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PRIMARY STRUCTURE OF CARBOXYPEPTIDASE II FROM MALTED BARLEY

by

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The primary structure of malt carboxypeptidase II has been determined. The enzyme is a dimer where each monomer is composed of two peptide chains, an A- and a B-chain, linked by disulphide bridges. The B-chain exists in two forms, both N-terminally blocked, which differ in position 38 and 39, one form containing Ala^{38}-Thr^{39}, the other containing Thr^{38}-Asn^{39} with carbohydrate attached to the asparagine side-chain. Fragments of the A- and B-chains were obtained by chemical cleavages with either cyanogen bromide, hydroxylamine or iodosobenzoic acid and by enzymatic cleavages with either trypsin or S. aureus V8 protease, sequenced and aligned to give the total sequence.

The A- and B-chains contain 260 and 159 amino acid residues, respectively. Glycosylated asparagines are found in positions 114, 125 and 257 of the A-chain, and positions 28, 34, 39 and 159 of the B-chain. Position 39 of the B-chain is only partially glycosylated (see above). Alignment of the sequence of the A-chain with the N-terminal part of carboxypeptidase Y revealed 28% homology. Similarly, the B-chain showed 21% homology with the C-terminal part of carboxypeptidase Y. The homology between malt carboxypeptidases I and II is 40% for the A-chains and 30% for the B-chains. The corresponding homologies between malt carboxypeptidase II and wheat carboxypeptidase II are 95% and 96%, respectively. No homology was observed with other proteins by a computer search of a sequence data base provided by the National Biomedical Research Foundation. A region of the A-chain was identical to the region around the essential seryl residue in position 146 of carboxypeptidase Y.

1. INTRODUCTION

According to MIKOLA (14), germinating barley contains five serine carboxypeptidases of complementary specificities and three of these, carboxypeptidase I, II and III have been isolated and characterised in this laboratory (4, 5, 6). These enzymes in combination with endopeptidases, very effectively produce free amino acids during germination by cleavage of reserve proteins in the endosperm. We have previously reported the sequence of malt carboxypeptidase I (18). This enzyme consists, like a number of serine carboxypeptidases from higher plants (2), of two identical subunits, each composed of two peptide chains, cross-linked by disulphide bridges (6). The amino acid sequence is homologous with that of carboxypeptidase Y from yeast (3, 13, 17), especially in the region around the reactive seryl residue. However, carboxypeptidase Y consists of a single peptide chain. In the present report, the primary structure of malt carboxypeptidase II has been determined, and it

Abbreviations: CABS-Sepharose = [N-(ε-aminocaproyl)-p-amino-benzyl]succinyl-Sepharose 4B; DPCC = diphenylcarbamyl chloride; EDTA = ethylenediaminetetraacetic acid, disodium salt; HPLC = high pressure liquid chromatography; PTH = phenylthiohydantoin; TFA = trifluoroacetic acid.

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is demonstrated that the sequence of this enzyme is almost identical with that of wheat carboxypeptidase II and homologous with that of malt carboxypeptidase I and carboxypeptidase Y.

2. MATERIALS AND METHODS

2.1. Materials

Malt carboxypeptidase II was prepared as previously described (6) from malted Gula barley (obtained from the Carlsberg Breweries, Denmark) by affinity chromatography on CABS-Sepharose.

Bio-Gels P-200, P-60, P-30 and P-6 were from Bio-Rad, USA. DPCC-treated trypsin and dithiothreitol were from Sigma, USA. S. aureus V8 protease was from Miles, USA. Carboxypeptidase Y was a product of Carlsberg Biotechnology, Denmark. 2-vinylpyridine was from Janssen Chimica, Belgium. All other chemicals were analytical or HPLC-grade from Merck, W. Germany.

2.2. Methods

2.2.1. Separation of peptide chains

After reduction and alkylation with 2-vinylpyridine of malt carboxypeptidase II as previously described (5), the A-chain (mol. weight 34,000) was separated from the B-chains (mol.weight 24,000-27,000) by gel filtration on Bio-Gel P-200 (5.0×88 cm) equilibrated with 5% (v/v) acetic acid.

2.2.2. Cleavage of peptide bonds

Chemical cleavage by cyanogen bromide and hydroxylamine and enzymatic digestion with trypsin of the reduced and alkylated chains was performed as previously described (18).

Cleavage at the carboxyl group of tryptophan residues in the A-chain was performed according to the procedure described by FONTANA et al. (8). The reaction was carried out in the dark at room temperature overnight. Tyramine hydrochloride (5 mg · ml^{-1}) was included as a scavenger in the reagent which consisted of o-iodosobenzoic acid (20 mg · ml^{-1}) in 4 M-guanidine hydrochloride and 80% acetic acid.

Digestion with S. aureus V8 protease (11) was performed at 25 °C in 0.1 M-ammonium bicarbonate and 2 M-urea at pH 7.8. The reduced and alkylated peptide chains were dissolved in 6 M-urea and diluted with 2 volumes 0.15 M-ammonium bicarbonate pH 7.8. Then enzyme was added and after 2-3 hours digestion the reaction was stopped by addition of TFA.

