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SPECIFICITY OF TWO ISOLATED WHEAT CARBOXYPEPTIDASES*

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Key Word Index—*Triticum aestivum*; Gramineae; wheat; proteolytic enzymes; carboxypeptidase; germination.

Abstract—The substrate specificity of two purified carboxypeptidases from germinated wheat has been examined. Both enzymes were active on a wide variety of carbobenzoxy substituted peptides but inactive with unsubstituted dipeptides. Neither enzyme was active upon endoprotease or amidase substrates and only low levels of esterase activity were evident. In time course studies, both enzymes gave rapid non-specific sequential release of amino acids, including proline, from the carboxyterminal of proteins and polypeptides of known amino acid sequence.

INTRODUCTION

Developing and germinating wheat possess several proteolytic enzymes with carboxypeptidase activity [1, 2]. Recently two of these enzymes have been isolated from germinated wheat [2] and shown to have properties similar to a number of previously isolated plant carboxypeptidases [3–8]. Preliminary studies indicated that both enzymes were highly active on hemoglobin and gluten and possessed broad specificities. The present study was undertaken to further characterize these enzymes with respect to their action on synthetic substrates and proteins of known amino acid sequence.

RESULTS AND DISCUSSION

The two isolated wheat carboxypeptidases from germinated wheat, designated carboxypeptidases A and B, were purified according to the method described in a previous paper [2]. Both enzymes were electrophoretically homogeneous and had sp. act. of *ca* 720 and 320 μmol glycyl–glycine/mg protein, respectively, for carboxypeptidase A and B. For all studies, enzyme solutions were adjusted to give proteolytic activities of 3 μmol glycyl–glycine/ml as determined by an automated fluorometric assay with hemoglobin substrate [9].

Action of wheat carboxypeptidases on synthetic substrates

Table 1 shows the relative rates of hydrolysis of carbobenzoxy (Z) peptides by the purified wheat carboxypeptidases. At pH 5.5 (optimum pH for Z–phe–ala hydrolysis by both enzymes) wheat carboxypeptidase A was most active on Z–phe–ala, Z–glu–tyr and Z–ala–phe while wheat carboxypeptidase, B was most active on Z–ala–phe and Z–phe–ala. With the exception of Z–gly–gly, both enzymes hydrolyzed all the substrates tested indicating a broad specificity similar to previously isolated plant carboxypeptidases [3, 5, 7, 8, 10, 11].

Table 1. Relative rates of hydrolysis of Z-peptides by wheat carboxypeptidases

Substrate	Relative rates of hydrolysis Carboxypeptidase A	Carboxypeptidase B
Z–phe–ala*	100	100
Z–ala–ala	27	48
Z–gly–ala	15	12
Z–ala–phe	71	136
Z–gly–phe	25	20
Z–val–phe	7	16
Z–ala–leu	4	4
Z–gly–leu	24	16
Z–gly–gly	0	0
Z–leu–gly	29	34
Z–leu–tyr	16	26
Z–glu–tyr	80	40
Z–ala–glu	5	8
Z–ile–leu–ala	22	14
Z–pro–leu–gly	76	52

* Actual rates of hydrolysis for Z–phe–ala were 0.052 and 0.061 μmol ala/min/ml respectively for carboxypeptidase A and B. Each tube contained enzyme, (activity = 0.15 μmol gly–gly/ml; 0.21 μg of carboxypeptidase A or 0.47 μg carboxypeptidase B) and substrate, 0.75 mg Z–dipeptide/ml, in acetate buffer, pH 5.5, 0.05 M.

However, comparison of relative hydrolysis rates of the two enzymes indicated some differences in catalytic properties.

Values of K_m, V_{max} and activation energies in the hydrolysis of Z–phe–ala were determined as 0.67 mM, 3.65 μmol/min (0.42 μg enzyme) and 9.10 kcal/deg/mol for carboxypeptidase A and 1.59 mM, 6.71 μmol/min (0.94 μg enzyme) and 9.42 kcal/deg/mol for carboxypeptidase B. Values of K_m were similar to those reported for watermelon [8], citrus fruit [12] and French bean [13] and lower than the value of 6.7 mM reported for barley [5].

