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Isolation and Characterization of Four Carboxypeptidases in *Canavalia lineata* Cotyledons

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Abstract: Four carboxypeptidases, CP1, CP2, CP3, and CP4 were isolated from the cotyledons of germinating seedlings of *Canavalia lineata* by sequential chromatography on the following four columns: 1) CM-cellulose, 2) Sephacryl S-300, 3) Procion red dye, and 4) Sephacryl S-200. A number of properties of the enzymes, such as substrate specificity, molecular weight, optimum pH, thermal stability, have been determined. Enzyme activities were measured using the Cbz(carbobenzoxy)-dipeptides containing phenylalanine at the penultimate position. The K_m values of four carboxypeptidases for Cbz-Phe-Ala were 0.50, 0.65, 1.30, and 1.35 mM, respectively. The inhibition studies indicated that the four carboxypeptidases were all serine type. Each of the carboxypeptidases with molecular weights of 145, 114, 105, and 104 kDa, respectively, had the optimum enzyme activity at pH 5.0~6.0. And they were sensitive to high temperature.
Key words: *Canavalia lineata*, carboxypeptidases, procion red, substrate specificity.

The degradation of storage proteins during seed germination has been studied for a long time, the starting points being the establishment of protein degradation and the detection of proteolytic activity in seeds. Proteolysis of storage proteins, which supply the embryonic axis with nitrogen compounds during the early stages of germination, is an essential process of seed germination (Shutov and Vaintraub, 1987). However, our knowledge of this process, and particularly of its initial stages, is not yet sufficient.

Recently we have been interested in the proteolysis of storage protein during the germination of *Canavalia lineata*. It was reported that storage proteins were hydrolyzed by the cooperative action of various proteolytic enzymes. So it was necessary to establish which enzymes play important roles during the germination of seeds and to purify and characterize one of these enzymes. We already have purified and characterized endopeptidases and aminopeptidases involved in germinating seeds of *C. lineata*, and suggested in part a proteolysis mechanism of those storage proteins (Koh *et al.*, 1991; Yoon and Kwon, 1993).

It has been reported that carboxypeptidases play an important role in the hydrolysis of reserve proteins in seeds (Mikola and Mikola, 1980; Kawamura and Yonezawa, 1982; Winsper *et al.*, 1984). However, isolation procedures for the carboxypeptidases from plants are generally cumbersome, and consequently these enzymes have been characterized only to a limited extent (Preston and Kruger, 1976; Umetsu *et al.*, 1981; Breddam and Sorenson, 1983). Most of the plant carboxypeptidases have been characterized as having a pH optimum around 5.0~6.0, inhibition by DFP (Diisopropylfluorophosphate), and broad specificity (Mikola and Mikola, 1980; Kawamura and Yonezawa, 1982). Five carboxypeptidases have been identified in germinating wheat and barley by references to differences in substrate specficities, molecular weights, and chromatographic profiles (Mikola, 1983; Mikola, 1986). In this paper we present the purification method and some of the enzymatic properties of carboxypeptidases isolated from cotyledons of *C. lineata*.

Materials and Methods

Plant material and chemicals

Seeds of *C. lineata* were collected at Cheju island, Korea and stored at 4℃. Decoated seeds were germinated in moist vermiculite at 30℃ in the dark. The cotyledons from seedlings grown for 6 days after sowing were harvested and stored at −20℃. CM-cellulose, Sephacryl S-300, Reactive RED 120, Sephacryl S-200 and all other chemicals were purchased from

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Sigma Chemical Co.(St. Louis, USA).

Crude extract preparation

Cotyledons harvested from seedlings at various germination stages were homogenized in 0.2 M sodium acetate buffer (pH 5.0) containing 50 μM DTT using a Waring blender. The homogenate was suspended for 30 min at 4℃ and centrifuged at 12,000×*g* for 30 min at 4℃. The supernatant was dialyzed against 25 mM sodium acetate buffer (pH 4.0) containing 50 μM DTT for 8 h and centrifuged at 12,000×*g* for 10 min. For determination of the enzyme activity at various stages of germination, the crude extracts were dialyzed against 50 mM sodium acetate buffer (pH 5.0) containing 50 μM DTT for 6 h.

