Purification and Some Properties of a Carboxypeptidase in Rice Bran

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Resting rice seeds contained a carboxypeptidase which hydrolyzed CPA(carbobenzoxyl-L-phenylalanyl-L-alanine) at pH 4 and this enzyme was tentatively termed CPAase-4. This enzyme is distributed in endosperm of rice grain, and its distribution pattern was similar to that of glutelin. CPAase-4 was purified from rice bran by chromatography on CM-Sephadex and gel filtration. The molecular weight of the enzyme was estimated to be 110,000. By SDS gel electrophoresis, the presence of two subunits (MW=35,000 and 21,000) was detected. The activity was inhibited when the enzyme had been preincubated with DFP at pH 7. The enzyme was optimally active for CPA at pH 4 and for acetyl-L-phenylalanine ethyl ester at pH 8, and was stable in a wide pH range (2.5~8.5).

As described in the preceding paper,¹⁾ resting rice seeds contained an enzyme, which was optimally active for CPA at pH 4, and this enzyme had been tentatively termed CPAase-4. CPA was the best substrate for many plant carboxypeptidases as far as examined.^{2~7)} The optimum pH of rice CPAase-4 was lower than those of most of plant carboxypeptidases, which were optimally active at pH 5~5.5. CPAase-4 activity in resting seeds did not increase, but rather decreased, during germination. This enzyme might act for the mobilization of storage proteins in the initial stage of germination. However, further characterization of CPAase-4 is needed to determine the physiological meanings of this enzyme.

The present paper describes the distribution of CPAase-4 in rice seeds, and the purification and some properties of the enzyme from rice bran.

MATERIALS AND METHODS

Plant materials. Rice seeds of low protein content (*Oryza sativa*, var. *Nihonbare*) were used throughout the experiments. Rice bran polished to various degrees and polished rice grain were kindly supplied from Okura Shuzo (Sake Brewing) Co., Ltd., Kyoto. The bran was passed through a sieve of 20 mesh to remove embryos. The polished rice grain was washed well with water to remove adsorbed bran and then air-dried.

Oligopeptide. BP-I, bitter peptide separated from the tryptic hydrolyzate of casein, was obtained in accordance with the method described by Matoba *et al.*⁸⁾ Its amino acid sequence is Gly-Pro-Phe-Pro-Ile-Ile-Val.

Chemical analysis. Dry weights of the samples were determined after drying at 105°C for 4 hr. Total nitrogen and total phosphorus were determined by micro-Kjeldahl⁹⁾ and Bartlett's procedure,¹⁰⁾ respectively. Total protein in bran and polished rice seeds was calculated by multiplying 6.25 times the nitrogen content. Protein contents in the extracts from bran and in the enzyme solution were determined by the method of Lowry *et al.*¹¹⁾

Enzyme assay The CPAase-4 activity for CPA was assayed at pH 4.0, 37°C, as described in a previous paper.¹⁾ Esterase activity was assayed at pH 5.2 by the pH-Stat method at 25°C.¹²⁾ Acid phosphatase was assayed at pH 5.2, 37°C, with *p*-nitrophenyl phosphate as the substrate as described in a previous paper.¹²⁾ Units of activities are expressed in μ mol of substrates hydrolyzed per min. Specific activities are expressed in units per mg of protein.

Abbreviations used: APEE, *N*-acetyl-L-phenylalanine ethyl ester; CPA, *N*-carbobenzoxyl-L-phenylalanyl-L-alanine; CPAase, CPA hydrolase; DEP, diisopropylfluorophosphate; IgG-L and -H, immunoglobulin G, L chain and H chain; PCMB, *p*-chloromercuribenzoic acid; SDS, sodium dodecyl sulfate; Tricine, tris(hydroxymethyl)methyl glycine; Z-, carbobenzoxyl-.