2.2.3. C-terminal sequence determination

C-terminal sequences of the entire A-chain and the CB4 fragment of the B-chain were determined by digestion with carboxypeptidase Y. The following conditions were used: 0.23 mM A-chain, 0.1 M-Mes, 1 mM-EDTA, 0.1% SDS, pH 7.0, 2.5 μM carboxypeptidase Y and 0.27 mM-CB4, 0.05 M-sodium acetate, pH 5.0, 7.7 μM carboxypeptidase Y. Aliquots were withdrawn during the reaction, pH was adjusted to 2.0 by addition of 0.5 M-HCl and the aliquots were applied directly to a Durrum D500 amino acid analyzer.

2.2.4. Separation of peptide fragments

Separation and purification of large peptide fragments were performed by gel filtration through Bio-Gel P-60 or P-30 in 10-30 % (v/v) acetic acid. Purification of small peptides was performed by reverse phase HPLC using equipment from Waters and wide pore C_{18} columns i.d. 4.6 mm and length 25 cm (J.T. Baker no. RP 7104-0 or Vydac no. 218TPb). Gradient elution was performed with linear gradients of acetonitrile in 0.1% (w/w) trifluoroacetic acid at a flow rate of 1.0 ml · min^{-1}. 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 in the same system but employing a more flat and narrow gradient of acetonitrile.

2.2.5. Amino acid sequence determinations

Peptides were sequenced either on a Beckman model 890C liquid phase sequencer as previously described (12) or on an Applied Biosystems model 470A gas-phase sequencer, using the

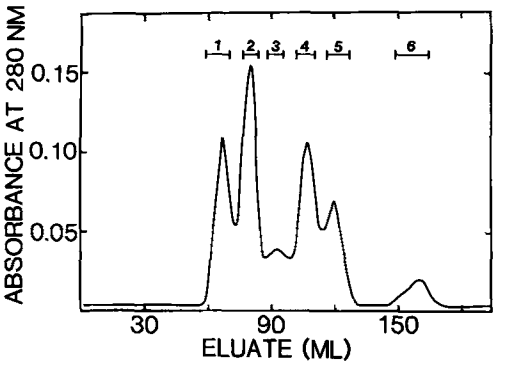

Figure 1. Separation of cyanogen bromide fragments of the A-chain (4 mg) on Bio-Gel P-60. The column (1.6×86 cm) was eluted with 30% acetic acid at a flow rate of 7 ml · h^{-1}. Fractions (1.5 ml) were pooled as indicated. Pool 6 was further purified by HPLC.

program provided by the company. 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 using a linear gradient from 10 to 45% methanol, (16). Amino acid compositions were determined as previously described (5).

2.2.6. Peptide nomenclature

Peptide fragments obtained by cleavage with cyanogen bromide, hydroxylamine, iodosobenzoic acid, trypsin or S. aureus V8 protease are designated CB, NG, W, T and E, respectively, followed by a number indicating their positions in the polypeptide chain from the N-terminus of the protein.

3. RESULTS

3.1. N-terminal sequences of A- and B-chains

The A- and B-chains, as obtained by gel chromatography of the reduced and alkylated enzyme, were subjected to N-terminal sequence determination. The A-chain was sequenced 37 cycles, while the B-chain was found to be N-terminally blocked. The blocking group was both acid and base labile. After its removal, the B-chain could be sequenced 25 cycles.

3.2. Cyanogen bromide fragments

According to the amino acid analysis, the A-chain of malt carboxypeptidase II contains four methionyl residues (6) corresponding to five cyanogen bromide fragments. Chromatography of the cyanogen bromide treated A-chain on Bio-Gel P-60 (Figure 1) produced six peaks. Pool 1 contained two peptides which later was demonstrated to originate from incomplete cleavage after two methionines. Pool 2 contained one peptide (CB3, 103 res.) which was sequenced 48 cycles. Pool 3 contained one peptide (CB4+5, 77 res.) originating from incomplete cleavage at Met^{248}. Pool 4 contained CB4 (65 res.), which was sequenced 53 cycles. Pool 5 contained CB2 (58 res.) which was sequenced 25 cycles and pool 6 was found to be a mixture of the N-terminal cyanogen bromide fragment (CB1, 22 res.) and the C-terminal cyanogen bromide fragment (CB5, 12 res.). After rechromatography by HPLC the complete sequence of CB5 could be determined.