As in the case of previously isolated plant carboxypeptidases [5, 10, 14], neither wheat enzyme appeared to hydrolyze the dipeptides pro–tyr, glu–ala, gly–phe,

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gly–asp, gly–glu and gly–gly. Neither did the enzymes show any endoproteolytic activity with azocasein, azocoll or hide powder azure after 12 hr incubation nor was any activity evident utilizing gelatin viscosity techniques. In addition, neither enzyme possessed amidase activity against α-*N*-benzoyl-L-arginine amide, α-*N*-benzoyl-L-arginine *p*-nitroanilide or glutaryl-L-phenylalanine *p*-nitroanilide.

Both isolated wheat carboxypeptidases appeared to have little esterase activity towards *N*-substututed amino acid ester substrates. Neither enzyme hydrolyzed α-*N*-benzoyl-L-arginine ethyl ester and only very low levels of hydrolysis were evident ($\Delta A < 0.01$ after 4 hr) with *N*-acetyl-DL-phenylalanine β-naphthyl ester. Hydrolysis of *N*-acetyl-DL-phenylalanine *p*-nitroanilide ester was slow with values of 8 and 7 nmol of *p*-nitroaniline released per hr, respectively, for wheat carboxypeptidase A and B. These results are somewhat surprising in view of previous studies showing that plant carboxypeptidases generally possess high esterase activity [8, 14–16]. However, Kubota *et al.* [7] have recently purified a carboxypeptidase from the exocarp of *Citrus natsudaidai* which also possesses low esterase activity. The low activities of carboxypeptidases of wheat and citrus [7] towards esterase substrates as compared to other plant carboxypeptidases supports Matoba and Doi's hypothesis [8] that although esterase and peptidase activities are catalyzed at the same active site (serine residue), the binding sites for the esterase and peptidase substrates are at different positions near the active site. Apparently with wheat and citrus carboxypeptidases, it would appear that the peptidase substrate binding site is much more efficient than the esterase binding site.

Action of wheat carboxypeptidases on polypeptides and proteins

Results of the action of the two purified wheat carboxypeptidases upon protein and polypeptide substrates of known amino acid sequence at pH 4.2, the optimum pH for hydrolysis of previously tested protein substrates [2],

Table 2. Release of carboxyterminal amino acids from glucagon by wheat carboxypeptidases

Amino acid sequence	Amino acid positions	Amino acid released (nmol/ml) Carboxypeptidase A 5 hr	11 hr	23 hr	Carboxypeptidase B 5 hr	11 hr	23 hr
Thr–CO_2H	1	65	90	120	93	111	114
Asn	2	71	84	110	96	121	116
Met	3	63	79	102	91	108	112
Leu	4, 16	59	85	126	93	124	184
Trp	5	15	45	63	40	70	92
Gln	6, 10	11	46	112	43	113	165
Val	7	7	36	78	38	80	121
Phe	8	9	37	81	25	77	113
Asp	9, 15	trace	16	78	8	49	151
Gln	—	—	—	—	—	—	—
Ala	11	0	9	44	0	29	76
Arg	12, 13	0	15	70	0	37	132
Arg	—	—	—	—	—	—	—
Ser	14, 19	0	9	64	0	32	113
Asp	—	—	—	—	—	—	—

Each tube contained enzyme (activity = 1 μmol gly–gly/ml; 1.4 μg carboxypeptidase A or 3.1 μg carboxypeptidase B) and glycagon, 143 nmol/ml assuming pure substrate, in acetate buffer, pH 4.2, 0.05 M.