Molecular weight. The molecular weight was estimated from the elution volume on Sephadex G-200 by the method of Andrews.¹³⁾

Gel electrophoresis. Disc polyacrylamide gel electrophoresis was carried out at pH 4.5 by the method of Reisfeld *et al.*¹⁴⁾ in 5% acrylamide gel. Instead of 0.35 M β -alanine, 0.35 M Tricine was used for the preparation of stock solution F. The pH of the solution F was adjusted to 4.5 with 1 M acetic acid. Electrophoretic run was performed in the direction of cathode at 3 mA per tube for 2 hr at 4°C. After electrophoresis of duplicate gels, the one was stained with Amido Black 10B, and the other was incubated with CPA at pH 4.0, 37°C, for 10 min. The latter was transferred into 1% ninhydrin solution containing 50% methyl cellosolve, 0.1 M citrate buffer, pH 5.2, and 0.01% SnCl_2 . Then, bands due to CPAase activity were visualized by heating the gel. Polyacrylamide gel electrophoresis in the presence of SDS was carried out by the procedure of Weber and Osborn.¹⁵⁾ Marker proteins and CPAase-4 were previously treated with 1% SDS and 2-mercaptoethanol in 25% glycerol at 100°C for 2 min and subjected to electrophoresis on

7% acrylamide gel in the presence of 0.1% SDS at 8 mA per tube for 6 hr. The gels were stained with Amido Black 10B, and protein bands were recorded with a Shimadzu Dual-Wavelength TLC Scanner CS-900.

RESULTS

Distribution of CPAase-4 in rice grains

The effect of salt concentration on the efficiency of extraction of CPAase-4 was examined prior to the investigation of the distribution of the enzyme in rice grains. CPAase-4 was extracted with 1 M NaCl more than with water and 0.1 M NaCl. Therefore, the distribution of proteins and enzymes in rice grain was examined with 1 M NaCl as extraction medium. Brans of different degrees of polishing were separated into four fractions from outside to inside, the different degrees of polishing being as follows; I (0~10%), II

FIG. 1. Distribution of Proteins, Phosphorus and Enzymes in Rice Grains.

Brans of rice grains polished in various degrees, I (0~10%), II (10~16%), III (16~22%), IV (22~27%) and residual kernels (27~100%) were analyzed. Polishing % indicates the proportion of bran to the whole grain by weight.

(a) $\square$, Total protein (Kjeldahl $\text{N} \times 6.25$) was measured after digestion of samples with H_2SO_4 ;

$\blacksquare$, Soluble protein in 1 M NaCl was measured by the method of Lowry *et al.*

(b) Total phosphorus was measured after digestion of samples with H_2SO_4 .

(c) CPAase-4 activity was assayed for the 1 M NaCl extract.

(d) Acid phosphatase activity was assayed for the 1 M NaCl extract.

All values are based on the dry weights of original materials. Extraction by 1 M NaCl was carried out as follows: 1 g of rice bran or kernels, 2 g of sea sand and 9 ml of 1 M NaCl were homogenized in a mortar with a pestle for 5 min. The homogenate was centrifuged for 20 min at 3000 rpm. Aliquots of the supernatant solution were used for protein determination and enzyme assays.

(10~16%), III (16~22%) and IV (22~27%). Polishing % in parentheses represent weight ratios of bran to unpolished grain. Fraction V (27~100%) is a residual kernel. This kernel had been washed well with water to remove the adsorbed bran. Total protein and total phosphorus were analyzed after degradation of samples by sulfuric acid. The soluble proteins in salt solution, CPAase-4 and acid phosphatase were analyzed after extraction with 1M NaCl as described in the caption of Fig. 1, in which the results are diagrammed. Total protein and proteins soluble in 1 M NaCl (albumins and globulins) were rich in Fraction I.

The difference between total protein and the soluble protein in 1 M NaCl, which gives a rough estimate of the amount of glutelin, was greater in Fraction II, III, and IV than in Fraction I. The soluble proteins in 1 M NaCl solution were very little in the kernels, and the insoluble proteins were predominant. Total phosphorus content was very high in the outer coat (Fraction I) and low inside the grains. CPAase-4 activity was higher in Fraction II than in Fraction I, and the general distribution pattern of this enzyme was similar to that of the insoluble proteins in 1 M NaCl. Though the CPAase-4 activity in the kernels was low, an appreciable amount of the activity was detected. The highest acid phosphatase activity was found in Fraction I, and its activity decreased inside the grains except for the kernels. The distribution pattern of acid phosphatase was similar to that

of total phosphorus except in the kernels.