Determination of enzymatic activity and protein concentration

The assay of carboxypeptidase activity was carried out by the method of Mikola and Kolehmainen (1972). The substrate solutions were prepared by dissolving Cbz-peptides in DMF (N,N-dimethylformamide) and finalizing to 50 mM sodium acetate buffer (pH 5.0) containing 0.5 mM Na-EDTA and 50 μM DTT. The enzymatic reaction was terminated by addition of TNBS (2,4,6-trinitro benzene sulfonic acid) reagent [TNBS, 0.2% (w/v) : sodium tetraborate, 5% (w/v)=4 : 1]. Color reaction was allowed to proceed for 1 h at 30℃ and terminated by adding 1 ml of 0.5 N HCl. The absorbance was read at 340 nm. A standard curve for the TNBS reaction was determined using L-alanine, and the carboxypeptidase activity was expressed as nanomoles of substrate hydrolyzed (carboxy-terminal amino acid liberated) per minute at 30℃.

Protein concentration was determined by the Bradford method (Bradford, 1976) using bovine serum albumin as a standard, and the protein concentration of purified enzyme preparation in each purification step was determined by absorbance at 280 nm.

Purification of carboxypeptidase

Step 1, CM-cellulose ion exchange chromatography. The dialyzed enzyme solution was chromatographed on a CM-cellulose column (2.6×40 cm) previously equilibrated with 25 mM sodium acetate buffer, pH 4.0, containing 50 μM DTT. The proteins were eluted from the column using a 0.4 liter linear gradient, from 25 to 600 mM sodium acetate buffer containing 50 μM DTT, pH 4.0. The fractions containing enzyme activity were pooled and the proteins were concentrated by ultrafiltration on a PM 30 membrane (Amicon) and used for the next step.

Step 2, Gel filtration chromatography (first). Gel filtration of the enzyme solution obtained from the ion exchange chromatography was performed on a column of Sephacryl S-300 (2.6×70 cm) with 25 mM sodium phosphate buffer (pH 6.5) containing 50 μM DTT. The fractions of prominent activity were pooled and used for the next step.

Step 3, Dye ligand chromatography. The active fractions pooled from gel filtration chromatography were dialyzed against 20 mM MES-KOH buffer (pH 5.5) containing 2 mM $MgCl_2$ and 50 μM DTT, and the resulting dialysate was fed into a Procion red dye column (0.7×10 cm) equilibrated with 20 mM MES-KOH buffer (pH 5.5) containing 2 mM $MgCl_2$ and 50 μM DTT, and then the column was washed with the same buffer (15 ml). Bound proteins were eluted with the following buffers in sequence that changed according to a stepwise gradient of varying buffer, pH, divalent ion or salt concentration; ① 20 mM MES-KOH buffer (pH 6.0, 2 mM $MgCl_2$, 50 μM DTT), ② 20 mM MES-KOH buffer (pH 6.5, 50 μM DTT), ③ 20 mM sodium phosphate buffer (pH 6.8, 50 μM DTT), ④ 20 mM sodium phosphate buffer (pH 6.8, 0.1 M NaCl, 50 μM DTT), ⑤ 20 mM sodium phosphate buffer (pH 6.8, 0.2 M NaCl, 50 μM DTT), ⑥ 20 mM sodium phosphate buffer (pH 6.8, 0.5 M NaCl, 50 μM DTT), ⑦ 20 mM sodium phosphate buffer (pH 6.8, 1 M NaCl, 50 μM DTT). Each of the active fractions was pooled and concentrated by ultrafiltration on a PM 30 membrane (Amicon).

Step 4, Gel filtration chromatography (second). Each of the active fractions obtained from dye ligand chromatography was loaded onto the column (1.6×70 cm) of Sephacryl S-200, separately. The column was eluted with 50 mM sodium phosphate buffer (pH 6.5) containing 50 μM DTT. The molecular weight of each carboxypeptidase was determined using standard proteins.

