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ACTION OF CARBOXYPEPTIDASE W ON OLIGOPEPTIDES CONTAINING CARBOXY-TERMINALLY AMIDATED PEPTIDES

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Key Word Index—*Triticum aestivum*; Gramineae; wheat; carboxypeptidase; serine-type carboxypeptidase; carboxyamidase; amidase; oligopeptide; carboxy-terminally amidated peptide.

Abstract—Carboxypeptidase W sequentially liberated amino acids from the carboxy-terminus of angiotensin I, bradykinin, delta sleep-inducing peptide and neurotensin, indicating that the sequential hydrolysis of peptides was limited by the occurrence of intermediates with the structure -Gly-X (X = L-amino acid), -Pro-X, -X-Gly and -X-Pro. The enzyme had carboxyamidase and/or amidase activities for the carboxy-terminally amidated peptides and then carboxypeptidase activity. Carboxyamidase activity of the enzyme for the carboxy-terminally amidated peptides tested was much lower than carboxypeptidase activity for Z-Glu-Tyr (Z = benzyloxycarbonyl). The enzyme essentially acted as a carboxyamidase for the long carboxy-terminally amidated peptides; an amidase became dominant for the substrates in the presence of a hydrophobic amino acid in the penultimate (P_1) or P_2 positions, especially the P_1 position, corresponding with the S_1 and S_2 sites of the enzyme.

INTRODUCTION

Serine carboxypeptidases (serine-type carboxypeptidase, EC 3.4.16.11) possessing active serine are widely distributed in higher plants, fungi and animal tissues. They have the ability to liberate acidic, neutral and basic amino acids, including proline, from the carboxy-termini of peptides and proteins under acidic conditions. Serine carboxypeptidases have been identified and characterised in many plants, such as French beans [1], germinated barley [2], germinated cotton seeds [3], watermelon [4], *Citrus* leaves [5], exocarp [6, 7] and wounded tomato leaves [8].

Wheat carboxypeptidase is considered to be one of the key enzymes playing an important role in the degradation of storage proteins at the initial stage of germination. Four carboxypeptidases, designated as I–IV, with isoelectric points of 4.8, 5.5, 6.0 and 6.5, respectively, and with the same M_r of 118 000, are present in resting wheat seeds [9, 10]. Enzyme III of the main fraction is termed carboxypeptidase W. The action for small *N*-acylpeptides [11] and the kinetic study for *N*-acylpeptides and oligopeptides [12] showed that carboxypeptidase W preferred substrates having aromatic or aliphatic amino acids in the P_1 position from their carboxy-termini.

The serine carboxypeptidases from yeast [13], fungi [14, 15] and plants [1, 6] acts on *N*-acylamino acid esters and/or amides. Furthermore, carboxypeptidase Y from yeast released the carboxy-terminal amino acid amide from carboxy-terminally amidated *N*-acyldipeptides [16]. We showed that the carboxypeptidases from fungi, i.e. *Aspergillus saitoi* [17] and *Absidia zychae* [18], had carboxyamidase and/or amidase activities for the large carboxy-terminally amidated peptides.

The present paper describes and discusses the amidase and carboxyamidase activities of serine carboxypeptidase from wheat, carboxypeptidase W for carboxy-terminally amidated peptides, together with its action on oligopeptides.

RESULTS

Figure 1 shows the result of sequential release of amino acids from angiotensin I (Asp-Arg-Val-Tyr-Ile-His-Pro-Phe-His-Leu). Leu, His and Phe were sequentially released from the carboxy-termini, followed by nearly equal amounts of Pro, Ile and Tyr. A small amount of Val was subsequently released. The sequence of angiotensin I could be read as -Val-(Tyr,

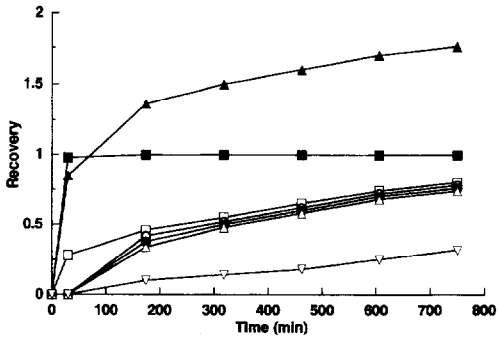

Fig. 1. Hydrolysis of angiotensin I by carboxypeptidase W. See text for experimental details. ■, Leu; ▲, His; □, Phe; ○, Pro; ●, Ile; △, Tyr; ▽, Val.