Purification of CPAase-4 from rice bran

Six kg of white brans (10~20% polishing) was suspended with 12 liters of *n*-hexane. After removal of hexane with filtration, the bran was air-dried and then homogenized with 30 liters of 0.1 M acetate buffer, pH 5.0, containing 0.1 M NaCl and 10 mM 2-mercaptoethanol. The homogenate was stirred for 1 hr and centrifuged for 20 min at 8,000×*g*. Ammonium sulfate was added to the supernatant solution (23 liters) to 40% saturation. The precipitates were removed by centrifugation, and ammonium sulfate was added to the supernatant solution to 55% saturation. The resulting precipitates were collected by centrifugation, dissolved in 0.01 M acetate buffer, pH 5.0, containing 10 mM 2-mercaptoethanol, and dialyzed against the same buffer. The dialyze (12.8 g protein in 400 ml) was applied to the duplicate columns of CM-Sephadex C-50 (5×50 cm) which had been equilibrated with 0.01 M acetate buffer, pH 5.0, containing 1 mM 2-mercaptoethanol. The

TABLE I. SUMMARY OF PURIFICATION PROCEDURE OF RICE BRAN CPAASE-4

Step	Protein (mg)	Activity (units)	Spec. act. (units/mg)	Yield (%)
Extraction	238,000	7,800	0.033	100
(NH ₄) ₂ SO ₄ 40~55% satn.	12,800	3,800	0.295	48
CM-Sephadex	35	995	28.7	13
Sephadex G-100	20	858	43.0	11
Sephadex G-200	7.2	400	55.6	5

FIG. 2. Chromatography of Rice Bran CPAase-4 on CM-Sephadex C-50.

The solution (170 ml) containing 1,300 units of enzyme and 4 g of proteins was applied to the CM-Sephadex column (700 ml: 5×50 cm) which had been equilibrated with 0.01 M acetate buffer, pH 5.0, containing 10 mM 2-mercaptoethanol. The column, was developed with the same buffer containing 0.3 M or 0.45 M NaCl as indicated in the figure. - - - -, protein (*A*₂₈₀);, NaCl concentration; ●—●, activity.

column was washed with the same buffer and then developed with the same buffer containing 0.3 M NaCl. The colored inert protein which had been adsorbed at the top of the column was gradually eluted during washing with the buffer containing 0.3 M NaCl (fractions 150 to 210 in Fig. 2). After the elution of this inert protein was completed, salt concentration in the buffer was elevated to 0.45 M and CPAase-4 was eluted. By this simple chromatography, the specific activity of CPAase increased about 100-fold. The active fractions were collected and precipitated with reverse dialysis against saturated ammonium sulfate solution. The precipitates were collected by centrifugation and further purified by gel chromatography on Sephadex G-100 and G-200. Figure 3 shows the final chromatogram with Sephadex G-200. A single symmetrical peak for activity and protein was obtained. The purification procedures are summarized in Table I. The enzyme was purified about 1000-fold from the crude extract by these procedures.

FIG. 3. Gel Chromatography of Rice Bran CPAase-4 on Sephadex G-200.

The enzyme solution (2 ml) contained 43 units of enzyme and 20 mg of protein. The column was packed with Sephadex G-200 (500 ml: 2.5×100 cm) and equilibrated with 0.01 M acetate buffer, pH 5.0, containing 0.1 M NaCl and 10 mM 2-mercaptoethanol. Proteins of known molecular weights were successively analyzed with the same column, and the peak positions are shown in the figure. - - - - -, protein (A_{280}), ●—●, activity.

Purity of the enzyme

A single peak observed in the chromatogram on Sephadex G-200 (Fig. 3) indicates the homogeneity of molecular size in the enzyme solution. Disc gel electrophoresis of the enzyme at pH 4.5 exhibited a sharp protein band, and the presence of CPAase activity was indicated in the same position (Fig. 4). From these findings, it can be concluded that the purified enzyme was homogeneous.

Molecular weight

Molecular weight of the enzyme was calculated to be 110,000 from the results shown in Fig. 3 by the method of Andrews.¹³⁾ SDS-polyacryl amide gel electrophoresis of the

FIG. 4. Disc Electrophoresis of Rice Bran CPAase-4.