Chromatography of the cyanogen bromide fragments of the B-chain on Bio-Gel P-60 yielded three peaks (Figure 2). Pool 1 contained a single peptide (CB3, 98 res.) which was se-

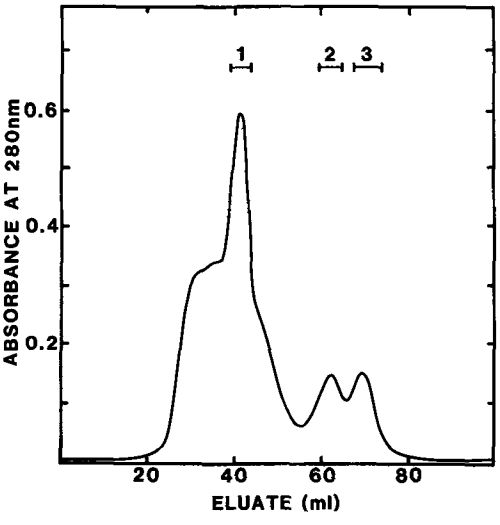

Figure 2. Separation of cyanogen bromide fragments of the B-chain (7 mg) on Bio-Gel P-60. The column (1.5×88 cm) was eluted with 30% acetic acid at a flow rate of 4 ml · h^{-1}. Fractions (1.3 ml) were pooled as indicated.

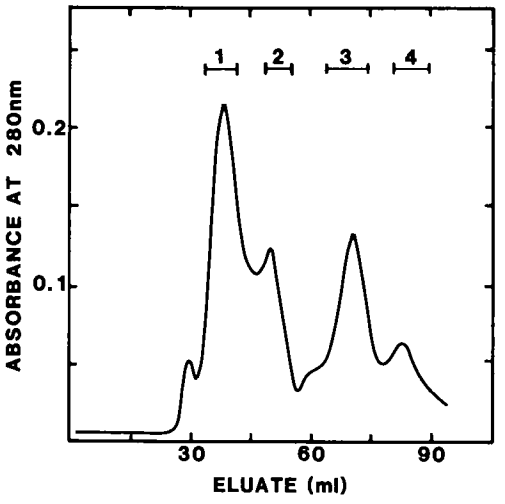

Figure 3. Separation of hydroxylamine fragments of the A-chain (5.0 mg) on Bio-Gel P-60. The column (1.5×88 cm) was eluted with 10% acetic acid at a flow rate of 7 ml · h^{-1}. Fractions (1.5 ml) were pooled as indicated.

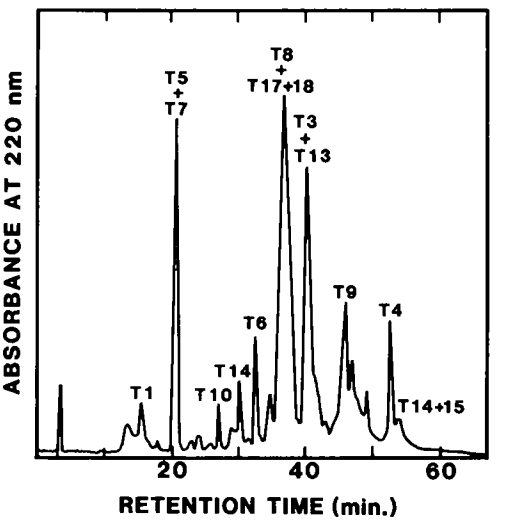

Figure 4. Fractionation of a tryptic digest of the A-chain by reverse phase HPLC using a Novapac 8NV C_{18} column (radial pac). The column was eluted over 60 min with a gradient from 0 to 54% acetonitrile in 0.1% TFA at a flow rate of 1.0 ml · min^{-1}. The eluate was monitored at 220 nm.

quenced 25 cycles. Pool 2 contained CB1 (33 res.) which was sequenced 25 cycles and pool 3 contained CB2 (22 res.) which was sequenced to the end and found to be polymorphous at two positions. The C-terminal cyanogen bromide fragment (CB4, 6 res.) could be isolated after reverse phase HPLC of the cyanogen bromide cleaved B-chain. It was sequenced 6 cycles. The C-terminal digestion with carboxypeptidase Y released no amino acids.

3.3. Hydroxylamine fragments

The fragments formed by cleavage of the A-chain with hydroxylamine could be purified by gel filtration on Bio-Gel P-60 (Figure 3). Pool 1 and 2 had identical N-terminal sequences (NG2) indicating that the cleavage at Asn^{186}-Gly^{187} was incomplete. Pool 2 was sequenced 33 cycles and provided the overlap between CB2 and CB3. Pool 3 contained NG3, the N-terminal sequence of which was known from the sequence of CB4. Pool 4 contained the already known N-terminal peptide, Ala^{1}-Asn^{58}.

Cleavage of the B-chain mixture with hydroxylamine and subsequent chromatography on Bio-Gel P-60 revealed only a single peak indicating the absence of N-G sequences in both forms of the B-chain.

3.4. Tryptic peptides

The amino acid composition of the A-chain of malt carboxypeptidase II indicated 3-4 Lys and 12 Arg residues (6), corresponding to 16-17 tryptic peptides. HPLC-chromatography of the digestion mixture revealed a complex chromatogram with fairly well resolved peaks (Figure 4). It was possible to identify several of the peptides from the amino acid analysis combined with the available sequence data. The remaining unknown peptides were sequenced as far as possible, in most cases by means of the gas-phase sequencer. Incomplete tryptic cleavages were observed between Lys^{180}- Gly^{181} and Lys^{217}-Asp^{218}. Sequencing of T4 provided the overlap between CB2 and NG2, while T17+18 gave the overlap between CB4 and CB5.

