Some Properties of Carboxypeptidases in Germinating Rice Seeds and Rice Leaves

Etsushiro DOI, Nobuhiko KOMORI, Teruyoshi MATOBA
and Yuhei MORITA

Research Institute for Food Science, Kyoto University, Uji, Kyoto 611

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Germinating rice contained three carboxypeptidases or carboxypeptidase-like enzymes which hydrolyzed CPA (carbobenzoxy-L-phenylalanyl-L-alanine) at different pH ranges. The three CPA hydrolases which acted optimally at pH 4, 5~5.5, and 7, were tentatively termed CPAase-4, CPAase-5, and CPAase-7 respectively. CPAase-4 was contained in resting seeds and endosperms of germinating seeds. Its activity was maximum in resting seeds and gradually decreased during germination. CPAase-5 was absent in resting seeds, and appeared in endosperms and seedlings in the later stages after germination. This enzyme was predominant in mature plant leaves. CPAase-7 was detected in the young shoots and young roots during a limited period of germination. Enzymatic properties of CPAase-5 partially purified from rice leaves were very similar to those of carboxypeptidases in various plants.

Carboxypeptidases having similar enzymatic properties have been detected in various plants including *Citrus*,^{1~4)} French beans,^{5,6)} barley,^{7~10)} cotton,^{11,12)} pine,¹³⁾ pineapple,¹⁴⁾ watermelon,^{15,16)} tomato,¹⁷⁾ charophyta,^{18,19)} and others,²⁰⁾ and in various organs including fruit,^{1~3,15~17)} leaves,^{4~8)} stems,¹⁴⁾ germinating seeds^{7~13)} and others.²⁰⁾ Most of these carboxypeptidases have been characterized by pH optimum around 5~5.5, inhibition by DFP, and broad substrate specificity. Almost all amino acid residues were released from polypeptides, but CPA was the best substrate for many plant carboxypeptidases as far as examined.^{7,10,14~16)} Physiological functions of these enzymes have been studied in germinating seeds,^{13,21,22)} and ripening fruit,¹⁷⁾ although no definite conclusions have been obtained.

A series of experiments was attempted by the authors to elucidate the nature and phys-

iological roles of carboxypeptidases in rice, especially in relation to mobilization of storage proteins during germination and to protein turnover in growing cells. This first paper describes the presence of three types of carboxypeptidases or carboxypeptidase-like enzymes in germinating rice seeds, and some of their properties compared with those of CPAase in mature leaves of rice.

MATERIALS AND METHODS

Plant materials. Seeds of the rice (*Oryza sativa*, L. Var. Nihonbare) were kindly supplied from the Head Farmstead (Takatsuki) of Agricultural Department, Kyoto University. The rice leaves (15~20 cm growth) were harvested from the field of Research Institute for Food Science, Kyoto University and stored at -25°C.

Germination. Rice seeds were sterilized by soaking them in 1% aqueous NaOCl for 30 min, washed, and steeped in water for 24 hr. After soaking, the seeds were incubated at 30°C in the dark unless otherwise indicated. The day when started the steeping of the seeds is taken as the zero day of germination. Endosperms, young shoots and young roots were separated from seeds of 3 or more days after germination, and kept -25°C for 24 hr. The frozen tissues obtained from 250 grains of seeds were homogenized in a mortar with additions of 20 to 50 ml of 0.1 M KCl and sea sands. The homogenate was used for various assays.

Abbreviations used: APEE, *N*-acetyl-L-phenylalanine ethyl ester; CPA, *N*-carbobenzoxy-L-phenylalanyl-L-alanine; CPAase, CPA hydrolase; DFP, diisopropyl-fluorophosphate; PCMB, *p*-chloromercuribenzoic acid; TPCK, L-(1-tosylamido-2-phenyl) ethyl chloromethyl ketone; Tricine, tris(hydroxymethyl)methyl glycine; Z-, carbobenzoxy-.

