Note

Characterization of Benzoyl-L-arginine *p*-Nitroanilide Hydrolase in Rice Embryo

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Proteolytic enzymes in plant seeds have been extensively studied in relation to mobilization of storage proteins of seeds.¹⁾ Most of them were optimally active at acidic pH, but amino peptidases and BAPAase which hydrolyzes benzoyl-L-arginine *p*-nitroanilide (BAPA) were at alkaline pH. BAPAase has been found in various plants,^{2~10)} but its enzymatic properties have not been well characterized. In the present note, partial purification and characterization of a BAPAase from the embryo of rice seeds are described.

BAPAase activity was assayed as follows. The reaction mixture containing the enzyme and 0.1M Tricine [N-tris(hydroxymethyl)methylglycine] buffer, pH 9.0, in a total volume of 0.99 ml was mixed with 0.01 ml of BAPA solution (50 mM in dimethyl sulfoxide). After reaction for 10 min at 37°C, 1 ml of 1M acetic acid was added to the reaction mixture, and the absorbancy at 400nm was measured. LPAase (L-leucine *p*-nitroanilide hydrolase) activity was assayed in a similar manner to BAPAase except for the use of 0.05M MOPS [3,(*N*-morpholino)propanesulfonic acid] buffer containing 1.0M Na acetate, pH 7.2, and LPA solution (100mM in dimethyl sulfoxide). A unit of BAPAase and LPAase is that amount which hydrolyzes 1 μ mol of BAPA and LPA, respectively, per min under the conditions described above. Esterase activity was assayed with a pH-Stat (Radiometer) and amidase activity, by colorimetric ninhydrin procedure. About a half of each BAPAase or LPAase activity in the whole seed of rice was detected in the embryo. Since the embryo occupies only 4% of the whole seed by weight, the specific activities of BAPAase and LPAase were very high in embryo. Partial purification of BAPAase was carried out from 600 g of defatted rice embryos. The embryos were homogenized with 2 liters of 0.01M Tris-HCl buffer, pH 7.2, containing 1M NaCl at room temperature. The extracts (374 units of BAPAase, 2530 units of LPAase and 6.7g of protein) were precipitated by the addition of ammonium sulfate to 50% saturation. The precipitate was collected by centrifugation, and washed with various concentrations of Ammonium sulfate solution. The washings with 45 to 37.5% saturation of ammonium sulfate

solution contained most of LPAase, and those with 35 to 30% saturation of ammonium sulfate solution contained BAPAase. The latter was used for further purification of BAPAase by gel chromatography on Sephadex G-150. The eluates containing BAPAase (95.2 units of BAPAase and 167 mg of proteins) were collected and used for the characterization of the enzyme. A part of the eluates was further purified by isoelectric focusing (IEF). The enzyme obtained after IEF (12.3 units of BAPAase and 5.98 mg of protein) was used for experiments described in Table I.

BAPAase (*pI*=4.9) and LPAase (*pI*=4.7) were separated by IEF indicating both of the activities derived from different enzymes. The BAPAase was stable at pH 7~8 for 1 hr at 37°C, and at pH 7.5 for 17 hr at 37°C. Optimum pH for the activity was observed at pH 9~9.5 at 37°C.

In addition to BAPA, the BAPAase hydrolyzed ethylester and amide derivatives of benzoyl-L-arginine in similar rates to BAPA (Table I). Esters of tosyl-L-arginine and tosyl-L-lysine were also hydrolyzed by the BAPAase. However, derivatives of L-tyrosine were not hydrolyzed by the enzyme. The enzyme activity was inhibited by DFP, antipain, leupeptin and tosyl-L-lysine chloromethylketone (TLCK) (Table II). These results indicate that the BAPAase is an enzyme similar to pancreatic trypsin. However, the BAPAase was not inhibited by soybean trypsin inhibitor. *p*-Chloromercuribenzoic acid inhibited the BAPAase, and the inhibited enzyme was reactivated by the addition of cysteine, indicating the BAPAase has characteristics of an SH-enzyme. Hydrolysis of casein, urea-denatured haemoglobin and heat-denatured albumin with BAPAase were examined by the method described by Kunitz.¹¹⁾ No hydrolysis of proteins was detected after reactions with the BAPAase (0.1 units per ml of the reaction mixture) for 20 hr at 37°C.

In conclusion, the present rice BAPAase is an enzyme optimally active at alkaline pH and it has characteristics both of a serine enzyme similar to trypsin and of an SH-

TABLE I. SUBSTRATE SPECIFICITY OF THE RICE BAPAASE

The enzyme solution purified by IEF (0.01~0.05 unit per ml of the reaction mixture) was used for the experiment. All activity assays were carried out at pH 8.0, 37°C.

Substrates	Relative activity (%)
0.5 mM Benzyl-L-Arg <i>p</i> -nitroanilide	100
0.5 mM Benzoyl-L-Tyr <i>p</i> -nitroanilide	0
3.0 mM Benzoyl-L-Arg amide	99.7
3.0 mM Benzoyl-L-Arg ethylester	149.3
3.0 mM Tosyl-L-Arg methylester	52.1
3.0 mM Tosyl-L-Lys methylester	42.0
3.0 mM Benzoyl-L-Tyr ethylester	0
3.0 mM Acetyl-L-Tyr ethylester	0

TABLE II. EFFECTS OF VARIOUS INHIBITORS AND ACTIVATORS ON THE BAPAAASE

Preincubation mixture contained the partially purified enzyme (0.0106 unit of BAPAAase and 18.6 μ g of protein), 0.1 M Tricine buffer, pH 8.0, and various inhibitors or activators in a total volume of 0.99 ml. After preincubation for 30 min at 37°C, BAPAAase activity was assayed by the addition of 0.01 ml of BAPA solution to the mixture as described in the text.

Additions	Concentrations ^a	Activity (%)
None	—	100
DFP	10 ⁻⁴ M	8.1
Antipain	2 μ g/ml	3.4
Leupeptin	2 μ g/ml	12.5
Soybean trypsin inhibitor	100 μ g/ml	99.2
TLCK	10 ⁻⁴ M	4.2
TPCK	10 ⁻⁴ M	87.9
EDTA	10 ⁻³ M	100
Iodoacetic acid	10 ⁻² M	64.2
PCMB	3 $\times$ 10 ⁻⁵ M	42.5
	10 ⁻⁴ M	11.5
Cysteine	10 ⁻³ M	104.2
	10 ⁻² M	101.6
2,3-Dimercapto-1-propanol	10 ⁻⁵ M	105.2
	10 ⁻⁴ M	109.6
PCMB ^b 3 $\times$ 10 ⁻⁵ M + cysteine 10 ⁻³ M		87.5
PCMB ^b 3 $\times$ 10 ⁻⁵ M + cysteine 10 ⁻² M		96.0

^a Concentrations are those in the final reaction mixtures.

^b The enzyme was preincubated with PCMB for 30 min at 37°C and then further incubated with cysteine for 10 min at 37°C.

TPCK (tosyl-L-phenylalanine chloromethyl ketone).

enzyme. However, hydrolysis of protein substrates was not detected in the present experiments. Absence of proteolytic activity of BAPAAase has been reported for the enzyme obtained from soybean,⁴⁾ peanuts⁶⁾ and wheat.¹⁰⁾ The action of the rice BAPAAase on peptide substrate is being investigated.

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