Amino acid analysis of the B-chain indicated 2 Lys and 12 Arg residues (6) consistent with 15 tryptic peptides. By HPLC-chromatography of the digestion mixture (Figure 5) several pure

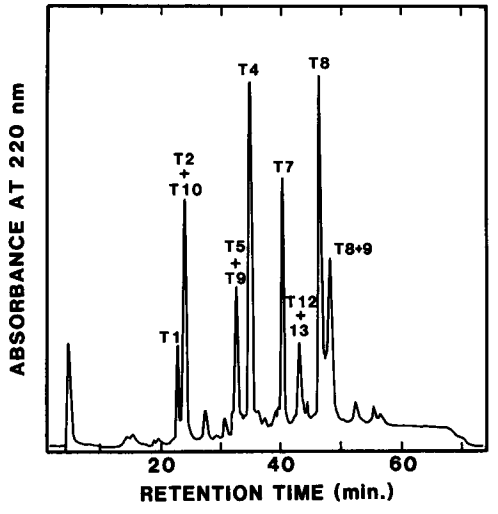

Figure 5. Fractionation of a tryptic digest of the B-chain by reverse phase HPLC using a wide pore C_{18} column (Bakerbond). The column was eluted over 60 min with a gradient from 0 to 54% acetonitrile in 0.1% TFA at a flow rate of 1.0 ml · min^{-1}. The eluate was monitored at 220 nm.

fragments were obtained and rechromatography by HPLC of a fraction obtained by Bio-Gel chromatography of a tryptic digest resulted in the separation of two T4 peptides (Figure 6) which differed only at two positions. This was the only indication of polymorphism in the B-chain. The sequences of the tryptic fragments T4 and T5 provided the overlaps between CB1 and CB2 and between CB2 and CB3, respectively. Sequencing of T12+13 revealed that the tryptic cleavage at Lys^{151}- Pro^{152} had been incomplete.

3.5. S. aureus V8 protease fragments

The entire A-chain was digested with S. aureus V8 protease. The resulting fragments were separated by reverse phase HPLC (Figure 7). Sequence analysis of E13 provided the overlap

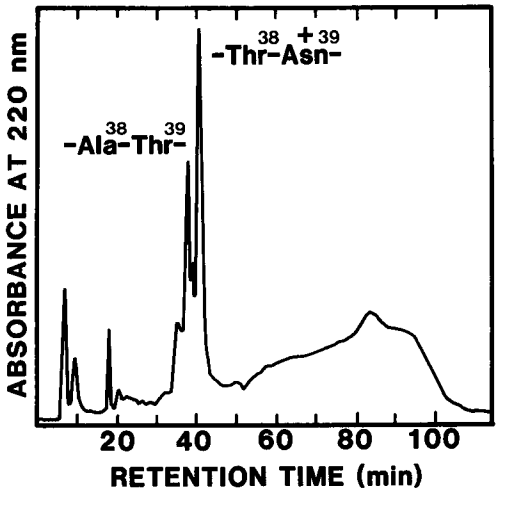

Figure 6. Rechromatography of a pool from Bio-Gel P-30 chromatography of a tryptic digest of the B-chain on a reverse phase wide pore C_{18} column (Bakerbond). The column was eluted over 80 min with a gradient from 2 to 45% acetonitrile in 0.1% TFA at a flow rate of 0.5 ml · min^{-1}. The eluate was monitored at 220 nm.

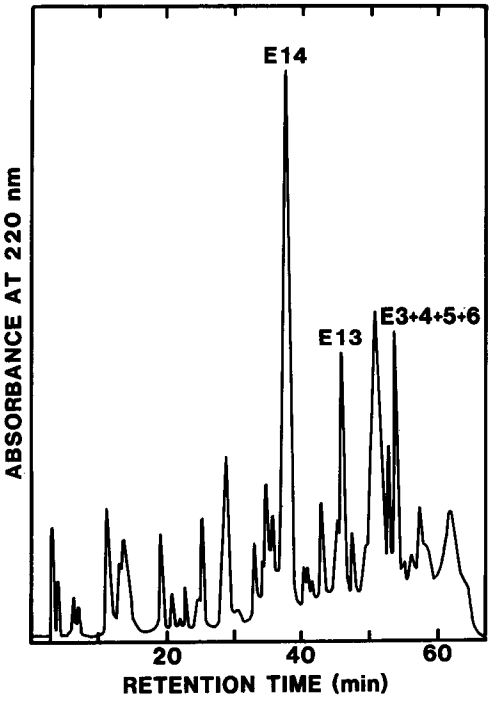

Figure 7. Separation of S. aureus V8 protease fragments from the A-chain by reverse phase HPLC using a wide pore C_{18} column (Bakerbond). The column was eluted over 60 min with a linear gradient from 14 to 41% acetonitrile in 0.1% TFA at a flow rate of 1.0 ml · min^{-1}. The eluate was monitored at 220 nm.