Inhibition studies

The various inhibitors presented in Table 2 were added to the purified enzyme and preincubated for 30 min at 30℃ prior to the addition of substrate. Remaining activities were assayed and compared with those of control.

Stability of carboxypeptidase

The heat stabilities of carboxypeptidases were investigated by preincubating the enzymes for 10 min at 30℃, 40℃, 50℃, 60℃, and 70℃, respectively.

Results and Discussion

Changes of carboxypeptidase activity during germination

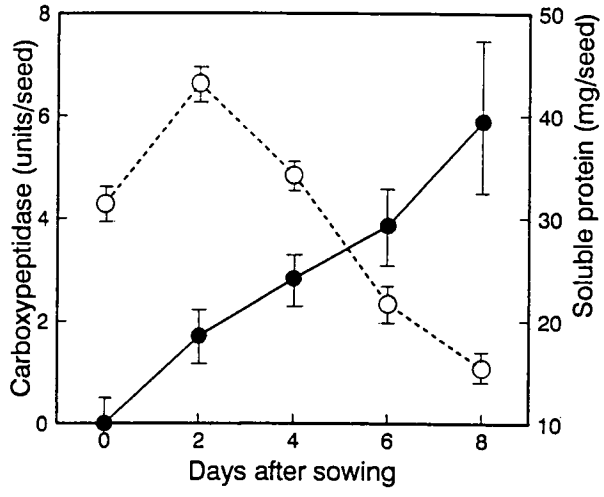

Fig. 1. The changes of carboxypeptidase activity and soluble protein content in cotyledons of *C. lineata* during germination at 27℃. The values for each points were obtained from 3 experiments. Carboxypeptidase activity: ○, soluble protein content; ●, Vertical bar indicates SD.

Changes of carboxypeptidase activity and soluble protein content in cotyledons of *C. lineata* during germination are shown in Fig. 1. Enzyme activity was not found in the resting seed of *C. lineata* but detected from the 2nd day, and it continued to increase until the 8th day of germination. However, its soluble protein content decreased with increasing carboxypeptidase activity. A similar pattern has been shown in cotton, mungbean, scots pine and pea (Ihle and Dure, 1972; Chrispeels and Boulter, 1975; Salmia and Mikola, 1976; Mikkonen, 1986).

In our previous studies, the activities of BAPNase (N-α-benzoyl-DL-arginine ρ-nitroanilide hydrolase) and aminopeptidase in cotyledons did not change distinctly during germination of *C. lineata* (Koh *et al.*, 1991; Yoon and Kwon, 1993). These results led us to presume that carboxypeptidase may play an important role in the hydrolysis of storage proteins and peptides during development of the cotyledon tissue of *C. lineata*.

When 70 to 80% of the storage protein of seeds has been degraded, how does the capacity of enzymes for protein degradation increase? There are different opinions about the nature of enzymes that hydrolyze the storage proteins-do they exist already in dormant seeds (Hara and Matsubara, 1980; Mikkonen, 1986) or are they synthesized de novo during germination (Abe *et al.*, 1977; Chrispeels and Boulter, 1975). It was reported that some proteases already existed in dormant seeds and they are inhibited by the action of their inhibitors (Shutov and Vaintraub 1987). On the other hand, other proteases such as acid vicilin peptidohydrolase in mung bean and carboxypeptidase