Table 3. Release of carboxyterminal amino acids from cyanoethylated egg white lysozyme by wheat carboxypeptidases

Amino acid sequence	Amino acid* position	Amino acid released (nmol/ml) Carboxypeptidase A 5 hr	11 hr	23 hr	Carboxypeptidase B 5 hr	11 hr	23 hr
Leu–CO_2H	1	13	23	27	12	18	28
Arg	2, 5	20	38	43	19	37	40
Cys–CN	3, 15	*	*	*	*	*	*
Gly	4, 13	10	18	31	10	16	27
Arg	—	—	—	—	—	—	—
Ile	6	7	13	19	3	8	11
Trp	7	4	8	14	trace	8	14
Ala	8	trace	6	12	trace	5	9
Gln	9	0	trace	6	0	trace	5
Val	10	0	0	trace	0	0	trace

Each tube contained enzyme (activity = 1 μmol gly–gly/ml; 1.4 μg carboxypeptidase A or 3.1 μg carboxypeptidase B) and cyanoethylated egg white lysozyme, 32 nmol/ml assuming pure substrate, in acetate buffer, pH 4.2, 0.05 M.
* Present but not determined.

are shown in Tables 2–5. Both wheat enzymes rapidly released carboxy-terminal amino acids sequentially from glucagon (Table 2) and cyanoethylated egg-white lysozyme (Table 3). The pattern of amino acid released indicated that both enzymes were non-specific with respect to amino acid sequence with the possible exception of the slower cleavage of trp–gln bonds in glucagon. Amino acids not in proximity to the carboxyterminal (not shown in Tables) were not released confirming the absence of endoproteolytic activity.

Similar results were obtained with the two proline containing polypeptides, bradykinin (Table 4) and carboxymethylated insulin B chain (Table 5). Both wheat carboxypeptidases gave rapid non-specific sequential cleavage of carboxyterminal amino acids. This included the ability to release proline at rates similar to other amino acids, and in the case of bradykinin there was evidence of the cleavage of the pro–pro bond, although at low rates probably due to the proximity of the amino terminal.

From the above results, both wheat carboxypeptidases appear similar to previously isolated plant carboxypeptidases from citrus fruit peel [3], cotton [14], citrus exocarp {7] and pineapple [16] in their non-specific hydrolysis of carboxyterminal peptide bonds of peptides and protein substrates. This property may make these enzymes useful in sequence studies. Recently both enzymes have been used to partially elucidate the sequence of wheat beta [17], alpha, (unpublished data) and alpha 2 (unpublished data) purothionin and may be especially useful in sequence studies involving isolated gluten proteins, a probable natural substrate.

EXPERIMENTAL

The two carboxypeptidases (designated A and B) were purified to electrophoretic homogeneity from 5-day germinated wheat (*Triticum aestivum* L. cv Manitou) as previously described [2]. Purified enzyme solns were dialyzed against 50 mM NaOAc pH 4.2 and stored at 4° until use.

Proteolytic activity. Activities of enzyme solns were determined by an automated fluorometric assay at 40° (pH 4) with hemoglobin as substrate [9]. Activity was expressed in terms of the equivalent concentration of glycyl–glycine required to give a fluorescence intensity equal to that of the hydrolysis products from a 20 min incubation period.

Action of enzymes on Z-peptides. Determination was by a modification of the method of ref. [5]. Enzyme soln (0.1 ml) was added to 2 ml substrate soln (15 mg Z-peptide in 20 ml 50 mM NaOAc pH 5.5) and incubated at 35°. At 0 (blank) and 100 min, 0.5 ml aliquots were removed and added to 2 ml of 0.4 M

Table 4. Release of carboxyterminal amino acids from bradykinin triacetate by wheat carboxypeptidases

Amino acid sequence	Amino acid released (nmol/ml) Carboxypeptidase A 5 hr	11 hr	23 hr	Carboxypeptidase B 5 hr	11 hr	23 hr
Arg–CO_2H	340	387	384	380	373	369
Phe	175	344	508	301	614	773
Pro	132	221	400	191	329	427
Ser	69	188	318	129	296	376
Phe	—	—	—	—	—	—
Gly	20	91	252	30	80	196
Pro	—	—	—	—	—	—
Pro	—	—	—	—	—	—
Arg–NH_2	—	—	—	—	—	—

Each tube contained enzyme (activity = 1 μmol gly–gly/ml; 1.4 μg carboxypeptidase A or 3.1 μg carboxypeptidase B) and bradykinin triacetate, 390 nmol/ml assuming pure substrate, in acetate buffer, pH 4.2, 0.05 M.