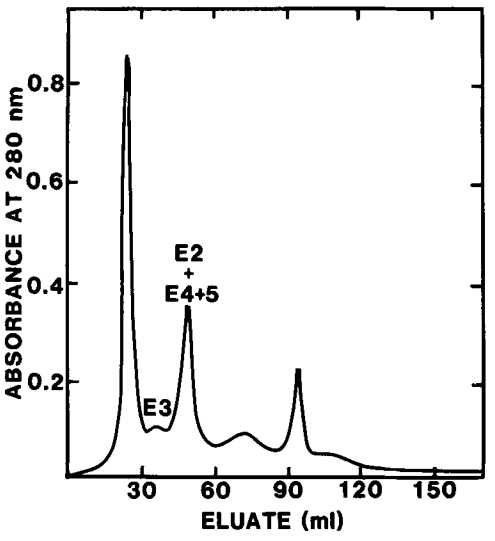

Figure 8. Separation of S. aureus V8 protease fragments of the B-chain (5 mg) on Bio-Gel P-30. The column (1.5×90 cm) was eluted with 10% acetic acid at a flow rate of 6 ml · h^{-1}.

between T13 and T14+15 and hence, the position of CB4 in the chain.

The B-chain was digested with V8 protease and the fragments were separated by gel filtration on Bio-Gel P-30 (Figure 8). Sequence analysis of E4+5 (cleavage at Glu^{131}-Val^{132} was incomplete) provided the overlap between T8 and T12+13. The sequence of E3 secured the overlap between T7 and T8.

3.6. Fragments obtained by cleavage with o-iodosobenzoic acid

The entire A-chain was cleaved with iodosobenzoic acid (8) and the fragments were separated by HPLC. Sequence analysis of W4 provided the overlap between T9 and T13.

3.7. Assignment of glycosylated asparagines

The A-chain of malt carboxypeptidase II contains 6 glucosamine residues (6). These were assigned to three glycosylated asparagines (Asn^{114}, Asn^{125} and Asn^{257}) from the absence of detectable PTH-amino acid in these positions after Edman degradation, the presence of a hydroxyamino acid two positions from the vacant position towards the C-terminal end, and finally by the amino acid analysis of T8 and CB5 indicating the presence of glucosamine and the excess of one or two Asx as compared with the composition obtained from the sequence determined. Similarly, the B-chain was found to contain glycosylated asparagines at positions 28, 34 and 159. Furthermore, one of the two forms of the B-chain contained a glycosylated asparagine at position 39.

4. DISCUSSION

Earlier investigations (6) have shown that malt carboxypeptidase II, like wheat carboxypeptidase II (7) and malt carboxypeptidase I (5, 18), is composed of two subunits where each subunit is constituted of two peptide chains, an A-chain and a B-chain, linked together by disulphide bridges. Determination of the amino acid sequence of serine carboxypeptidase II from barley malt revealed that the A-chain contained 260 amino acid residues (Figure 9). Earlier studies (6) indicated that the B-chain existed in two forms which differed about 3,000 daltons in molecular weight due to different contents of carbohydrate. This is supported by the present study where it is shown that the B-chain is polymorphous at positions 38 and 39: one form with the sequence Thr^{38}-Asn^{39} with carbohydrate attached and the other with the sequence Ala^{38}-Thr^{39} without carbohydrate. No evidence of polymorphism at other positions has been found. The two forms of the B-chain which contain 159 amino acid residues (Figure 10) were N-terminally blocked. The nature of the blocking group has not been investigated but it was observed that the group was released after exposure to acidic as well as basic conditions. The presence of two forms of the B-chain indicates that barley contains at least two genes each coding for one form of the B-chain.

The amino acid compositions of the A- and B-chains as calculated from the sequences are in good agreement with the compositions determined by amino acid analysis (Table I). The A-chain is N-glycosylated at 3 asparagines at positions 114, 125 and 257. The glycosylation