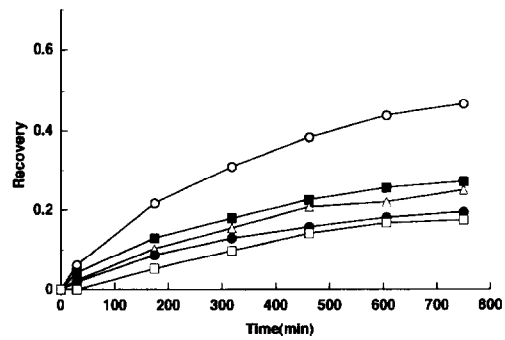

Fig. 3. Hydrolysis of [D-Ala^2, Met^5]-enkephalinamide by carboxypeptidase W. See text for experimental details. ○, Met-NH_2 + Met; □, Met; △, NH_3; ●, Met-NH_2; □, Phe.

Ile, His)-Pro-Phe-His-Leu. When the enzyme acted on bradykinin (Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg), Arg, Phe, Pro and Ser were sequentially released from carboxy-termini and the sequence could be read as -Phe-Ser-Pro-Phe-Arg. Acting on delta sleep-inducing peptide (Trp-Ala-Gly-Gly-Asp-Ala-Ser-Gly-Glu), Glu at carboxy-termini was slowly released with a release ratio of 29% after an incubation time of 766 min and, subsequently, Gly, Ser and Ala. When the enzyme acted on neurotensin (Pyr-Leu-Tyr-Glu-Asn-Lys-Pro-Arg-Arg-Pro-Tyr-Ile-Leu), Leu, Ile and Tyr were sequentially released. The fourth Pro was not released from the intermediate with the structure -Arg-Arg-Pro.

When carboxypeptidase W hydrolysed the carboxy-terminally amidated peptides, amidated amino acids (carboxyamidase activity) and/or ammonia (amidase activity) were released from carboxy-termini; then, amino acids (carboxypeptidase activity) were sequentially released. The course of eledoisin-related peptide (Lys-Phe-Ile-Gly-Leu-Met-NH_2) hydrolysis is shown in Fig. 2. Carboxy-terminal Met-NH_2 was released, followed by sequential increases of Leu, Gly and Ile. After prolonged incubation, no ammonia and Met were detected in the reaction mixture. The enzyme acted only as a carboxyamidase for eledoisin-related peptide, followed by carboxypeptidase activity. Figure 3 shows the course of [D-Ala^2, Met^5]-enkephalinamide (Tyr-D-Ala-Gly-Phe-Met-NH_2) hydrolysis by the enzyme. After 174 min of incubation, carboxy-terminal Met-NH_2 and Met were liberated in the ratio of 2:3. An increase in ammonia from the carboxy-terminal amide was also detected after prolonged incubation. These results indicated that the enzyme had both carboxyamidase and amidase activities for the carboxy-terminal Met-NH_2 of [D-Ala^2, Met^5]-enkephalinamide. The enzyme also had both carboxyamidase and amidase activities for CCK-tetrapeptide (Trp-Met-Asp-Phe-NH_2) and FMRF-amide (Phe-Met-Arg-Phe-NH_2) to release Phe-NH_2 and ammonia, followed by releasing amino acids sequentially. Only ammonia was liberated from Z-Gly-Phe-NH_2 by the enzyme. A comparison between carboxyamidase and amidase activities of carboxypeptidase W for the carboxy-terminally amidated peptides is shown in Table 1, with data for other serine carboxypeptidases. Carboxypeptidase W differs from *A. saitoi* and *A. zychae* carboxypeptidases in the ratios of carboxyamidase and amidase activities for the amidated peptides. In particular, the enzymes form *A. saitoi* and *A. zychae* had no carboxyamidase and amidase activities for a small peptide, Z-Gly-Phe-NH_2, while carboxypeptidase W had amidase activity for it, liberating ammonia. Table 2 shows the relative activities of carboxypeptidase and carboxyamidase by carboxypeptidase W, with data for other serine carboxypeptidases. Carboxyamidase activity of carboxypeptidase W was lower than carboxypeptidase activity for the carboxy-terminally amidated peptides tested. Carboxypeptidase W from wheat resembled the enzyme from *A. saitoi* in the rate of carboxyamidase activity for the carboxy-terminally amidated peptides.

