PRIMARY STRUCTURE AND ENZYMATIC PROPERTIES OF CARBOXYPEPTIDASE II FROM WHEAT BRAN

by

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Wheat carboxypeptidase II has been isolated from wheat bran by affinity chromatography and its enzymatic properties and amino acid sequence has been determined. The enzyme is a dimer of molecular weight around 110,000 with each monomer composed of two peptide chains, an A-chain and a B-chain, linked by disulfide bridges. The A-chain exists in two forms, one, the A'-chain, being three amino acids shorter at the N-terminus, and consequently two different subunits, i.e. A-B and A'-B, and three different dimers, i.e. A'-B/A'-B, A'-B/A-B and A-B/A-B, are found. These different forms could be separated by ion exchange chromatography due to the A- and A'-chains differing by a single charge.

Fragments of the A'- and B-chains, obtained by chemical cleavages with either cyanogen bromide or hydroxylamine and by enzymatic cleavages with either trypsin or *S. aureus* V8 protease, were sequenced and aligned to give the complete sequences of the two chains. The A'- and B-chains contain 260 and 160 amino acid residues, respectively. Three glycosylated asparagines are found in each chain. Alignment of the A- and B-chains with the corresponding chains of the known carboxypeptidases from germinated barley (malt) showed 95% homology with carboxypeptidase II and 37% homology with carboxypeptidase I. Similarly, the A-chain of wheat carboxypeptidase II exhibits 29% homology with the N-terminal part of carboxypeptidase Y and the B-chain exhibits 21% homology with the C-terminal part of carboxypeptidase Y.

Like other serine carboxypeptidases wheat carboxypeptidase II exhibits peptidase activity with an acidic pH-optimum and esterase, amidase and peptidyl amino acid amide hydrolase activities with a basic pH optimum. The three different forms of the enzyme hydrolyse peptides with slightly different K_m and k_{cat} values but identical k_{cat}/K_m values. The specificity of the enzyme is identical to that of carboxypeptidase II from germinated barley: it exhibits a preference for basic and hydrophobic amino acid residues in the P_1 and/or P'_1 positions.

Abbreviations: Bicine = N,N-bis(2-hydroxyethyl)glycine; BS = benzyl succinic acid; Bz = benzoyl; CABS-Sepharose = N-(6-aminocaproyl)-p-amino-benzylsuccinyl-Sepharose 4 B; Ches = 2(N-cyclohexylamino)ethane sulfonic acid; DPCC = diphenylcarbonyl chloride; EDTA = ethylenediamine tetraacetic acid, sodium salt; FA = furylacryloyl; Hepes = N-2-hydroxyethylpiperazine N'-2-ethanesulfonic acid; HPLC = high performance liquid chromatography; Mes = 2-(N-morpholino)ethanesulfonic acid; p-HMB = parahydroxy-mercuribenzoate; PTH = phenylthiohydantoin; TFA = trifluoroacetic acid. The binding site notation is that of SCHECHTER and BERGER (24). Accordingly, the binding site for the C-terminal amino acid residue of the substrate is denoted S'_1 and those for the amino acid residues in the amino-terminal direction away from the scissile bond are denoted $S_1, S_2, \dots, S_i$. Amino acid residues in the substrate are referred to as $P_1, P_2, \dots, P_i$ and P'_1 in correspondence with the binding site.

1. INTRODUCTION

Ion exchange chromatography of extracts from germinated barley grains has indicated the presence of five different serine carboxypeptidases, termed carboxypeptidases I to V (25). Carboxypeptidases I and II have been purified and it has been shown that they are similar with respect to physical-chemical properties, i.e. both enzymes are dimers, but exhibit different specificities (7, 10, 11, 12). The carboxypeptidase system in germinated wheat grains corresponds to that of barley grains and it has been shown that carboxypeptidase I is absent from resting wheat grains but increases during germination whereas carboxypeptidase II is abundant in resting grains and decreases during germination (24). The carboxypeptidase isolated from wheat bran (32, 33) has been suggested to be carboxypeptidase II (24). However, while this enzyme may be fractionated into three components by ion exchange chromatography (32), indicating charge heterogeneity, the corresponding enzyme from germinated barley elutes as a single peak from an ion exchange column (12). The structural basis of this difference has been clarified in the present investigation.

2. MATERIALS AND METHODS

2.1. Materials

CABS-Sepharose and FA-Phe-Leu-OH were prepared as previously described (5, 11). All other substrates were from Bachem, Switzerland. Wheat bran was obtained from Vejle Dampmølle, Denmark, D,L-benzylsuccinic acid from Burdick and Jackson, USA. Bio-Gels were from Bio-Rad, USA, DPCC-trypsin was from Sigma, USA, *S. aureus* V8 protease was from Miles, USA, and carboxypeptidase Y was from Carlsberg Biotechnology, Denmark. 2-vinylpyridine was purchased from Janssen Chimica, Belgium. All other reagents and solvents were of analytical purity and obtained from Merck, W. Germany.

2.2. Methods

2.2.1. Determination of enzymatic activity and protein concentration

Carboxypeptidase activity was routinely assayed at 25 °C by following the hydrolysis of

FA-Phe-Leu-OH spectrophotometrically at 337 nm using a Cary Model 219. The following assay mixture was used: 25 µl 8 mM substrate dissolved in methanol was added to 965 µl 0.05 M-sodium acetate, 1 mM-EDTA, pH 4.5 (the established pH optimum for the hydrolysis of FA-Phe-Leu-OH by the purified enzyme), followed by addition of 10 µl enzyme solution. One unit was defined as the amount of enzyme necessary to release one micromole of leucine per min at 25 °C.