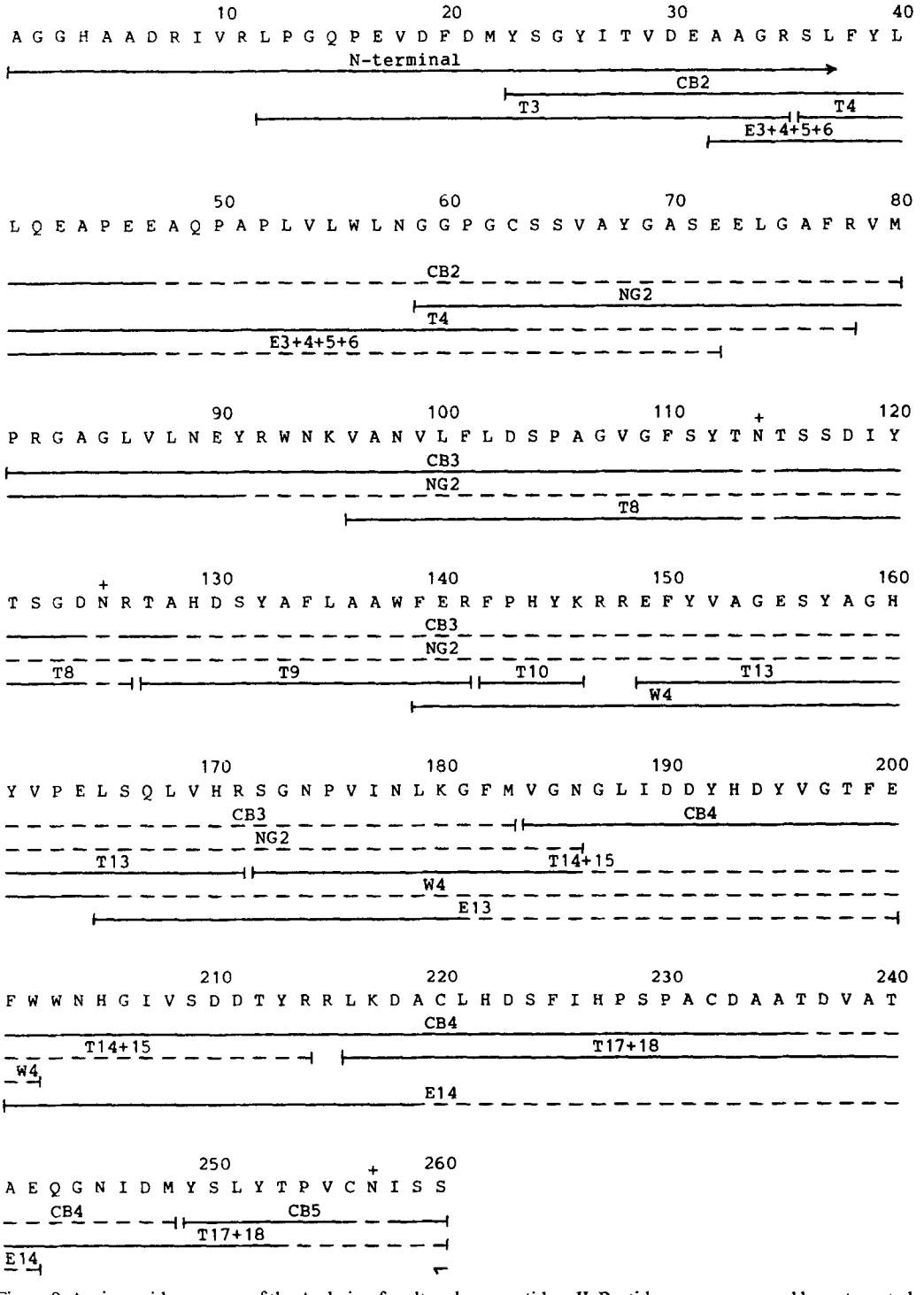

Figure 9. Amino acid sequence of the A-chain of malt carboxypeptidase II. Peptides were sequenced by automated Edman degradation (full line) and digestion with malt carboxypeptidase II (←). Each peptide fragment is indicated by a full line for sequenced residues followed by a broken line for residues 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; W, iodosobenzoic acid fragments. $\overset{+}{N}$ are glycosylated asparagines.