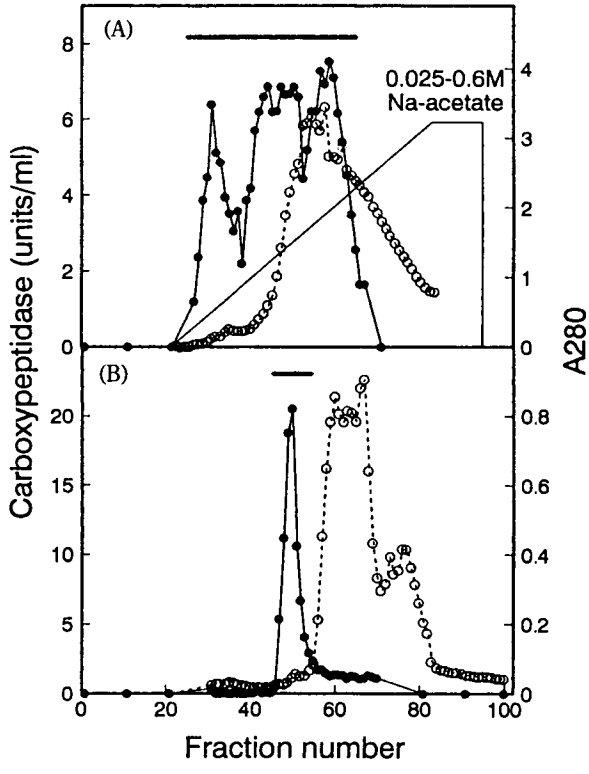

Fig. 2. Chromatography of the carboxypeptidases of *C. lineata* cotyledons on the CM-cellulose (A) and the Sephacryl S-300 column (B). Carboxypeptidase activity: ○, protein content: ●.

I in barley were newly synthesized (van der Wilden *et al.*, 1980; Ranki *et al.*, 1990). In this experiment, activity of carboxypeptidase was not detected in the resting seeds, however, it could not be determined whether the enzymes already existed with some inhibitors in the seeds or were synthesized *de novo*.

Enzyme purification

For the carboxypeptidase purification, dialyzed crude extract was loaded to a CM-cellulose column at first. Fig. 2 shows the elution profile from the CM-cellulose column and the Sephacryl S-300 column. More than three peaks overlapped on a CM-cellulose column(Fig. 2, A). but when the pooled enzyme fractions were next applied to a Sephacryl S-300 column, only one peak of enzyme activity was detected (Fig. 2, B). This result showed that carboxypeptidases of *C. leneata* have similar solubilities and molecular weights. The active fractions obtained from the Sephacryl S-300 column were pooled and chromatographed on a Procion red dye column (Fig. 3). Four peaks were detected when the column was eluted with 20 mM MES-KOH buffer (pH 6.5), 20 mM sodium phosphate buffer (pH 6.8) containing 0.1 M NaCl, 20 mM sodium phosphate buffer (pH 6.8) containing 0.2 M NaCl, and 20 mM sodium phosphate buffer (pH 6.8) containing 0.5 M NaCl, re-

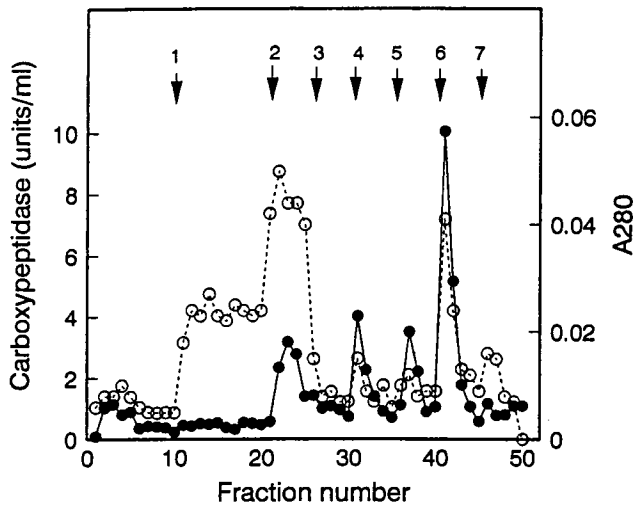

Fig. 3. Separation of the carboxypeptidases of *C. lineata* cotyledons on the Procion red dye column. Arabic figures indicate different elution buffers as mentioned in Materials and Methods. Carboxypeptidase activity: ○, protein content: ●.

spectively and the peaks were nominated as CP1, CP2, CP3, and CP4 according to the elution order and then each of the active fractions was chromatographed on a Sephacryl S-200 column, with no further separation of carboxypeptidases (data not shown).