The protein concentration was determined spectrophotometrically at 280 nm using $A_{280}(1\%) = 24$, determined from the amino acid composition and sugar content of the purified enzyme as obtained from CABS-sepharose.

2.2.2. Purification of wheat bran carboxypeptidase

The carboxypeptidase from wheat bran was purified by a method combining the previously used procedure for the purification of this enzyme (32) with the affinity chromatographic procedures used for the purification of malt carboxypeptidases I and II (11, 12). 11 kg wheat bran was added to 60 l 3% acetic acid, 0.05 M-NaCl and then poured into a stainless steel vessel (diameter: 60 cm, height: 80 cm), equipped with a filter bottom with 0.75 mm holes. After 16 hours at 4 °C the wheat bran had swelled and formed a bed with a height of 20 cm. The vessel was then opened in the bottom and the "column" was eluted with approximately 90 liters 3% acetic acid, 50 mM-NaCl. The extract ("eluate") was concentrated approximately 10 fold by ultrafiltration, using equipment from the Danish Sugar Refineries and a membrane with a cut-off around a molecular weight of 20,000. 230 g ammonium sulfate was added per liter concentrate (38% saturation) and after 16 hours at 4 °C the precipitate was removed by centrifugation. To the supernatant was added 238 g ammonium sulfate per liter (70% saturation) and after 16 hours at 4 °C the precipitate containing the enzyme was obtained by centrifugation. This precipitate could be stored for at least two weeks at 4 °C.

The ammonium sulfate precipitate was dissolved in 10 mM-acetic acid, 40 mM-NaCl and

subjected to diafiltration, using a Pellicon Cassette System from Millipore, equipped with a PTGC 00005 membrane with a cut-off around molecular weight of 10,000. When the conductivity had decreased to that of the buffer, pH was adjusted to 6.0 with 2 N-NaOH. After two days the enzyme had crystallized and the crystals were collected by centrifugation and redissolved in 0.02 M-acetic acid, 0.1 M-NaCl, pH 4.0 by stirring overnight. The solubility was approximately $1.5 \text{ mg} \cdot \text{ml}^{-1}$, i.e. $100 \text{ U} \cdot \text{ml}^{-1}$. After centrifugation to remove insoluble material the solution was applied to a CABS-Sepharose column (5×22 cm, i.e. approximately 65 U per ml resin) equilibrated with 0.02 M-sodium acetate, 0.1 M-NaCl, pH 4.0. Essentially all enzymatic activity was bound to the column which was washed with approximately 5 l 0.05 M-sodium acetate 0.1 M-NaCl, pH 4.0 until A_{280} of the eluate was below 0.02. The enzyme was eluted with 1 mM-benzylsuccinic acid, 0.05 M-sodium acetate, 0.1 M-NaCl, pH 4.0. The affinity resin was regenerated with 0.01 M-Tris, 3 M-NaCl, pH 8.5, followed by 0.05 M-sodium acetate, 0.1 M-NaCl, pH 4.0.

The eluate from the affinity column was adjusted to 0.5 M-NaCl by addition of solid NaCl, concentrated approximately four fold by ultrafiltration using an Amicon cell, equipped with a YM 10 membrane with a cut-off of a molecular weight around 10,000 and then dialysed against 0.05 M-acetic acid, pH 6.0, thus crystallizing the enzyme as previously described (32). The crystals were removed by centrifugation at 18,000 g for one hour in a Sorvall centrifuge. They could be kept frozen at -18 °C without loss of enzymatic activity. The crystals were dissolved in 0.05 M-acetic acid, 0.1 M-NaCl, pH 4.0 by stirring the suspension overnight at 4 °C.

Alternatively, the carboxypeptidase could be separated into three forms by ion exchange chromatography. The eluate from the affinity column was diluted with one volume 0.02 M-acetic acid, pH 4.0 and applied ($150 \text{ U} \cdot \text{ml}^{-1}$ gel) to a CM-52-cellulose column (2.5×18 cm) equilibrated with 0.1 M-sodium acetate buffer, pH 5.0. The column was washed with one volume of this buffer, and the elution was performed with a gradient from 0.1 to 0.6 M-sodium acetate

buffer, pH 5.0, using a total of 16 column volumes. The carboxypeptidases in each of the pooled fractions was crystallized as described above.

2.2.3. Homogeneity of isolated enzyme

The homogeneity of the isolated carboxypeptidases was tested by polyacrylamide electrophoresis at pH 4.3 according to the procedure of REISFELD (26) and by SDS gel electrophoresis (see section 2.2.4).

The Pharmacia FPLC System equipped with a Mono S cation exchanger was used to fractionate native carboxypeptidases, using a linear gradient and as A-buffer: 0.05 M formic acid, pH 3.5, and as B-buffer 0.05 M formic acid, 0.5 M-NaCl, pH 3.5. Denatured (dissociated) carboxypeptidases were tested in the corresponding system with 6 M-urea included in the buffers.

2.2.4. Molecular weight determination

The molecular weight was determined in a Spinco Model E analytical ultracentrifuge using the Yphantis miniscus depletion method as modified by CHERVENKA (13) in a 0.01 M-sodium acetate buffer, $\mu = 0.2$ at 14,290 rev. per min. Equilibrium was reached after 16 hours. The partial specific volume, 0.73, was calculated from the amino acid composition (14) and sugar content (18).