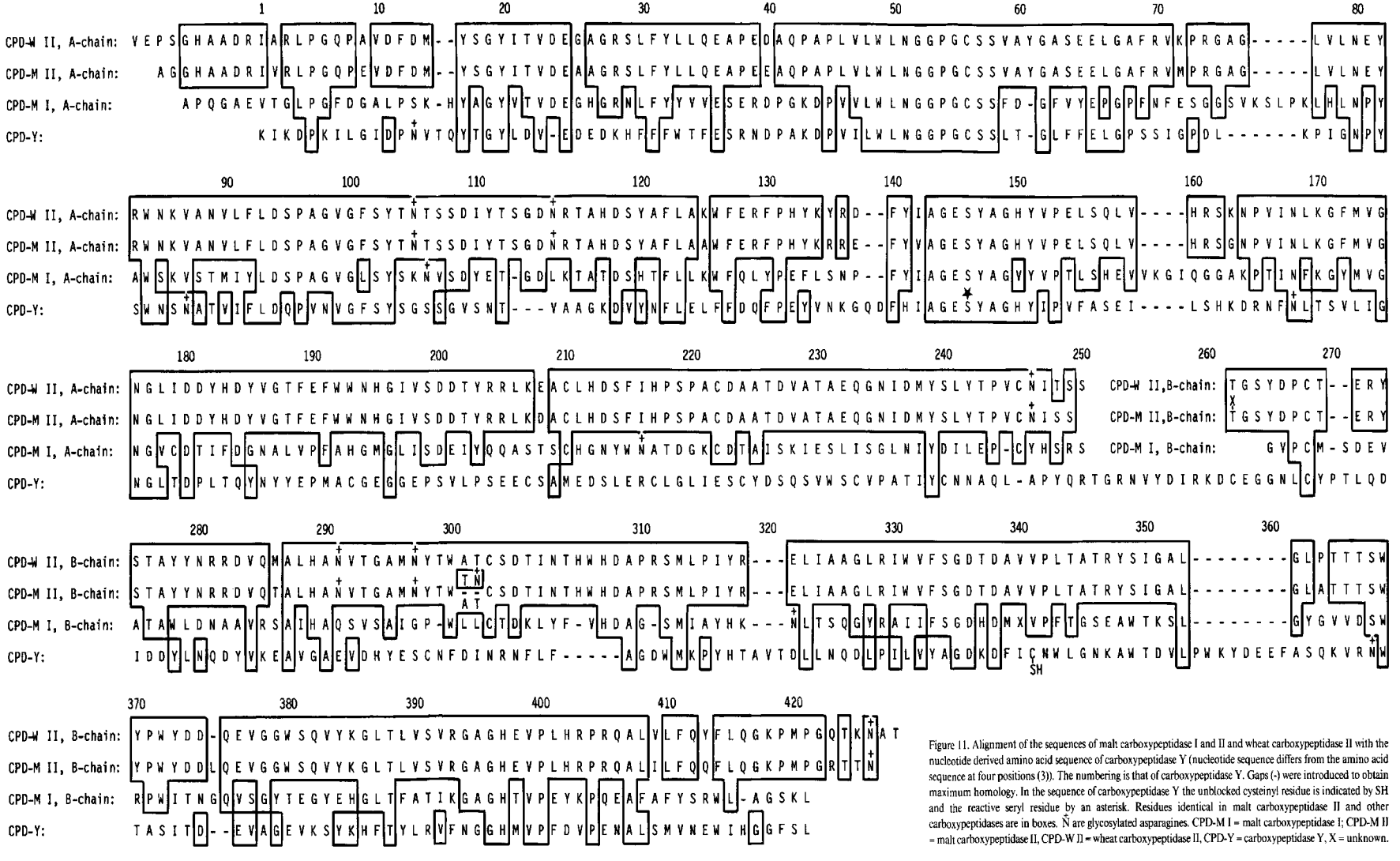

Figure 11. Alignment of the sequences of malt carboxypeptidase I and II and wheat carboxypeptidase II with the nucleotide derived amino acid sequence of carboxypeptidase Y (nucleotide sequence differs from the amino acid sequence at four positions (3)). The numbering is that of carboxypeptidase Y. Gaps (-) were introduced to obtain maximum homology. In the sequence of carboxypeptidase Y the unblocked cysteinyl residue is indicated by SH and the reactive seryl residue by an asterisk. Residues identical in malt carboxypeptidase II and other carboxypeptidases are in boxes. $\hat{N}$ are glycosylated asparagines. CPD-M I = malt carboxypeptidase I; CPD-M II = malt carboxypeptidase II, CPD-W II = wheat carboxypeptidase II, CPD-Y = carboxypeptidase Y, X = unknown.

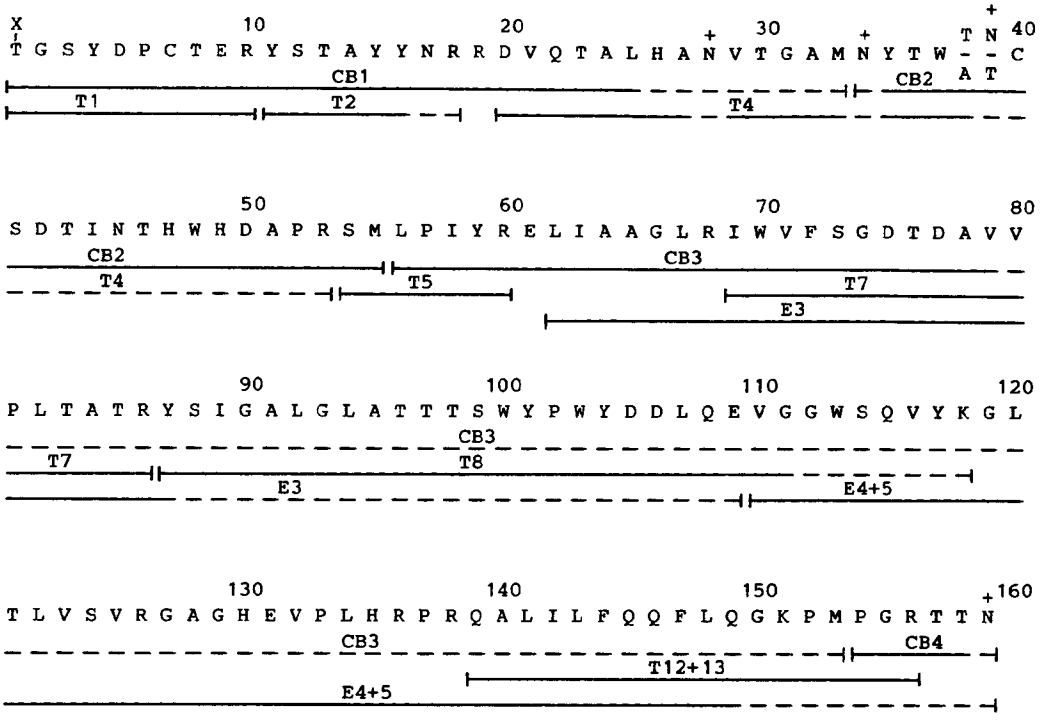

Figure 10. Amino acid sequence of the B-chain of malt carboxypeptidase II. Polymorphism is seen in position 38 and 39. X is the N-terminal blocking group. See legend for Figure 9.

sites in the the B-chain are asparagines 28, 34, 39 and 159, position 39 only in the form of the B-chain with Asn at position 39. No unused N-glycosylation sites were found. The presence of glycosylated asparagine as C-terminal amino acid residue in the B-chain is unusual since the condition of glycosylation, i.e. a hydroxyamino acid residue two positions in the C-terminal direction, is not fulfilled. This implicates a proteolytic processing subsequent to glycosylation. This position of the carbohydrate moiety is consistent with the lack of released amino acids by digestion with carboxypeptidase Y.