Table 5. Release of carboxyterminal amino acids from carboxymethylated insulin B chain by wheat carboxypeptidases

Amino sequence	Amino acid position	Amino acid released (nmol/ml) Carboxypeptidase A 5 hr	11 hr	23 hr	Carboxypeptidase B 5 hr	11 hr	23 hr
Ala–CO_2H	1	15	21	28	16	26	26
Lys	2	13	17	24	13	17	25
Pro	3	10	14	22	8	16	23
Thr	4	8	15	24	7	13	20
Tyr	5, 15	8	13	22	8	15	22
Phe	6, 7	15	20	33	13	21	30
Phe	—	—	—	—	—	—	—
Gly	8, 11	6	12	25	4	8	12
Arg	9	trace	5	9	trace	3	7
Glu	10	trace	4	7	0	3	8

Each tube contained enzyme (activity = 1 μmol gly–gly/ml; 1.4 μg carboxypeptidase A or 3.1 μg carboxypeptidase B) and carboxymethylated insulin B chain, 34 nmol/ml corrected for presence of ammonium sulfate, in acetate buffer, pH 4.2, 0.05 M.

H_3BO_3 (Ph 9) to terminate the reaction. Amino acid release was measured by fluorometric analysis following reaction with fluorescamine [18]. For non-substituted peptides, enzyme and substrate solns were incubated for 300 min at pH 4.5 and 5.5 as described above.

Endoproteolytic activity. Measurement was by the release of bound dye from azocasein, azocoll and hide powder azure at pH 6 by the method of ref. [19]. Products were determined by the increase in A at 440 nm (azocasein) or 520 mn (azocoll and hide powder azure) following suitable incubation times. Gelatin viscosity measurements were carried out as previously described [20].

Action of enzymes on amidase and esterase substrates. Amidase activities of the enzymes were tested with α-*N*-benzoyl-L-arginine amide at pH 5.5 by the method of ref. [15], α-*N*-benzoyl-L-arginine *p*-nitroanilide at 8.6 by the method of ref. [20], and glutaryl-L-phenylalanine *p*-nitroanilide at pH 5.2 by the method of ref. [15]. Esterase activity was determined with α-*N*-benzoyl-L-arginine ethyl ester at pH 5.2 and 7 (30°, 0.2 ml enzyme) by the method of ref. [20], *N*-acetyl-DL-phenylalanine β-naphthyl ester at pH 5.2 (30°, 0.1 ml enzyme) by the method of ref. [15] and *N*-acetyl-DL-phenylalanine *p*-nitroanilide ester at pH 6.2 (30°, 0.1 ml enzyme) by the method of ref. [15].

Action of enzymes on proteins and peptides. Glucagon, bradykinin triacetate and egg white lysozyme (Grade 1, 3 × crystallyzed) were obtained from Sigma and insulin-reduced carboxymethylated B-chain was obtained from Mann Research Labs. Egg white lysozyme was reduced and cyano-ethylated by the method of ref. [21], washed with 95% EtOH, dissolved in H_2O and lyophilized. Protein (5 mg) was suspended in 8 ml of 50 mM NaOAc buffer pH 4.2 and 2 ml of enzyme soln, which had been concentrated to give proteolytic activities of 5 μmol gly–gly/ml, was added. Solns were then incubated at 35° and 2 ml aliquots were removed at various times and inactivated with 2 ml of 10% sulfosalicylic acid pH 2.2, centrifuged and frozen until further use. Amino acid compositions of these solns were then determined as previously described [2].

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