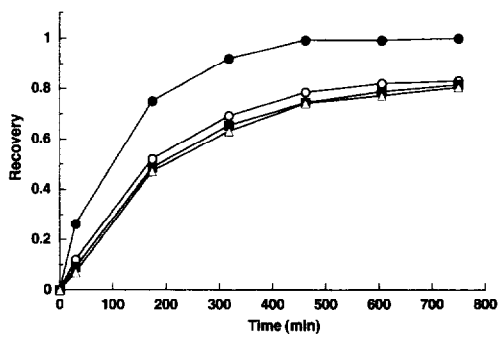

Fig. 2. Hydrolysis of eledoisin-related peptide by carboxypeptidase W. See text for experimental details. ●, Met-NH_2; ○, Leu; □, Gly; △, Ile.

Discussion

When carboxypeptidase W was incubated with angiotensin I and bradykinin, no endopeptidase activity could be detected. The sequence of carboxy-terminal amino acid through to nearly the fifth amino acid of the substrates tested could be read. Our previous papers [11, 12] indicated that carboxypeptidase W preferred substrates having aromatic and aliphatic amino acids, such as Tyr, Phe and Leu, in the P_1 position to substrates having Glu and Ala in the P_1

Table 1. Comparison between carboxyamidase and amidase activities of serine carboxypeptidases towards carboxy-terminally amidated peptides

Substrate	Split site by the enzyme S_4 P_4	$-S_3$ $-P_3$	$-S_2$ $-P_2$	$-S_1$ $-P_1$	$\downarrow S'_1$ $-P'_1$	Relative rate of hydrolysis by CPase W	*Aspergillus saitoi* CPase*	*Absidia zychae* CPaseZ-1†
Eledoisin-related	-F	-I	-G	-L	$-MNH_2$	1	0.65	0.8
peptide	-I	-G	-L	-M	$-NH_2$	0	0.35	0.2
[D-Ala^2, Met^5]-	Y	-DA	-G	-F	$-MNH_2$	0.4	0	0.6
enkephalinamide	-DA	-G	-F	-M	$-NH_2$	0.6	1	0.4
CCK-tetrapeptide‡		W	-M	-D	$-FNH_2$	0.9	1	1
	W	-M	-D	-F	$-NH_2$	0.1	0	0
FMRF-amide		F	-M	-R	$-FNH_2$	0.3	0.16	0.9
	F	-M	-R	-F	$-NH_2$	0.7	0.84	0.1
Z-Gly-Phe-NH_2			Z	-G	$-FNH_2$	0	0	0
		Z	-G	-F	$-NH_2$	1	0	0

Arrow indicates site split by enzyme. CPase, carboxypeptidase.

*,† Data from refs [17] and [18], respectively.

‡Relative rates of hydrolysis by *A. saitoi* CPase and *A. zychae* CPase Z-1 were measured using gastrin-related peptide (Aoc-W-M-D-F-NH_2) as substrate. One-letter symbols for amino acids are: DA, D-Ala; D, Asp; F, Phe; G, Gly; I, Ile; L, Leu; M, Met; R, Arg; W, Trp; Y, Tyr.

Table 2. Relative activities of carboxypeptidase and carboxyamidase in serine carboxypeptidases

Substrate						Relative activity CPase W	*Aspergillus saitoi* CPase*	*Absidia zychae* CPase Z-1†
			Z	-E	$\downarrow$Y	1000	1000	1000
		W	-M	-D	$-FNH_2$	74	82	99
K	-F	-I	-G	-L	$-MNH_2$	34	12	164
		F	-M	-R	$-FNH_2$	7	27	266
	Y	-DA	-G	-F	$-MNH_2$	2	0	34

Arrow indicates site split by enzyme. Rate of hydrolysis of Z-E-Y is arbitrarily taken to be 1000. CPase, carboxypeptidase.

*, † Data from refs [17] and [18], respectively.

E, Glu. Other one-letter symbols of amino acids are described in Table 1.

position, and did not prefer substrates having Gly and Pro in the P_1 position. The present results of the actions for oligopeptides showed that the enzyme had the same preference for amino acids in the P_1 position of substrates and that sequential hydrolysis of substrates was limited by the occurrence of intermediates with the structure -Gly-X, -Pro-X, -X-Gly and -X-Pro.

