P-Ia can hydrolyze preferably peptide bonds adjacent to the carboxyl group of tyrosine and seems to be similar to pepsin rather than papain. These results can well explain the high release rates of aromatic amino acids during corn germination and ascertain the contribution of Pa-I to the degradation of zein.¹⁾

Collating the data obtained from the present study, we can postulate: (1) that P-Ia hydrolyzes zein preferably at the peptide bond adjacent to the carboxyl group of aromatic amino acid and (2) that resulted peptides are attacked by carboxypeptidases to liberate effectively aromatic amino acids.

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