The results of a typical purification are shown in Table 1. The enzymes were purified approximately 283, 171, 475, and 109-fold, respectively (Table 1). The highest specific activity was shown in CP3 (167 U/mg protein), and the highest enzyme yield was detected in CP4 (12.9%). Several carboxypeptidases were isolated with DEAE-ion exchange chromatography (Mikola, 1983; Mikola, 1986), however, the DEAE medium was not effective in this experiment (data not shown). The fractionation of extracts by CM-cellulose and Sephacryl S-300 failed to separate carboxypeptidases. Four carboxypeptidases were separated efficiently with a Procion red dye column (Fig. 3). Several carboxypeptidases were separated using a DEAE and [N-(ε-aminocaproyl)-p-aminobenzyl] succinyl-Sepharose column which has been used in some limited plant materials (Umetsu *et al.*, 1981; Breddam and Sorenson, 1983; Mikola, 1983; Mikola, 1986).

Enzymatic properties

The relative rates of hydrolysis of 14 N-blocked peptides by four carboxypeptidases were listed in Table 2. The enzymes hydrolyzed preferentially peptides containing phenylalanine in the penultimate position, such as Cbz-Phe-Ala, Cbz-Phe-Leu, and Cbz-Phe-Met (Table 2). CP1 and CP2 cleaved Cbz-Phe-Met about 2.4 and 1.9 times faster than Cbz-Phe-Ala, CP3 and CP4 preferentially cleaved peptides including COOH-terminal alanine or leucine in the case of penultimate phenylalanine. However, the enzymes had low activities for the peptides containing glycine or proline in the penultimate position. But the enzymes did not resemble carboxypeptidase IV and V of wheat and barley, which act only on substrates containing proline in their penultimate position (Mikola, 1983; Mikola, 1986). The four enzymes preferred Cbz-Gly-Tyr to Cbz-Gly-Phe and Cbz-Gly-Met. Various activities were shown in peptides containing alanine in the penultimate position. The enzymes cleaved Cbz-Ala-Ala and Cbz-Ala-Met more efficienly than other substrates containing alanine in the penultimate position. And Cbz-Ala-Val was cleaved faster by CP2, CP3, and CP4 than by CP1. Cbz-Pro-Leu-Gly was more rapidly cleaved by CP1 and CP2 than by CP3 and CP4. Each specific penultimate amino acid had a significant effect on the hydrolysis rate. These results suggested that the four carboxypeptidases

Table 1. Purification of four carboxypeptidases from cotyledons of *C. lineata*

Purification step		Total protein (mg)	Total activity (U)[a]	Specific activity (U/mg protein)	Purificaton fold	Enzyme yield (%)
Crude extract		808	1012	1.3	1	100
CM-cellulose		315	1045	3.3	2.5	103
Sephacryl S-300		14	656	50	37	65
Procion red dye	(CP1)	2.78	300	108	83	29.6
	(CP2)	0.58	64	111	85	6.4
	(CP3)	0.68	149	220	169	14.8
	(CP4)	1.40	141	101	78	14.0
Sephacryl S-200	(CP1)	0.32	118	368	283	11.6
	(CP2)	0.36	80	222	171	7.9
	(CP3)	0.14	86	617	475	8.5
	(CP4)	0.92	130	142	109	12.9

[a]CBZ-Phe-Ala was used as a substrate. 1 unit=nmol alanine produced/min.

Table 2. Hydrolysis of Cbz-peptides by four carboxypeptidases of *C. lineata* cotyledons

Substrate	Relative activity[a] (%)			
	CP1	CP2	CP3	CP4
Cbz-Phe-Ala	100	100	100	100
Cbz-Phe-Leu	160	114	89	150
Cbz-Phe-Met	242	190	93	110
Cbz-Gly-Phe	3	3	3	5
Cbz-Gly-Met	4	3	1	6
Cbz-Gly-Tyr	16	13	12	21
Cbz-Pro-Phe	3	0	5	1
Cbz-Pro-Ala	3	0	2	1
Cbz-Ala-Phe	14	9	13	20
Cbz-Ala-Ala	25	70	79	90
Cbz-Ala-Val	3	25	25	31
Cbz-Ala-Met	23	20	34	61
Cbz-Gly-Gly-Phe	4	0	0	0
Cbz-Pro-Leu-Gly	109	115	30	5

[a]Each value obtained from 2 triplicate experiments.