The subunit size was estimated by SDS polyacrylamide gel electrophoresis in 1 mm thick slab gels with the SDS-phosphate continuous buffer system (34). The size of the peptide chains was estimated by polyacrylamide gel electrophoresis after reduction with dithiothreitol. All electrophoresis experiments were performed in a Pharmacia gel electrophoresis apparatus GE-2/4 LS.

2.2.5. Analytical procedures

Samples were hydrolysed in 6 M-HCl at 110 °C in vacuo for 24, 48 and 72 hours. The evaporated hydrolysates were analysed on a Durrum D 500 amino acid analyser. Tryptophan contents were estimated by the method of GOODWIN and MORTON (19). Half-cystine was determined as

cysteic acid after performic acid oxidation (20). Free thiol groups were measured by means of Ellman's reagent in 5 M-guanidine hydrochloride at pH 8.0 (17). An approximate estimation of the carbohydrate content was obtained by means of the phenol-sulfuric acid method of DUBOIS et al. (15), using glucose as standard. The content of hexosamine was determined on the amino acid analyser after hydrolysis for 5, 8 and 17 hours in 6 M-HCl at 110 °C in vacuo. Glucosamine hydrochloride was used as standard.

Peptides were sequenced either on a Beckman 890 C liquid phase sequencer as previously described (22) or on an Applied Biosystems model 470A gas phase sequencer. The phenylthiohydantoin amino acid derivatives from the gas phase sequencer were identified on-line by reverse phase HPLC using equipment from Applied Biosystems, while those from the liquid phase sequencer were identified with a Hewlett-Packard HPLC system as previously described (28).

C-terminal sequences of the A'- and B-chains were determined by digestion with malt carboxypeptidase II and carboxypeptidase Y according to the general procedures described in ref. 9. The reaction mixture consisted of 0.15 mM-peptide (E7 from the A'-chain and CB5 from the B-chain), 0.5 µM malt carboxypeptidase II, 0.5 µM-carboxypeptidase Y, 0.05 M-sodium acetate, 1 mM-EDTA, pH 4.5. Aliquots were withdrawn during the reaction, pH was lowered to 2.0 by addition of 0.5 M-HCl and the aliquots were applied directly to a Durrum D 500 amino acid analyser.

2.2.6. Separation of A, A' and B-chains

Reduction and alkylation of wheat carboxypeptidase with 2-vinylpyridine produced three different peptide-chains termed A-, A'- and B-chains. The A- and A'-chains were separated from the B-chain by chromatography on Biogel P-200 (5×100 cm), equilibrated with 5% acetic acid, as previously described for malt carboxypeptidase I (11). The lyophilized preparation of the combined A-chains (50 mg) was dissolved in 0.05 M-sodium formate, 0.15 M-NaCl, 6 M-urea, pH 3.5 and separated by ion

exchange chromatography on Fractogel TSK-SP-650 (S) (1.6×60 cm), equilibrated with the same buffer. The elution was accomplished with a linear gradient from 0.15 to 0.30 M-NaCl in 0.05 M-sodium formate 6 M-urea, pH 3.5, using a total of 2000 ml buffer.

2.2.7. Cleavages of chains

Cyanogen bromide cleavages of the reduced and alkylated chains were performed in the following way: 5 mg peptide was dissolved in 0.5 ml 70% formic acid and bubbled with nitrogen for 10 min. 25 mg CNBr was added and the solution purged with nitrogen. The reaction mixture was stirred in the dark for 20 hours at 22 °C, followed by bubbling with nitrogen for three hours to remove the reagent. The peptides were separated by gel filtration (see section 2.2.8).

Cleavage at Asn-Gly bonds in peptides (2-5 mg · ml⁻¹) was effectuated with freshly prepared 2 M-hydroxylamine in 6 M-guanidine hydrochloride, adjusted to and kept at pH 9.0 with 4.5 M-LiOH (3). After 4 hours at 45 °C the reaction was stopped by adding glacial acetic acid at 10% (v/v) and the mixture was desalted on Bio-Gel P-6 eluted with 10% acetic acid.

Tryptic digestions of peptide chains were performed in a pH stat in 3 M-urea at 30 °C and pH 8.0. In a typical experiment 5 mg peptide was dissolved in 1 ml 6 M-urea and 1 ml water and 5 µl 0.1 M-CaCl₂ was added. pH was adjusted to 8.0 with 50 mM-NaOH and 50 µl DPCC-treated trypsin in 1 mM-HCl (1 mg/ml) was added (1:100 w/w). pH was kept at 8.0 by titration with 50 mM-NaOH. After 30 min 50 µl trypsin was again added, the reaction continued for another 30 min and then stopped by addition of 10 µl TFA. The peptide fragments were separated by HPLC as described in section 2.2.8.

Digestion with *S. aureus* V 8 protease was performed at 25 °C in NH₄HCO₃, pH 7.8 to effect cleavage at the carboxyl side of glutamic acid residues (21). 5 mg A'-chain was dissolved in 1.0 ml 6 M-urea and 2.0 ml 0.1 M-NH₄HCO₃, 2 mM-EDTA, pH 7.8, and 125 µg enzyme was added. After 2 hours the reaction was stopped by addition of 10 µl TFA and fractionated by HPLC (see section 2.2.8). 5 mg B-chain was dissolved in 1.0 ml 6 M-guanidine hydrochloride and 2.0

ml 0.1 M-NH₄HCO₃, 2 mM-EDTA, pH 7.8, and 500 µg of enzyme was added. The reaction was stopped after 66 hours and the fragments separated as described above.