(a) Cationic run at pH 4.5 in 7.5% polyacrylamide gel (10 μ g protein), 3 mA per tube for 2 hr. The protein band was visualized with Amido Black 10B. (b) Same as (a), but the band was visualized with ninhydrin reagent after enzymatic reaction with CPA. The gel considerably shrunk on heating in ninhydrin solution.

FIG. 5. SDS-polyacrylamide Gel Electrophoresis of Rice Bran CPAase-4.

The purified CPAase-4 and marker proteins were previously reduced and denatured as described in MATERIALS AND METHODS. Electrophoretic run was carried out in 7% acrylamide gel containing 0.1% SDS for 6 hr (8 mA per tube). Protein bands were visualized with Amido Black 10B and recorded with a densitometer. Peak positions of marker proteins are indicated in the figure.

reduced enzyme gave two protein bands (Fig. 5). Molecular weights of the two bands were estimated to be 35,000 and 21,000, and the amount of each protein calculated from Fig. 5 was approximately proportional to its molecular weight. The results of these experiments indicate that the native enzyme is a tetramer and is composed of two homologous subunits each. The pH of the isoelectric point of the purified enzyme was determined to be 7.8 by isoelectric focusing as described in a preceding paper.¹⁾

Effects of pH on the enzyme activity and stability

The optimum pH of the purified enzyme for CPA hydrolysis was found to be 4.0 (Fig. 6a). Crude enzyme solution which had been extracted from rice bran showed the same pH-dependence of the activity as the purified enzyme. Esterase activity for APEE was most enhanced at pH 8.0. The purified enzyme was very stable and could be stored

FIG. 6. The pH-Dependences of the Activity (a) and the Stability (b) of Rice Bran CPAase-4.

(a): ●—●, peptidase activity for CPA of the purified CPAase-4; △—△, peptidase activity for CPA of the crude extract from rice bran; ○—○, esterase activity for APEE of the purified CPAase-4. The crude extract was prepared from bran (10–20% polished) with 0.1 M NaCl as described in Fig. 1. Peptidase activity was assayed in the reaction mixture (1 ml) containing the purified enzyme (0.18 μ g) or the crude extract (5.8 mg), 0.05 M acetate, 0.05 M Tricine, 0.5 M sucrose and 3.3 mM CPA at various pH, 37°C, for 30 min. Esterase activity was assayed in the reaction mixture (2.5 ml) containing the purified enzyme (0.9 μ g), 10 mM APEE and 0.5 M sucrose at various pH, 25°C.

(b): ●—●, stability of the purified enzyme after 60 min at 37°C, ○—○, stability of the purified enzyme after 30 min at 50°C.

The purified CPAase-4 (0.18 μ g) was preincubated in 100 μ l of the solution containing 0.05 M acetate, 0.05 M Tricine and 0.5 M sucrose at various pH. After the preincubation, the residual CPAase activity was assayed at pH 4.0, 37°C, for 30 min.

several months at 5°C without loss of activity. The stability of the enzyme examined at 37°C for 60 min and 50°C for 30 min, is shown in Fig. 6b. The enzyme was stable in a wide pH range (2.5 to 8.5) at 37°C, and in the neutral and acidic pH range (3 to 6) at 50°C. In the critical pH ranges on both acidic and alkaline sides, activation rather than inactivation of enzyme activity was observed after preincubation at 37°C.

Effect of inhibitors

Effects of various inhibitors on the purified

TABLE II. EFFECTS OF INHIBITORS ON RICE BRAN CPAase-4

The enzyme (0.29 μ g) was preincubated in 0.5 ml of 0.1 M acetate buffer, pH 4.0, containing 1.0 M sucrose and various inhibitors for 30 or 60 min at 37°C. After the preincubation, 0.2 ml of water and 0.3 ml of 10 mM CPA were added to the mixture, and the activity was assayed for 10 min.