Table 3. Effects of protease inhibitors on the four carboxypeptidases activities of *C. lineata* cotyledons

Addition	Concentration (mM)	Relative activity[a] (%)			
		CP1	CP2	CP3	CP4
Control		100	100	100	100
PMSF	1	12	23	38	49
	2	2	16	17	27
Iodoacetate	2	91	109	114	117
1,10-Phenanthroline	1	93	107	95	108
	2	100	114	94	101
Pepstatin A	0.2	92	99	86	91
TPCK	0.1	100	114	92	105
	0.2	104	81	99	106
TLCK	0.1	101	119	109	104
	0.2	108	97	111	107
ZPCK	0.1	108	56	74	90
	0.2	100	41	49	89
DTT	1	116	98	92	101
	2	104	94	121	95

[a]Cbz-Phe-Ala was used as a substrate.
Each value obtained from 2 triplicate experiments.

of *C. lineata* cotyledons had different substrate preference and thus could hydrolyze a wide variety of peptides. The effects of substrate concentration on the reaction rates at pH 5.0 were determined for Cbz-Phe-Ala. The Km values of four carboxypeptidases were CP1 : 0.50, CP2 : 0.65, CP3 : 1.30, and CP4 : 1.35 mM. The affinities of CP1 and CP2 for Cbz-Phe-Ala were higher than those of CP3 and CP4.

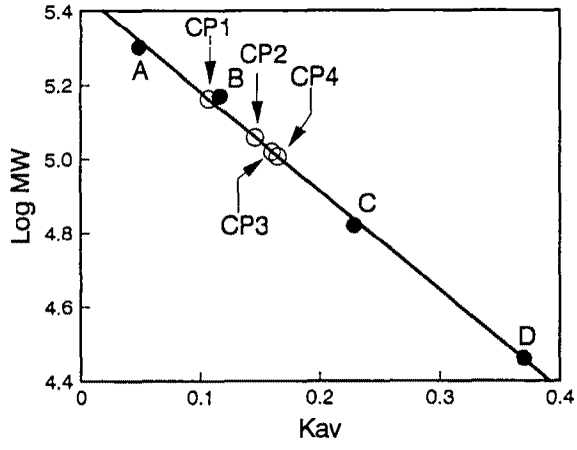

Fig. 4. Molecular weight determinations of four carboxypeptidases (CP1, CP2, CP3, and CP4) of *C. lineata* cotyledons by using β-amylase (200 kDa, A), alcohol dehydrogenase (150 kDa, B), BSA (66 kDa, C), and carbonic anhydrase (29 kDa, D) as standard proteins. Molecular weight of each carboxypeptidases was determined in separate experiments.

Inhibition studies

The most effective inhibitor of the carboxypeptidases of *C. lineata* cotyledons was PMSF (phenylmethylsulfonylfluoride), which inhibits serine type proteases, so it was suggested that the enzymes were all serine type (Table 3). These results are similar to the fact that most plant carboxypeptidases are serine carboxypeptidases, which are inactivated by DFP (diisopropylfluorophosphate) or PMSF (Preston and Kruger, 1976; Mikola, 1983; Umetsu *et al.*, 1981). The enzymes (CP1, CP2, CP3, CP4) were not inhibited by EDTA (data not shown) or 1,10-phenanthroline, which indicates that, like the cotton carboxypeptidases (Ihle and Dure, 1972), they were not metallo-carboxypeptidases.