Serine carboxypeptidase is known to have carboxyamidase and/or amidase activities for carboxy-terminally amidated peptides. *Aspergillus saitoi* serine carboxypeptidase showed carboxyamidase activity only for gastrin-related peptide and amidase activity in preference to carboxyamidase activity for FMRF-amide and [D-Ala^1, Met^5]-enkephalinamide (Table 1) [17]. For *A. saitoi* serine carboxypeptidase, the P_3 and P_4 positions of substrates were important for the hydrolysis of carboxy-terminally amidated peptides. Whether *A. zychae* serine carboxypeptidases acted as a carboxyamidase or an amidase was dependent on the hydrophobicity in the P_3 and P_2 positions and the bulkiness of the amino acid side-chains in the P_1 and P'_1 positions of substrates [18].

Carboxypeptidase W had both carboxyamidase and amidase activities for [D-Ala^2, Met^5]-enkephalinamide, CCK-tetrapeptide and FMRF-amide, only carboxyamidase activity for eledoisin-related peptide and only amidase activity for Z-Gly-Phe-NH_2 (Table 1). The enzyme acted as an amidase for [D-Ala^2, Met^5]-enkephalinamide, CCK-tetrapeptide, FMRF-amide and Z-Gly-Phe-NH_2 with the relative rates of hydrolysis of 0.6, 0.1, 0.7 and 1.0, respectively. When the enzyme acted as an amidase for CCK-tetrapeptide, FMRF-amide and Z-Gly-Phe-NH_2, the P_1 position of the substrates was Phe. The presence of hydrophobic amino acids in the P_1 position of substrates is considered to be important in the appearance of amidase activity of carboxypeptidase W. The

enzyme functioned as an amidase for [D-Ala^2, Met^5]-enkephalinamide in the presence of Phe in the P_2 position, indicating that the hydrophobicity in the P_2 position of substrates was also important in the appearance of amidase activity. Carboxypeptidase W acted as a carboxyamidase for [D-Ala^2, Met^5]-enkephalinamide having D-Ala in the P_3 position and as an amidase for a P_3-lacking substrate, such as Z-Gly-Phe-NH_2. This indicates that the S_3 site of the enzyme, corresponding with the P_3 position of substrates, had flexibility in the enzyme reaction. These results lead us to believe that the enzyme acts essentially as a carboxyamidase for long carboxy-terminally amidated peptides because of the presence of bulky amino acid amide in the P'_1 position and an amidase becomes dominant for substrates bearing a hydrophobic amino acid in the P_1 and/or P_2 positions, especially the former, corresponding with the S_1 and S_2 sites of the enzyme. This preference of hydrophobic amino acid in the P_1 and P_2 positions for the carboxy-terminally amidated peptides was consistent with our results obtained from kinetic studies using *N*-acylpeptides and oligopeptides [12] and with results of crystallographic analysis of peptide aldehyde complexes with wheat serine carboxypeptidase II [19].

EXPERIMENTAL

Materials. Z-Gly-Tyr, Z-Gly-Phe-NH_2, angiotensin I, bradykinin, delta sleep-inducing peptide, neurotensin, eledoisin-related peptide, [D-Ala^2, Met^5]-enkephalinamide, CCK-tetrapeptide and FMRF-amide were purchased from the Peptide Institute Inc. (Osaka, Japan). All other chemicals used were of reagent grade.

Carboxypeptidase W from wheat. Carboxypeptidase W from bread and common wheat (*Triticum aestivum* L.) was prepd according to ref. [9]. The enzyme used was homogenous on polyacrylamide gel electrophoresis at pH 4.0 and by analytical ultracentrifugation. Carboxypeptidase W was assayed as reported previously, using Z-Glu-Tyr as substrate [9]. One katal was the amount of enzyme required to liberate 1 mol tyrosine a second from Z-Glu-Tyr at pH 4.0 and 30°.

Hydrolysis of oligopeptides and C-terminally amidated peptides. One nkat of carboxypeptidase W was incubated with 100 nmol of substrate at 30° in 1 ml of 50 mM acetate buffer (pH 3.0). The released amino acids, NH_3 and amino acid amides in 50 μl of reaction mixts were analysed directly and continuously using an automatic amino acid analyzer [17].

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