Addition	Concentration ^a (M)	Residual activity after	
		30 min (%)	60 min (%)
None	—	100	100
DFP	2×10^{-4}	101	98
	2×10^{-8}	104	103
	2×10^{-4b}	95	78
	2×10^{-9b}	14	9
Iodoacetamide	2×10^{-2}	95	98
	2×10^{-1}	63	56
Iodoacetic acid	2×10^{-2}	79	88
	2×10^{-1}	53	50
PCMB	1.2×10^{-5}	100	91
	1.2×10^{-4}	88	89
HgCl ₂	1×10^{-4}	91	92
	1×10^{-3}	90	88
EDTA	2×10^{-4}	93	89
	2×10^{-3}	96	98
2-Mercaptoethanol	2×10^{-3}	106	102
	2×10^{-2}	101	103

^a Concentrations are those in preincubation mixture.

^b Preincubation was carried out at pH 7.

rice bran CPAase-4 were examined (Table II). DFP, the most potent inhibitor for various plant carboxypeptidase, did not affect rice bran CPAase-4 at pH 4. However, marked inhibition was observed after preincubation with 2×10^{-3} M DFP at pH 7. Sulfhydryl reagents such as iodoacetamide, iodoacetic acid, PCMB, and HgCl₂ partly inhibited the activity after preincubation with high concentrations of them. EDTA and reducing reagent did not affect the activity.

Substrate specificity of CPAase-4

The action of rice bran CPAase-4 on an oligopeptide, BP-I, was examined as shown in Fig. 7. A degradative release of amino acids from the C-terminal of the peptide was observed. Release of proline was detected. This result indicates that CPAase-4 has a

FIG. 7. Hydrolysis of an Oligopeptide, BP-I, by Rice Bran CPAase-4.

The purified enzyme (5.74 μ g) was incubated in 200 μ l of reaction mixture containing 2 mM BP-I, 0.05 M acetate buffer, pH 4.0, at 37°C. Aliquots of 20 μ l were withdrawn from the reaction mixture after 0, 5, 15, 45 and 90 min, and analyzed by thin layer chromatography on Silica gel G which was developed with phenol-water (75:25). Released amino acids were visualized by ninhydrin reaction. Broken line indicates the yellow spot. The amino acid sequence of BP-I is Gly-Pro-Phe-Pro-Ile-Ile-Val.

TABLE III. RELATIVE RATES OF HYDROLYSIS OF PEPTIDES AND ESTER BY RICE BRAN CPAase-4, RICE LEAF CPAase-5, AND BARLEY CARBOXYPEPTIDASES

Hydrolysis of peptides by the purified CPAase-4 was assayed at pH 4.0, 37°C, in the presence of 3.3 mM Z-Phe-Ala, Z-Glu-Tyr or 2 mM Z-Phe-Phe. Esterase activity was assayed at pH 8.0, 25°C, in the presence of 10 mM APEE. Values for other enzymes are cited from the following literature; rice leaf CPAase-5,¹⁾ barley carboxypeptidases.^{2,3,10)}

Substrates	Rice		Barley carboxypeptidase	
	Bran CPAase-4	Leaf CPAase-5	I	II
Z-Phe-Ala	100	100	100	100
Z-Phe-Phe	13 ^a	30	8	54
Z-Glu-Tyr	23	4	4	6
APEE	1.5	23	30	—

^a Precipitation of the substrate occurred during incubation at pH 4.

substrate specificity as a carboxypeptidase. Relative hydrolytic rates for some peptides and an amino acid ester are shown in Table III. Hydrolysis of Z-Phe-Phe was lower than those of for Z-Phe-Ala and Z-Glu-Tyr. However, the value for the hydrolysis of Z-Phe-Phe at pH 4.0 is least reliable because of precipitation of this peptide at pH 4. Esterase activity for APEE is very low compared to those for other plant carboxypeptidases.

DISCUSSION

As described in the preceding paper,¹⁾ germinating rice contained three kinds of CPAase and two of them, CPAase-4 and -5, were the most active at acidic pH. CPAase-5 was observed in endosperms and seedlings at the later stage of germination, and CPAase-4 was found in the resting seeds. Among many carboxypeptidases known in various tissues of various plants, those of barley,^{2,3,16~18)} cotton^{19,20)} and pine²¹⁾ have been reported to be present in germinating plants. Almost all of these enzymes were the most active at pH around 5 or 6, except for the pine enzyme²¹⁾ which was active on CPA at pH 4.5 or 4.5~3.8 depending on the kind of buffer. The activity of these carboxypeptidases was little detected in resting seeds and increased during germination. Germinated plants have been used for the preparation and characterization of these enzymes. In this regard, these known enzymes are similar to rice CPAase-5 and different from CPAase-4. CPAase-4 described in the present paper is the first enzyme whose purification and characterization have been made from resting seeds.