Total nitrogen. Nitrogen content was determined by the micro-Kjeldahl method.²³⁾

Free amino acids. Total amounts of free amino acids were determined by using the gasometric methods which measure the CO_2 evolved by the reaction of free amino acids and chloramin T, as described by Matoba *et al.*²⁴⁾

Activity assay. CPAase activity was determined at 37°C in terms of alanine released from CPA by a colorimetric ninhydrin procedure. The reaction mixture (1 ml) contained enzymes, 0.05 M acetate or Tricine buffer, 0.5 M sucrose, and 3.3 mM CPA.¹⁴⁾ Unit of activity is defined as the enzyme which yields 1 μmol of alanine per min. Specific activity is expressed as units per mg protein. Protein content was determined by the method of Lowry *et al.*²⁵⁾ Esterolytic activity was determined with a pH-Stat (Radiometer, PHA 943/REA 300/ABU 12, Denmark). The reaction mixture (2.5 ml) containing 0.01 M APEE, 0.1 M sucrose and the enzyme, was incubated at 25°C. The unit of esterolytic activity is expressed in terms of μmol of APEE hydrolyzed per min.

Partial purification of leaf CPAase. Ninety gram of rice leaves (15~20 cm growth) stored at -25°C, were broken in a Waring blender with 450 ml of 0.5 M sucrose solution containing 0.1 M KCl for a few minutes. The broken tissue was further homogenized in a mortar with a pestle. The homogenate was clarified by centrifugation and filtration. Ammonium sulfate was added to this solution (80% saturation). The precipitate formed was collected by centrifugation, dissolved in 0.02 M Na-acetate buffer, pH 5.2, and dialyzed against the same buffer. The dialyzate was put on the column of DEAE-Sephadex A-50 (3.7 $\times$ 130 cm) which had been equilibrated with 0.02 M Na-acetate buffer, pH 5.2. The column was developed by linear gradient elution with increasing concentrations of KCl (0.1 to 0.5 M) in the same buffer. The chromatogram gave a single peak of the activity. The active fractions were collected and precipitated by an addition of ammonium sulfate. The precipitate was dissolved and dialyzed against 0.02 M Na-acetate buffer, pH 5.2. The dialyzate (2.1 ml) was further purified by a gel filtration on Sephadex G-150 (1.6 $\times$ 100 cm) which had been equilibrated with 0.02 M Na-acetate buffer, pH 5.2 (Fig. 4). The active fractions were collected and concentrated by a collodion-bag (SM 13200 Sartorius-Membranfilter GmbH, Germany). The obtained solution was used for the experiments for enzyme characterization.

Isoelectric focusing. This was carried out in a small column with a capacity of 30 ml as described by Doi and Ohtsuru.²⁵⁾ The pH gradient was obtained with

carrier ampholites for pH 3.5 to 10 (LKB-Produktor AB, Sweden). Electrofocusing was attained at 800 V for 72 hr.

RESULTS

Changes in nitrogen and amino acid contents during germination

Changes in fresh weight, protein and free amino acid contents during germination of rice cultivated in the present conditions are indicated in Fig. 1. Apparent germination was observed after 2 days, and young shoots and young roots were separated from endosperms after 3 days. The decrease in total nitrogen in endosperms was observed after 3 days in accordance with the increase in free amino acid content in endosperms and seedlings (young shoots and young roots). Only 10 μmol of amino acids were detected in resting seeds, and about 100 μmol of amino acids were found in endosperms 11 days after germination.

FIG. 1. Changes in Fresh Weight (a), Total Nitrogen (b), and Free Amino Acid Contents (c) during Germination.

During 0 to 2 days, whole seeds were analyzed altogether (●-●). After 3 days, endosperms (●-●), young shoots (Δ-Δ), and young roots (▲-▲) were analyzed separately.

Change in enzymatic activity during germination

Most of known carboxypeptidases detected in plants are active at pH 5~5.5. However, the presence of carboxypeptidases which act at different pH ranges are probable at different stages of germination. The pH dependence of CPAase activities on homogenates obtained from resting seeds, and endosperms and young shoots of different stages of germination, was examined. The homogenate obtained from resting seeds showed the maximum activity at pH 4.0 (Fig. 2, a, filled circle). The activity decreased at neutral pH, and no peak of the activity was observed at pH 5.0 and 5.5. The homogenate obtained from the endosperms after 6 days showed the maximum activity at pH 5.0, though the activity at pH 4 was about 90% of the activity at pH 5.0 (Fig. 2, a, open circle). The homogenate obtained from young shoots after 4 days, showed the maximum activity

at pH 7.0 (Fig. 2, b, open triangle). The homogenate obtained from young shoots after 12 days showed the maximum activity at pH 5.0~5.5 (Fig. 2, b, filled triangle). These results indicate the presence of at least three kinds of CPAase which are optimally active at pH 4, pH 5~5.5, and pH 7 in germinating rice seeds. Thus, these three enzymes were tentatively termed CPAase-4, CPAase-5, and CPAase-7, respectively. The presence of CPAase-4 in resting seeds and germinated endosperms, CPAase-5 in germinated endosperms and seedlings at later stage, and CPAase-7 in seedlings at earlier stage were observed. Changes of these three enzyme activities during germination were determined by the assays at pH 4, 5.5, and 7 (Fig. 3). CPAase activity at pH 4 in endosperms changed little during first 3 days and then gradually decreased at the later stage, though about a half of