2.2.8. Separation of peptides

Separations of large peptide fragments were performed by gel filtration through Bio-Gel P-60 or P-30 in 10 – 30% (v/v) acetic acid. Small peptide fragments were separated by reverse phase HPLC, using a Waters chromatograph equipped with a 0.46×25 cm wide pore column (J.T. Baker no. RP 7104-0) and a linear gradient of CH₃CN in 0.1% TFA. The flow rate was 1 ml · min⁻¹ and the eluate was monitored at 220 nm supplemented with fluorescence detection at 375 nm after excitation at 280 nm to detect tryptophan-containing peptides. Inhomogeneous fractions were rechromatographed using a Vydac no. 218TPb column and the same elution system as described above.

2.2.9. Peptide nomenclature

Peptide fragments obtained by cleavage with cyanogen bromide, hydroxylamine, trypsin and *S. aureus* V 8 protease are designated CB, NG, T and E, respectively, followed by a number indicating their positions in the polypeptide chain from the N-terminus of the protein.

2.2.10. Stability of wheat bran carboxypeptidases

The stability of the three types of carboxypeptidases were studied by diluting the top fractions of peak I, II and III from CM-cellulose into five volumes of the following buffers containing 0.1 M-NaCl and 1 mM-EDTA: 0.05 M-sodium formate, pH 2.7 and 3.5; 0.05 M-sodium acetate, pH 4.5; 0.05 M-Mes, pH 7.5; 0.05 M-Bicine, pH 9.0 and 9.5; 0.05 M-Ches, pH 9.5, 9.7, and 9.8. The resulting pH was measured and the enzyme solutions were incubated at 25 °C. The activity towards FA-Phe-Leu-OH (see section 2.2.1) was followed as a function of time.

2.2.11. Enzymatic properties of the wheat bran carboxypeptidases

The pH dependence of the activities of the wheat bran carboxypeptidases towards FA-Phe-

Leu-OH, FA-Phe-OMe, FA-Phe-NH₂ and FA-Phe-Gly-NH₂ was determined in 0.05 M buffer, 0.5 M-NaCl, 1 mM-EDTA. The following buffers were used: acetic acid, pH 3.5 – 6.5; Mes, 6.0 – 7.2; Hepes, 7.5 – 8.6.

The kinetic parameters for the hydrolysis of FA-Phe-Leu-OH and FA-Ala-Lys-OH in 0.05 M-sodium acetate, 1 mM-EDTA, pH 4.5 were determined from Lineweaver-Burk plots. The hydrolysis of a series of Z-Ala-X-OH and Bz-X-OMe substrates (X = amino acid residue) was performed in 0.05 M-sodium acetate, 1 mM-EDTA, pH 4.5 and 0.05 M-Hepes, 1 mM-EDTA, pH 8.0, respectively, and monitored at 230 and 254 nm, respectively. 25 µl of a 20 mM substrate solution in methanol was added to 965 µl of the buffer, followed by 10 µl enzyme. The hydrolysis was followed to completion and k_{cat}/K_m values were determined from the integrated form of the Michaelis-Menten equation.

3. RESULTS

3.1. Purification of wheat bran carboxypeptidase

Wheat bran was swelled in 3% acetic acid, 50 mM-NaCl, packed in a column and the next day eluted with the same medium (see section

Figure 1. Extraction of wheat bran by elution from column. The bars indicate fractions collected during the elution. Hatched areas indicate enzymatic activity towards FA-Phe-Leu-OH (see section 2.2.1) and unhatched areas the absorbance at 280 nm.

Table I. Purification of carboxypeptidases from wheat bran

	ml	A ₂₈₀	U/ml	U _{Total}	U/mg
Concentrated extract	7000	72	8.0	56,000	0.11
Supernatant after 38% ammonium sulfate	7300	37	7.7	56,000	0.21
Dissolved ammonium sulfate precipitate	3700	26	13	48,000	0.50
Dissolved crystals	760	4.6	37	28,000	8.0
Eluate from CABS- Sephadex	930	0.75	23	21,000	30

2.2.2). The carboxypeptidase activity in the eluate decreased rapidly with the elution volume whereas the absorbance at 280 nm decreased much more slowly (see Figure 1). All solubilized activity was eluted in a volume corresponding to only approximately 1.5 times the volume of column bed, and the specific activity of the pooled fractions was 2-3 fold higher than obtained in a corresponding experiment using a

traditional extraction procedure (11, 12). The inclusion of 50 mM-NaCl in the extraction medium served to reduce the volume necessary to elute all enzyme. This extract was concentrated and subjected to ammonium sulfate fractionation. The precipitate obtained at 70% saturation of ammonium sulfate was dissolved in and diafiltered against 10 mM-sodium acetate, 40 mM-NaCl, pH 3.8 (Table I), the NaCl preventing adsorption of the enzyme to the ultrafiltration membrane. Crystallization of the enzyme was achieved by a pH change and further purification was obtained by an affinity chromatographic procedure utilizing CABS-Sephadex as affinity matrix and the inhibitor benzylsuccinic acid as eluent (see Figure 2). The carboxypeptidase was finally crystallized (see section 2.2.2). The overall yield of this procedure was 40%.