Most of proteins in plant seeds have been known to be distributed in subcellular granules which are commonly called protein bodies. Protein bodies and aleurone grains have been used as synonyms in dicotyledonous plants. However, these two terms are used for different organelles in monocotyledonous plants.^{22~24)} Aleurone grains are con-

tained in cells of aleurone layer, which is composed of one or two layers of cells which lies inside the pericarp.²³⁾ On the other hand, protein bodies are distributed in endosperms, especially in the periphery of endosperms.^{24,25)} Under electron microscopy, many protein bodies are found in the cells adjacent to aleuronelayer,²⁵⁾ though some protein bodies are detected inside starchy endosperms. Aleurone grains contain phytic acid and serve as the storage granules for phosphorus.^{22,25)} Albumin and globulin would be the major protein components in aleurone grains.^{22,26~28)} Taking into account these facts, the distribution patterns of proteins and enzymes in Fig. 1 are interpreted as follows. Fraction I is thought to be chiefly derived from aleurone layer, because phosphorus and soluble proteins in 1 M NaCl (albumin and globulin) were rich in this fraction. Fraction II is thought to be derived from protein bodies-rich endosperms, because the protein which was insoluble in salt solution (glutelin) was rich in this fraction. Fractions III and IV contained similar amounts of glutelin and decreasing amounts of phosphorus compared with fraction II. Fraction V (kernels) contained glutelin as the major protein component and very little phosphorus. The highest CPAase-4 activity was observed in fraction II. The distribution pattern of CPAase-4 is similar to that of glutelin. This result suggests the presence of CPAase-4 in or adjacent to protein bodies. Most of acid phosphatase activity was detected in fraction I. Distribution patterns of acid phosphatase and CPAase-4 were different from each other in rice grain, although both enzymes have been used as the marker enzymes with which search for lytic compartments in plant tissues.^{12,29,30)}

CPAase-4 was purified from rice bran by relatively simple procedures. Because of basic nature of the enzyme protein ($pI=7.8$),¹⁾ CPAase-4 was separated from large amounts of inert acidic proteins by chromatography on CM-Sephadex. The molecular weight of CPAase-4 (110,000) is similar to that

of rice leaf CPAase-5 (120,000).¹⁾ By SDS-polyacryl amide gel electrophoresis, the subunit structure of CPAase-4 was clarified. The enzyme appears to be composed of two identical polypeptide chains, whose molecular weights are 35,000 and 21,000. Cotton seed carboxypeptidase, whose molecular weight is 85,000, have been shown to be composed of three non-identical polypeptide chains.²⁰⁾ No other plant carboxypeptidases have been clarified as to their subunit structures.

The pH-dependence of the purified enzyme is the same as that of crude extract from rice bran and also compatible with that of the extract obtained from whole seeds referred to in the preceding paper (Fig. 2 in ref. 1). These results indicate that CPAase-4 is the only CPAase which exists in resting rice seeds. The mechanism of the activation of CPAase-4 in alkaline and acidic pH ranges was not elucidated in the present study.

CPAase-4 activity was inhibited by DFP and some of sulfhydryl inhibitors as well as rice CPAase-5 and other plant carboxypeptidases. However, CPAase-4 was less sensitive to these inhibitors than other carboxypeptidases.

The presence of CPAase-4 in dormant seeds may indicate that this enzyme acts for the hydrolysis of storage proteins in the initial stage of germination. The presence of proteinase in rice seeds which is optimally active at pH 3.0 for rice glutelin has been reported.³¹⁾ The degradation of proteins with the cooperation of such exo- and endo-peptidase is supposed to occur within the acidic region of cells. To elucidate the mechanism of degradation of storage proteins during germination of seeds, further knowledge is required about the sites of intracellular localization of these acid exo- and endo-peptidases.

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