FIG. 2. The pH Dependence of CPA Hydrolysis by Extracts Obtained from Germinating Seeds of Various Stages.

(a) Resting whole seeds (●—●) and endosperms of 6 days (○—○).

(b) Young shoots of 4 days (△—△) and those of 12 days (▲—▲).

Plant tissues were homogenized and extracted with 0.1 M KCl. The reaction mixture contained 0.5 ml of the buffer solution containing 0.1 M Na-acetate, 0.1 M sucrose, 0.2 ml of the tissue extract and 0.3 ml of 10 mM CPA. The buffer solution and the tissue extracts were adjusted to various pH by 0.1 N NaOH or 0.1 N HCl.

FIG. 3. Changes in CPAase Activities during Germination.

(a) Whole seeds (0 to 2 days) and endosperms (3 to 12 days).

(b) Young shoots (3 to 12 days). (c) Young roots (3 to 12 days).

Preparations of 0 to 11 days were obtained from the plants cultured in the dark. The preparation of 12 days was obtained from the plants cultured under the light after 10 days. CPAase activities were assayed in 0.05 M acetate buffer at pH 4.0 (△—△) and at pH 5.5 (○—○), and in 0.05 M Tricine buffer at pH 7.0 (□—□).

the original activity remained after 11 days of germination. CPAase activity at pH 5.5 in endosperms was very little in resting seeds and increased after 3 days. Increase in CPAase activity at pH 5.5 was also observed in young shoots and young roots at the later stage of germination. From 4 to 7 days after germination, CPAase activity at pH 7 was higher than that of CPAase activity at pH 5.5 in both young shoots and roots. Then, CPAase activity at pH 7 decreased and that at pH 5 was predominant at later stage of germination. These tendencies were remarkable in the seedlings grown under the light condition. Leaves and roots obtained from the plants cultured under the light for 3 weeks contained only CPAase-5.

FIG. 4. Gel Filtration of Leaf CPAase-5 on Sephadex G-150.

The enzyme solution (2.1 ml) contained 14 units of enzyme and 4.3 mg of proteins. The Sephadex G-150 column (200 ml:1.4×100 cm) was equilibrated and developed with 0.02 M Na-acetate buffer, pH 5.2, containing 0.1 M KCl. Proteins of known molecular weight were analyzed by the same column successively and their peak positions are shown on the figure. ---, protein (A_{280}); ●—●, activity

Properties of leaf CPAase-5

CPAase-5 is predominant in mature leaves, and this enzyme seems to belong the most common type of carboxypeptidases in plants. A partially purified enzyme preparation was

TABLE I. SUMMARY OF PURIFICATION OF LEAF CAPASE-5

Enzyme activity was assayed at pH 5.2, 37°C as described in MATERIALS AND METHODS

Step	Total protein (mg)	Total activity (units)	Spec. activity (unit/mg)	Yield (%)
Extraction	490	69	0.14	100
(NH ₄) ₂ SO ₄ pptn.	63	38	0.64	54
DEAE-Sephadex	4.7	15	3.24	22
Sephadex G-150	0.4	9	22.7	13

obtained from rice leaves as described in MATERIALS AND METHODS. Final purification was achieved by the gel filtration on Sephadex G-150 (Fig. 4). Over 150 fold purification was attained through the procedures. Table I shows the summary of purification procedures. The following experiments were carried out by using this puri-

FIG. 5. The pH Dependence of the Activity (a) and the Stability (b) of Rice Leaf CPAase-5.

(a) The effect of pH on peptidase activity (●—●) was measured in the reaction mixture containing enzyme (1.7 μ g), 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 measured in the mixture containing enzyme (8 μ g) 10 mM APEE and 0.5 M sucrose at various pH, 25°C.