Figure 2. Purification of wheat bran carboxypeptidase by chromatography on CABS-Sephadex. The dissolved crystals were applied to the CABS-Sephadex column (5×22 cm) and washed with approximately 5 l 0.05 M-NaH₂PO₄, 0.1 M-NaCl, pH 4.5. The elution was performed with 1 mM-BS included in the same buffer. Flow rate: 250 ml · hour⁻¹, fraction size: 25 ml, A₂₈₀: (●), activity towards FA-Phe-Leu-OH (▲).

3.2. Primary structure of wheat bran carboxypeptidase

SDS polyacrylamide gel electrophoresis showed two bands of mobilities corresponding to molecular weights 37,000 and 26,000, respectively, in the presence of dithiothreitol, and one band (MW: 55,000) in the absence of dithiothreitol. This suggested that wheat bran carboxypeptidase is composed of two chains, linked by disulfide bridges. After reduction and pyridylethylation these chains could be separated by chromatography on Bio-Gel P-200 (data not shown). Sequence analysis indicated only a single peptide chain in the fraction containing the smaller chain (B-chain), but two peptide

Table II. Amino acid composition of wheat bran carboxypeptidase

Amino acid	Residues per molecule					
	From amino acid analysis ^{a)}			From amino acid sequence		
	A' (per 28800)	B (per 17900)	Dimeric form (per 93400)	A'	B	(A'-B)×2
Asp	} 31	} 13	} 86	19	} 8	} 54
Asn				12		
Thr	11 ^{c)}	16 ^{c)}	57 ^{c)}	11	17	54
Ser	23 ^{c)}	9 ^{c)}	57 ^{c)}	21	9	60
Glu	} 19	} 11	} 58	12	} 4	} 32
Gln				5		
Pro	14	9	54	14	10	48
Gly	25	13	71	23	13	72
Ala	26	13	76	25	14	78
Val	17 ^{d)}	11 ^{d)}	58 ^{d)}	17	11	56
Met	3	4	13	3	4	14
Ile	9 ^{d)}	5 ^{d)}	28 ^{d)}	10	5	30
Leu	20	14	68	20	13	66
Tyr	16	10	57	18	11	58
Phe	13	4	31	13	3	32
His	9	5	29	9	5	28
Lys	8	3	16	7	3	20
Arg	11	11	48	12	10	44
Trp	n.d.	n.d.	21 ^{g)}	5	6	22
Cys	4 ^{f)}	2 ^{f)}	10 ^{e)}	4	2	12
Glc-NH ₂	n.d.	n.d.	25	6 ^{b)}	6 ^{b)}	24 ^{b)}
Total amino acids				260	159	838

a) Based on the calculated molecular weight of the protein parts as obtained from the amino acid sequence. The third column (dimer) refers to the complete mixture of carboxypeptidases as obtained from the affinity column.

b) Based on the assumed content of two glucosamines per carbohydrate moiety. c) Extrapolated to zero time. d) Value after 72 hours hydrolysis. e) Determined as cysteic acid after performic acid oxidation (20). f) Determined as pyridylethylcysteine. g) Determined spectrophotometrically by the method of GOODWIN and MORTON (19).

chains in approximately equal quantities in the fraction containing the larger chain (A-chain). The preparation of A-chain was applied to a Fractogel cation exchange column and separated into two peaks by elution with a salt gradient (data not shown). Each of these fractions were subjected to 37 steps of Edman degradation. The fraction eluting at the lowest concentration of NaCl contained two peptide chains in different quantities, the major component, A (80%) with the N-terminal sequence VEPSGHAADRI-, and the minor component, A' (20%) with a sequence starting at position 4 but otherwise

identical, i.e. SGHAADRI-. The fraction eluting at the highest salt concentration contained only the latter sequence. Attempts to further purify the fraction eluting first from the ion exchange column failed. The amino acid compositions of the two homogeneous chains, i.e. the A'- and B-chains, are listed in Table II. Both contain neutral sugar and glucosamine. The values are based on the calculated molecular weight of the protein part as determined from their primary structures.

The A'- and B-chains were cleaved with cyanogen bromide, hydroxylamine, trypsin and

Figure 3. Amino acid sequences of the A- and A'-chains of wheat bran carboxypeptidase. Peptides were sequenced by either automated Edman degradation (–) or by digestion carboxypeptidase (←). Each peptide fragment is indicated by a full line for sequenced residues followed by a broken line for residues not sequenced in that particular fragment. Peptide fragments are designated by the following nomenclature: CB, cyanogen bromide fragments; NG, hydroxylamine fragments; T, tryptic fragments; E, *S. aureus* V8 protease fragments. N are glycosylated asparagines.

S. aureus V8 protease, and most of the fragments were isolated by HPLC or gel chromatography. The peptides were sequenced (up to 47 cycles of Edman degradation) and aligned with overlaps as shown in Figures 3 and 4. The overlap between T10 and T11 in the A'-chain was obtained by digestion of E7 with a mixture of carboxypeptidase Y and malt carboxypeptidase II. Digestion of CB5 from the B-chain and the complete A'-chain with malt carboxypeptidase II identified the four amino acid residues at the C-terminus of the B-chain and serine at the C-terminus of the A'-chain. The amino acid

composition is in good accordance with that obtained by amino acid analysis of the isolated chains. The glucosamine and neutral sugar content of wheat bran carboxypeptidase II was assigned to Asn¹¹⁶, Asn¹²⁷ and Asn²⁵⁹ of the A'-chain and Asn²⁸, Asn³⁴ and Asn¹⁵⁸ of the B-chain.