To explore the possible roles of the histidine residue in carboxypeptidases, the effects of chloromethyl ketone compounds such as TLCK (N-α-p-tosyl-L-lysine chloromethyl ketone), TPCK (N-tosyl-L-phenylalanine chloromethyl ketone) and ZPCK (N-Cbz-L-phenylalanine chloromethyl ketone) were tested. TPCK and TLCK had no effects on the enzyme activities. However, ZPCK inhibited CP2 and CP3 activity more than 50%. These inhibition patterns were similar to those of carboxypeptidase Y of yeast (Hayashi *et al.*, 1975). And it has also been reported that carboxypeptidase Y of yeast and carboxypeptidase I, II, and V of barley were inhibited by pHMB (p-hydroxymercuribenzoic acid), which is a SH-specific proteinase inhibitor (Hayashi *et al.*, 1975; Mikola, 1983), but the four carboxypeptidases in this experiment were not inhibited by pHMB (data not shown). In this respect, the carboxypeptidases of *C. lineata* were different from those of yeast and barley.

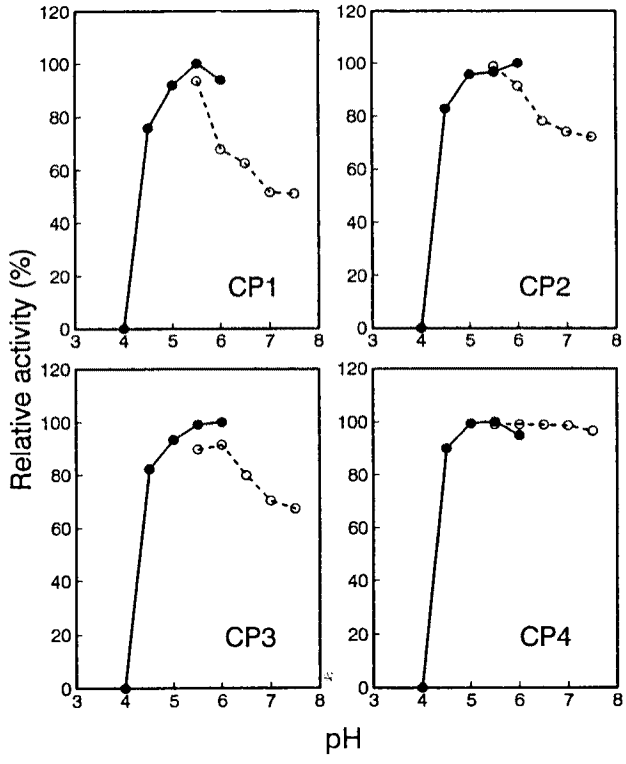

Fig. 5. Effects of pH on the activity of four carboxypeptidases. The activities were estimated in the presence of 1.5 mM Cbz-Phe-Ala. Buffers employed; 50 mM sodium acetate buffer (pH 4.0~6.0) : ○, 50 mM sodium phosphate buffer (pH 5.5~7.5) : ●.

Physical chemical characterization

The molecular weight of each carboxypeptidase was determined by a Sephacryl S-200 gel filtration chromatography utilizing standard proteins, separately. The results were plotted in Fig. 4. The apparent molecular weights of CP1, CP2, CP3, and CP4 were 145, 114, 105, and 104 kDa, respectively. In spite of these different molecular weights, the carboxypeptidases could not be separated in a Sephacryl S-300 column (Fig. 2, B). These results are consistent with the finding that carboxypeptidases purified from plant materials have molecular weights in the range of 85-120 kDa (Ihle and Dure, 1972; Doi *et al.*, 1980; Umetsu *et al.*, 1981; Breddam *et al.*, 1985). However, CP1 showed a slightly higher molecular weight, being similar to those of carboxypeptidase IV and V of barley and wheat (Mikola and Mikola, 1980; Mikola, 1983; Mikola, 1986).

The pH optima for the four carboxypeptidases are shown in Fig. 5. The carboxypeptidases had slightly different pH optima : CP1, 5.5 ; CP2, 5.0-6.0 ; CP3, 5.5-6.0, while CP4 had the same activity from 5.0 to 7.5. However, the reasons for the differences were not determined.