(b) The effect of pH on the stability was measured after preincubation for 60 min at 37°C (●—●), and for 30 min at 50°C (○—○). The enzyme (1.7 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 37°C, pH 5.2, for 30 min.

fied enzyme preparation unless otherwise indicated. Molecular weight was estimated to

TABLE II. EFFECTS OF INHIBITORS ON
LEAF CPAASE-5

The enzyme (0.86 μ g) was preincubated in 0.5 ml of 0.1 M pH 5.2 acetate buffer containing 1.0 M sucrose and various inhibitors for 30 min or 60 min at 37°C. After preincubation, 0.2 ml of water and 0.3 ml of 10 mM CPA was added to the mixture and the activity was assayed.

Reagent	Concentration ^a (mm)	Relative activity after	
		30 min (%)	60 min (%)
None	—	100	100
DFP	0.2	11	4
	2.0	0	0
Iodoacetamide	20 0	57	63
	200 0	18	11
Iodoacetic acid	20.0	89	79
	200 0	66	67
PCMB	0.01	84	61
	0.2	5	5
HgCl ₂	0.1	54	47
	1.0	6	0
TPCK	0.02	93	70
	0.2	68	53
EDTA	0.2	98	90
	2.0	95	96
<i>o</i> -Phenanthroline	0.2	93	102
	2 0	98	99
2-Mercaptoethanol	2.0	100	112
	20 0	103	98

^a Concentrations are those in the preincubation mixtures.

be 120,000 from the gel filtration data on Saphadex G-150 (Fig. 4). Isoelectric point was estimated to be 6.0 by isoelectric focusing (Fig. 6). Optimum pH of peptidase activity for CPA and esterase activity for APEE are 5.2 and 7.5 respectively (Fig. 5, a). The enzyme was stable between pH 4 and 6 (Fig. 5, b). Inhibition of the activity with various reagents is shown in Table II. DFP inhibited the activity completely. Sulfhydryl reagents such as iodoacetamide, PCMB, and HgCl₂ inhibited the activity in the presence of high concentration of the inhibitors. Metal sequestering reagents showed little effects on the activity. The relative activities against some peptides and an amino acid ester are shown in Table III. The values for some plant carboxypeptidases taken from literatures are also given in Table III. CPA was a good substrate for rice leaf CPAase-5 as well as for other plant carboxypeptidases.

Identity of CPAase-5 in leaf and in endosperms

Several enzymatic properties of CPAase-5 prepared from rice leaf were similar to those carboxypeptidases in other plants. However, identity of CPAase-5 in leaf with the enzyme in endosperms had not been ascertained. This was achieved with comparison of isoelectricfocusing patterns of CPAase-5 from leaves and that from endosperms after 11 days of germination (Fig. 6). The pattern for

TABLE III. RELATIVE RATES OF HYDROLYSIS OF PEPTIDES AND ESTER BY
RICE LEAF CAPASE-5 AND OTHER PLANT CARBOXYPEPTIDASES

Hydrolysis of peptides by the leaf CPAase-5 was carried out at pH 5.2, 37°C, in the presence of 3.3 mM of Z-Phe-Ala or Z-Glu-Tyr or 2 mM of Z-Phe-Phe. Esterase activity was assayed at pH 7.0, 25°C, in the presence of 10 mM APEE. Values for the other enzymes are cited from the following literatures; watermelon,^{15,16)} pineapple¹⁴⁾ and barley.^{7,9,10)}

	Rice leaf	Watermelon fruit	Pineapple stem	Barley malt	
	CPAase-5	F-II		I	II
Relative rates					
Z-Phe-Ala	100	100	100	100	100
Z-Phe-Phe	30	33	34	8	54
Z-Glu-Tyr	4	6	8	4	6
APEE	20	18	19	30	—
MW	120,000	110,000	—	90,000	—
Opt. pH	5.2–5.5	5.0–5.5	5.2	5.2	4–5.5

FIG. 6. Isoelectric Focusing of Rice Leaf CPAase-5(a), Bran CPAase-4(b), and Endosperm CPAases in Germinating Seeds(c).

(a) The crude leaf CPAase-5 solution (35 mg protein) which had been precipitated with ammonium sulfate from extracts was applied for electrofocusing.

(b) The purified rice bran CPAase-4 (1.4 mg Protein)²⁷⁾ was applied for electrofocusing.

(c) The extract obtained from endosperms of 11 days was precipitated with ammonium sulfate (80% satun). The precipitate was dialyzed against 0.1 M acetate buffer, pH 5.5. The dialyzate (2.5 mg protein) was applied for electrofocusing.