The N-terminal sequence analysis of the A-chain suggested that it was three amino acids longer at the N-terminus, but otherwise identical to the A'-chain. To further investigate this, cyanogen bromide fragments from the mixture of A- and A'-chains, as obtained from gel filtra-

Figure 4. Amino acid sequence of the B-chain of wheat bran carboxypeptidase. See legend for Figure 3.

Figure 5. Separation of wheat bran carboxypeptidase into three forms by ion exchange chromatography. The eluate from CABS-sepharose was applied to a CM-52 cellulose column (2.5×18 cm), equilibrated with 0.1 M-sodium acetate pH 5.0. The elution was performed with a gradient from 0.1 to 0.6 M-sodium acetate, pH 5.0 (---). A_{280} (●), and the activities towards FA-Phe-Leu-OH (Δ) and FA-Ala-Lys-OH (■) were measured. Fraction size: 25 ml. Flow-rate: 80 ml · hour⁻¹.

tion chromatography on Biogel P-200 (see above), were isolated and sequenced. Five fragments were obtained and two of these reflected the different N-termini of the A- and A'-chains. The residual three fragments showed no heterogeneity, and the A-chain most likely differs from the A'-chain only by three additional amino acids at the N-terminus (see Figure 3).

3.3. Subunit structure of wheat bran carboxypeptidase

The eluate from the affinity column was applied to an ion exchange column (CM-cellulose) and separated into three components by elution with a salt gradient (Figure 5). The ratio of the activities towards FA-Phe-Leu-OH and FA-Ala-Lys-OH and the specific activities are essentially identical in the three peaks. The eluted fractions were pooled as indicated and crystallized (see section 2.2.2). Each fraction appeared homogeneous by FPLC ion exchange analysis in the absence of denaturing agents (see section 2.2.3). SDS polyacrylamide gel electrophoresis showed the presence of two peptide chains of molecular weights 37,000 and 26,000

in all three fractions and sedimentation equilibrium centrifugation of the enzymes eluting in fractions II and III both gave molecular weights around 105,000. This indicated that the enzymes are dimers with each monomer composed of an A-chain and a B-chain, linked by disulfide bridges. N-terminal sequence analysis showed that fraction I contained the A- and B-chains in equal quantities, fraction III the A'- and B-chains in approximately equal quantities, and fraction II the A- and A'-chains in equal quantities and the B-chain in a quantity corresponding to the sum of the two A-chains. These results suggested that the dimeric enzymes eluting in fractions I and III are composed of two identical monomers, i.e. A-B/A-B in fraction I and A'-B/A'-B in fraction III. The composition of the enzyme eluting in fractions I, II and III was further studied by FPLC chromatography in the presence of 6 M-urea. Fraction II contained two components eluting at 0.4 M and 0.5 M-NaCl, in approximately equal quantities. Sequence analysis indicated that the first component contained the A-chain and the B-chain and the second component the A'-chain and the B-chain. In contrast, fractions I and III comprised single components eluting at 0.4 M and 0.5 M-NaCl, respectively. The enzyme eluting in fraction II is therefore composed of two different monomers, i.e. A-B/A'-B.

3.4. Enzymatic properties of wheat bran carboxypeptidases

All three forms of wheat bran carboxypeptidase were stable at 25 °C in the pH range 3.6 to 8.7; at pH 8.9 their activities decreased with time ($t_{1/2}$ values of 30 min for all three derivatives). Thus, all three forms of the enzyme exhibit identical stability.

The kinetic parameters for the hydrolysis of FA-Ala-Lys-OH and FA-Phe-Leu-OH by the three forms of wheat bran carboxypeptidase were determined (Table III). It is seen that the enzyme in fraction I hydrolyses both substrates with lower K_m and k_{cat} values than the enzyme in fraction III and that the enzyme in fraction II exhibits intermediary values. However, for both substrates k_{cat}/K_m is essentially independent of the enzyme used.

Table III. Kinetic parameters for the hydrolysis of peptide substrates by wheat bran carboxypeptidases

Substrate		Enzyme		
		A-B/ A-B	A'-B/ A'-B	A'-B/ A'-B
FA-Phe-Leu-OH	k_{cat}	10700	11600	14600
	K_m	0.39	0.51	0.59
	k_{cat}/K_m	27000	23000	25000
FA-Ala-Lys-OH	k_{cat}	8600	11000	13000
	K_m	0.17	0.23	0.26
	k_{cat}/K_m	51000	48000	50000

k_{cat} values are in min^{-1} , K_m values in mM and k_{cat}/K_m values in $\text{min}^{-1} \cdot \text{mM}^{-1}$. Assays were performed in 0.05 M-sodium acetate, 1 mM-EDTA, pH 4.5 at 25 °C.

The pH profiles for the peptidase, esterase, amidase and peptidyl amino acid amide hydrolyase activities of the wheat bran carboxypeptidases towards FA-Phe-Leu-OH, FA-Phe-OMe, FA-Phe-NH₂ and FA-Phe-Gly-NH₂, respectively, were determined. With all three forms the hydrolysis of FA-Phe-Leu-OH is optimal at pH 4.5 whereas the rates of hydrolysis of the other three substrates increase from essentially zero at pH 4.0 to the maximum values at pH > 7. The side-chain specificity was therefore only studied with the hybrid form (A'-B/A-B), eluting in fraction II from the ion exchange column (see Figure 5). The specificity with respect to the P₁ position of peptide substrates was determined at pH 4.5. The highest k_{cat}/K_m values were obtained for substrates containing C-terminal amino acid residues with positively charged side-chains, i.e. Lys og Arg, medium values for those with hydrophobic side-chains, i.e. Ile, Met, Phe, Val and Ala, and low values for those with hydrophilic side chains, i.e. Gly, Pro, Asn, Asp and Ser (Table IV). The specificity with respect to the P₁ position of ester substrates was investigated at pH 8.0 using a series of N-blocked amino acid esters (Table IV). The highest k_{cat}/K_m is obtained for Bz-Phe-OMe and this value is 15-30 times higher than the values obtained with substrates with an aliphatic side-chain with an unbranched β -carbon, i.e. Leu and Met. The substrates with a branched β -carbon, i.e. Val and