The final enzyme preparations were stable, so that we could store them at 4℃ for several months without loss of activity. However, the purified enzymes were sensitive to high temperature, losing as much as 90% of their original activities at 70℃. Especially, the activity of CP1 drastically decreased at temperatures above 30 ℃ (Fig. 6).

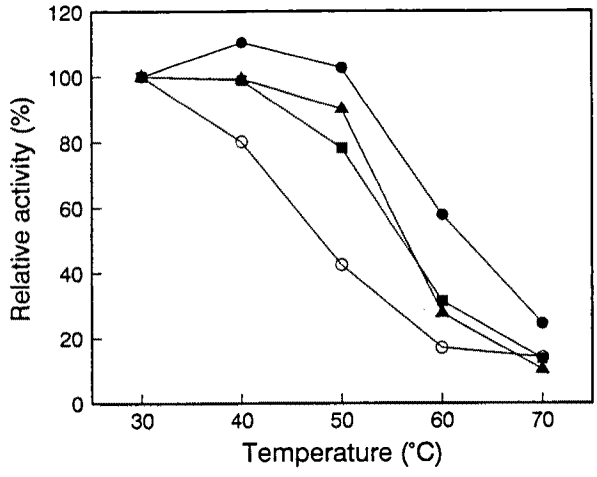

Fig. 6. Effects of temperature on the carboxypeptidase activity of *C. lineata* cotyledons. CP1 : ○, CP2 : ●, CP3 : ■, CP4 : ▲.

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References

Abe, M., Arai, S. and Fujimaki, M. (1977) *Agric. Biol. Chem.* **41**, 887.

Bradford, M. M. (1976) *Annal. Biochem.* **72**, 248.

Breddam, K. and S rensen, S. B. (1983) *Carlsberg Res. Commun.* **48**, 217.

Breddam, K., Sϕrensen, S. B. and Ottesen, M. (1985) *Carlsberg Res. Commun.* **50**, 199.

Chrispeels, M. J. and Boulter, D. (1975) *Plant Physiol.* **55**, 1031.

Doi, E., Komori N., Matoba, T. and Morita, Y. (1980) *Agric. Biol. Chem.* **44**, 85.

Hara, I. and Matsubara, H. (1980) *Plant Cell Physiol.* **21**, 219.

Hayashi, R., Bai, Y. and Hata, T. (1975) *J. Biol. Chem.* **250**, 5221.

Ihle, J. N. and Dure, L. S. (1972) *J. Biochem.* **247**, 5034.

Kawamura, Y. and Yonezawa, D. (1982) *Agric. Biol. Chem.* **46**, 767.

Koh, S. C., Hwang, I. D. and Kwon, Y. M. (1991) *Korean Biochem. J.* **24**, 578.

Mikkonen, A. (1986) *Physiol. Plant.* **68**, 282.

Mikola, L. and Kolehmainen, L. (1972) *Planta* **104**, 167.

Mikola, L. and Mikola, J. (1980) *Planta* **149**, 149.
Mikola, L. (1983) *Biochim. Biophys. Acta* **747**, 241.
Mikola, L. (1986) *Plant Physiol.* **81**, 823.
Preston, K. R. and Kruger, J. L. (1976) *Plant Physiol.* **58**, 516.
Ranki, H., Sopanen, T. and Voutilainen, R. (1990) *Plant Physiol.* **93**, 1449.
Salmia, M. A. and Mikola, J. J. (1976) *Physiol. Plant.* **36**, 388.
Shutov, A. D. and Vaintraub, I. A. (1987) *Phytochemistry* **26**, 1557.
Umetsu, H., Abe, M., Sugawara, Y., Watanabe, T. and Ichishima, E. (1981) *Food Chem.* **7**, 125.
Van der Wilden, W., Gilkes, N. R. and Chrispeels, M. J. (1980) *Plant Physiol.* **66**, 390.
Winsper, M. J., Preston, K. R., Rastogi, V. and Oaks, A. (1984) *Plant Physiol.* **75**, 480.
Yoon, J. W. and Kwon, Y. M. (1993) *Mol. Cells* **3**, 83.