The pH gradient was made by using 1% carrier ampholine (pH 3.5–10.0) in a 30 ml of isoelectric-focusing column. After electrofocusing for 72 hr at 800 V, the content in the column was fractionated (1 ml/fraction) and pH in each fraction was determined. Ten μl of aliquot of each fraction was subjected to CPAase activity assay at pH 4.0 (○—○) and 5.2 (●—●) for 30 min (a and b) or 24 hr (c).

CPAase-4 purified from rice bran³⁷⁾ is also shown in Fig. 6, b. The pI value for leaf CPAase-5 was estimated to be 6. CPAase-5 activity in endosperms (filled circles in Fig. 6, c) was also electrofocused to pH 6, which indicates the identity in isoelectric points of endosperm CPAase-5 and leaf CPAase-5. The purified CPAase-4 from rice bran is an basic protein, whose isoelectric point is 7.8. This enzyme activity in the endosperms decreased at the later stage of germination (Fig. 3). However, its presence in endosperms

even 11 days after germination is indicated by isoelectric focusing (open circles in Fig. 6, c).

DISCUSSION

During the germination of rice seeds the nitrogen contents of the endosperms decreased while those of growing seedlings (shoots and roots) increased (Table I). The accumulation of free amino acids in endosperms and seedlings which accompanies the decrease of nitrogen in the former indicates that the alterations in nitrogen content are caused by a hydrolysis of the storage proteins in the endosperms followed by a transport of the product to the developing seedlings. Large increase in amino acid contents was observed in germinating endosperms (Fig. 1). The gasometric method used for the determination of amino acid content in the present experiments allows to measure the only free amino acids.²⁴⁾ Therefore, undoubtedly some exopeptidases were working for the mobilization of the storage proteins.

As described in the introduction, carboxypeptidases having similar properties are widely distributed in various organs of various plants. They are inhibited by DFP and most active around pH 5 to 5.5 and so on. Partially purified CPAase-5 from rice leaves has all of these properties and its molecular weight is similar to those of other carboxypeptidases (Table III). Remarkable similarities of rice leaf CPAase-5 and other carboxypeptidases are observed in the relative hydrolytic rates for some peptides and an amino acid ester (Table III). These results indicate that rice leaf CPAase-5 belongs to the most common type of carboxypeptidase in plants. The presence of two or more isozymes of carboxypeptidase have been reported in exocarp of *Citrus* fruit,²⁷⁾ germinating barley,^{7,8,10)} and sarcocarp of watermelon.¹⁵⁾ These isozymes are somewhat different in substrate specificities, though they are most active around pH 5 to 5.5. During the course of purification of rice leaf CPAase-5, no other

active fractions were observed on the chromatographic patterns of DEAE-Sephadex and Sephadex G-150 except for the main fraction of the activity. Single isoelectric point was observed after isoelectric focusing of the crude enzyme preparation. These results indicate the presence of single type of CPAase in rice leaf used in the present experiments.

In contrast to the facts described above, germinating rice contained enzymes which split CPA at pH 4 and 7 other than CPAase-5. CPAase-4 was detected in resting seeds and in germinating endosperms. The enzyme activity did not increase during germination and rather decreased. CPAase-4 activity assayed at the later stage of germination may be overlapped with the activity of CPAase-5 at pH 4. However, the presence of CPAase-4 even after 11 days of germination was ascertained by isoelectric focusing. CPAase-7 activity was detected during a limited period in young shoots and in young roots. Such CPAase which is active at neutral pH has never been reported in plants.

CPAase-4 is a distinct enzyme from CPAase-5 and other plant carboxypeptidases in respect to its optimum pH and isoelectric point. The pI value for rice CPAase-5 is 6, that for the pineapple enzyme is 4.3,²⁶⁾ those for the watermelon enzymes are 4.4 and 5.0,¹⁶⁾ and that for the *Citrus* enzyme is 4.3.^{28, 29)} The presence of active CPAase-4 in resting seeds would allow the immediate onset of protein degradation upon inhibition of the seeds, and would serve for the mobilization of proteins in the initial stage of germination.

CPAase-7 is a first CPAase which is known to act at neutral pH. Its appearance in early stage of seedlings suggests the role of this enzyme for the rapid turnover of proteins in developing cells. More details about the properties of CPAase-4 and CPAase-7 will be described in the succeeding papers.

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