Table IV. Specificity of carboxypeptidase II from wheat bran and malted barley

	k_{cat}/K_m ($\text{min}^{-1} \cdot \text{mM}^{-1}$)	
	Carboxypeptidase II from	
	wheat bran	malt
Bz-Gly-OMe	< 5	< 5
Bz-Ala-OMe	29	19
Bz-Val-OMe	8.7	7.1
Bz-Ile-OMe	11	7.5
Bz-Leu-OMe	540	310
Bz-Met-OMe	92	160
Bz-Phe-OMe	9200	5700
Bz-Pro-OMe	< 5	< 5
Bz-Asp-OMe	< 5	< 5
Bz-Lys-OMe	2900	2200
Bz-Arg-OMe	2700	950
Bz-His-OMe	88	76
Bz-Thr-OMe	< 5	< 5
Z-Ala-Gly-OH	< 5	11
Z-Ala-Ala-OH	1000	560
Z-Ala-Val-OH	4100	1800
Z-Ala-Ile-OH	5900	3300
Z-Ala-Met-OH	7400	3200
Z-Ala-Phe-OH	4200	2100
Z-Ala-Pro-OH	11	6
Z-Ala-Asp-OH		71
Z-Ala-Asn-OH	200	62
Z-Ala-Lys-OH	52000	25000
Z-Ala-Arg-OH	59000	18000
Z-Ala-His-OH	3100	1100
Z-Ala-Ser-OH	200	150

Assay conditions: 0.5 mM substrate, 2.5% CH₃OH, 0.05 M-sodium acetate pH 4.5, 1 mM-EDTA (for Z-Ala-X-OH substrates) or 0.025 M-Hepes, 1 mM-EDTA, pH 8.0. (Bz-X-OMe substrates). The rate of hydrolysis was measured spectrophotometrically at 230 nm or 254 nm and 25 °C and followed to more than 95% completion. From the progression curves k_{cat}/K_m values were determined utilizing the integrated form of the Michaelis-Menten equation. The values for the wheat bran enzyme are based on the calculated molecular weight of the monomer (57000).

Ile, are hydrolysed with very low rates and those with Pro, Gly, Thr and Asp are hydrolysed with negligible rates. The N-blocked amino acid ester substrates with positively charged side-chain, are hydrolysed with high rates.

4. DISCUSSION

Serine carboxypeptidases are isolated from fungi as monomers, from higher plants usually as dimers and from mammalian tissues as dimers or even higher degrees of association. The enzymes from malt are the most extensively characterized dimeric forms: carboxypeptidase I contains two identical subunits (11) and carboxypeptidase II contains two subunits of different molecular weights due to different contents of carbohydrate (12). The enzyme from wheat bran is also a dimeric enzyme with each monomer containing two peptide chains, an A-chain and a B-chain of approximate molecular weights 37,000 and 26,000, respectively, linked by disulfide bridges. The A-chain exist in two forms, A and A', of approximately equal abundance, the latter being three amino acids shorter at the N-terminus. The A'-chain appears not to be formed during the isolation of the enzyme since variations in the duration of the isolation procedure from one to five weeks had very little influence on the ratio of A- to A'-chains. It is therefore probable that both forms exist in the wheat bran and by implication, in the resting wheat grain. The two types of A-chains result in two types of subunits, A-B and A'-B and consequently, three types of dimers, A-B/A-B, A'-B/A-B and A'-B/A'-B. These are isolated in the approximate ratio of 1:2:1, consistent with a random association of the subunits, but it is unknown whether this has taken place in the wheat bran or during isolation of the enzyme. However, when a solution of the hybrid form of the enzyme, containing two types of subunits, was subjected to the low pH (3.5) of the extraction medium none of the two other forms were seen, as analysed by FPLC ion exchange chromatography in the absence of urea, indicating that under these conditions no redistribution of the monomers had taken place. In addition, conditions which caused dissociation of the hybrid dimer, e.g. in 6 M-urea at pH 3.5, resulted in a simultaneous and irreversible loss of the enzymatic activity.

The A'- and B-chains of the carboxypeptidase from wheat bran contain 260 and 160 amino acid residues, respectively. The glucosamine and neutral sugar is attached to three asparagines in the A'-chain and three in the

B-chain. No unused N-glycosylation sites are found and O-glycosylation is absent. Unlike carboxypeptidase Y (2, 4) and malt carboxypeptidase I (11) but like malt carboxypeptidase II (12) wheat bran carboxypeptidase contains no free cysteinyl residues. The six cysteinyl residues are therefore organized as three disulfide bridges.

A data base computer search demonstrated that the amino acid sequence of wheat bran carboxypeptidase is homologous with that of other serine carboxypeptidases (see ref. 31): when due allowance is made for gaps the A'-chain shows 41% and 95% identity with the A-chains of carboxypeptidases I and II from malt (30, 31) respectively, and 29% identity with the N-terminal portion of the single-chain carboxypeptidase Y from yeast (8, 23, 29). The B-chain shows 30% and 96% identity with the B-chains of carboxypeptidases I and II, respectively, and 21% identity with the C-terminal portion of carboxypeptidase Y. The high homology with malt carboxypeptidase II is illustrated in Table V, listing the positions of divergence. Three amino acid residues in carboxypeptidase Y have been shown to be associated with the activity of the enzyme (Table VI). Ser¹⁴⁶ is the essential seryl residue and the region around this amino acid residue is highly conserved in all four carboxypeptidases. Cys³⁴¹ is situated at the S_i binding site of carboxypeptidase Y. This amino acid residue and the region around it is not conserved in any of the carboxypeptidases of plant origin. The region around Met³⁹⁸, located in the S_i binding site of carboxypeptidase Y, is fairly conserved in all four enzymes. However, Met³⁹⁸ itself is replaced with a threonyl residue in malt carboxypeptidase I, and in malt carboxypeptidase II and wheat bran carboxypeptidase with a glutamyl residue. Enzymatic studies with malt carboxypeptide II have indicated that a negative charge in the S_i binding site of malt carboxypeptidase II is responsible for the binding of the positively charged side-chains of arginyl and lysyl residues at the P_i position of peptide substrates (7). The glutamyl side-chain at position 398 of both malt carboxypeptidase II and the wheat bran enzyme, which exhibit similar enzymatic properties (see below), could possibly function as such a binding site. Consis-

Table V. Positions of divergence in the primary structures of wheat bran carboxypeptidase and malt carboxypeptidase II

Position	Carboxypeptidase from	
	wheat bran	malt
A-chain:		
1	Val	-
2	Glu	-
3	Pro	Ala
4	Ser	Gly
12	Ala	Val
19	Ala	Glu
34	Gly	Ala
49	Asp	Glu
82	Lys	Met
139	Lys	Ala
149	Tyr	Arg
151	Asp	Glu
154	Ile	Val
175	Lys	Gly
220	Glu	Asp
261	Thr	Ser
263	Ser	-
B-chain:		
1	Thr	N-blocked Thr
23	Met	Thr
38	Ala	Ala/Thr ^{b)}
39	Thr	Thr/Asn ^{b)}
95	Pro	Ala
106-107 ^{a)}	-	Leu
141	Val	Ile
145	Tyr	Gln
155	Gln	Arg
157	Lys	Thr
159	Ala	-
160	Thr	-

The numbering of Figures 3 and 4 is used. ^{a)} 106-107 represents a deletion in the wheat bran enzyme relative to that from malt. ^{b)} The two amino acids represent polymorphism in the B-chain of carboxypeptidase II from malt.

tent with this the methionyl or threonyl residues present at position 398 of carboxypeptidase Y and malt carboxypeptidase I, respectively, render peptides with C-terminal arginine or lysine poor substrates of these enzymes. The only histidyl residue which is conserved in all four enzymes is that in position 397 (see Table VI). Since the serine carboxypeptidases probably employ the catalytic mechanism of the serine endopeptidases (6) this residue may correspond to the essential histidyl residue of the catalytic triad (1).

All the serine carboxypeptidases characterized in this laboratory have been demonstrated to catalyse the release of ammonia and amino acid amides from peptide amides and alcohols from peptide esters, i.e. they exhibit amidase, peptidyl amino acid amide hydrolase and esterase activities, respectively, in addition to their peptidase activities. The three forms of the wheat bran enzyme also exhibit these activities and with identical pH dependencies: the peptidase activity is optimal around pH 4 - 4.5 and the activities towards substrates with blocked C-terminus above pH 7. These optima are essentially identical to those obtained with malt carboxypeptidases I and II (7, 11).

The three forms of wheat bran carboxypeptidase exhibit slightly different enzymatic properties. The A-B/A-B form hydrolyses the peptide substrates FA-Phe-Leu-OH and FA-Ala-Lys-OH with lower k_{cat} and K_m values than the A'-B/A'-B form, and the hybrid form exhibits intermediary values. However, for both substrates k_{cat}/K_m is independent of the enzyme, suggesting that all three forms exhibit the same specificity, especially when it is considered that the two substrates are of widely different structures. The k_{cat}/K_m values for the hydrolysis of a series of Z-Ala-X-OH and Bz-Y-OMe substrates show the following preference of the hybrid form for

Table VI. Homologies with catalytically important regions of carboxypeptidases Y

	146	341	398
Carboxypeptidase Y	-AGESYAGH-	-KDFICNWLG-	-NGGHHMVPFDVP-
Carboxypeptidase I from malt	-AGESYAGV-	-HDMXVPFTG-	-GAGHTVPEYKP-
Carboxypeptidase II from malt	-AGESYAGH-	-TDAVVPLTA-	-GAGHEVPLHRP-
Carboxypeptidase from wheat bran	-AGESYAGH-	-TDAVVPLTA-	-GAGHEVPLHRP-

The numbering is that of carboxypeptidase Y

X: Lys > Arg > Ile, Met > Phe, Val > His > Ala > Ser > Asp, Asn > Gly, Pro and for Y: Phe > Lys > Arg > Leu > Met > His > Ala > Val, Ile > Pro, Asp, Gly, Thr. This substrate preference is essentially identical to that of malt carboxypeptidase II (7, 11) and since the two enzymes structurally also are highly homologous (see above) they probably are functionally equivalent. The enzyme from wheat bran should, henceforth be termed wheat carboxypeptidase II as already suggested by MIKOLA, based on ion exchange chromatography (24).

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