Each subunit of the enzyme contains 6 cysteinyl residues consistent with the presence of 3 disulphide bridges. Like in wheat carboxypeptidase II, but unlike carboxypeptidase Y and malt carboxypeptidase I, no free sulphydryl group is present.

Comparison of the amino acid sequence of malt carboxypeptidase II with those of other proteins only demonstrated homology with malt carboxypeptidase I, wheat carboxypeptidase II and carboxypeptidase Y. The alignment, with due allowance for gaps, of the amino acid sequences of these four serine carboxypeptidases is shown in Figure 11. The A-chains of the cereal carboxypeptidases show regions of high homology with the N-terminal part of carboxypeptidase Y (Figure 11). Similarly, the B-chains of the cereal carboxypeptidases show homology with the C-terminal part of carboxypeptidase Y. The percentages of homology are shown in Table II. The sequences of malt carboxypeptidase II and wheat carboxypeptidase II are almost identical (95-96% homology) suggesting that they are functionally equivalent. The homology with malt carboxypeptidase I is significantly lower but it is higher than that with carboxypeptidase Y (see Table II). This indicates, as would be expected, that the three cereal carboxypeptidases mutually are more closely related than each of

Table I. Amino acid composition of A- and B-chains of malt carboxypeptidase II

Amino acid	Residues per molecule					
	A-chain			B-chains		
	Sequence analysis		Amino acid analysis (per 28,800)[a]	Sequence analysis		Amino acid analysis (per 17,900)[a]
Aspartic acid	18	30	29.5	8	13-14	13.2
Asparagine	12			5-6		
Threonine	10		10.5	18		17.1
Serine	20		19.6	9		8.2
Glutamic acid	14	19	19.4	4	11	11.8
Glutamine	5			7		
Proline	14		14.4	9		9.8
Glycine	24		24.3	13		13.6
Alanine	26		26.1	13-14		14.6
Cysteine	4		4.1	2		2.2
Valine	19		19.4	10		10.3
Methionine	4		3.7	3		2.7
Isoleucine	9		8.7	6		5.9
Leucine	20		20.5	14		14.5
Tyrosine	17		18.0	10		10.1
Phenylalanine	13		13.3	3		3.3
Histidine	9		8.8	5		5.1
Lysine	4		3.3	2		1.8
Arginine	13		11.7	11		10.7
Tryptophan	5		5.3	6		5.4
Total	260		260.6	159		160.3

[a]Based on the calculated mol.wt. of the protein part.

them is related to carboxypeptidase Y.

Two regions in the four enzymes are highly conserved, i.e. the regions 48-58 and 143-149 in the carboxypeptidase Y numbering. The essential Ser^{146} of carboxypeptidase Y (10) is located in the latter region and the conservation of this residue in all four carboxypeptidases suggests that it is the active site serine in these enzymes as well. The sequence around the reactive seryl residue in three serine carboxypeptidases from citrus fruits (9, 15) exhibit higher homology with the corresponding regions of serine endopeptidases like trypsin, chymotrypsin, elastase and plasmin than the cereal carboxypeptidases and carboxypeptidase Y. This supports the earlier suggestion (18) that two separate groups of plant serine carboxypeptidases exist.

The conserved region 48-58 is not present in the serine endopeptidases and it does not contain aspartic acid or histidine, indicating that it is not a part of the catalytic triad in the active site (1). However, the presence of three glycyl and one prolyl residue in this region suggest a highly twisted peptide chain most reasonably important for the three-dimensional structure of the enzymes. This is consistent with the presence of Cys in position 56, which participates in an S-S bridge with another Cys-residue in the molecule.

Earlier indications that His^{397} is the most probable candidate to the histidyl residue functioning as an acid/base catalyst in the active site (18) is supported from this histidine being the only histidine conserved in all four enzymes.

It is not clear which of the aspartic acid residues participate in the active site, since five aspartic acid residues are conserved in all four

Table II. Amino acid sequence homolgy (%) between serine carboxypeptidases

Peptide chain	A-chain CPD-M II (260 residues)	A-chain CPD-W II (263 residues)	A-chain CPD-M I (266 residues)
A-chain CPD-W II	95		
A-chain CPD-M I	40	41	
CPD-Y (421 residues)	28	29	29
Peptide chain	**B-chain CPD-M II (159 residues)**	**B-chain CPD-W II (160 residues)**	**B-chain CPD-M I (148 residues)**
B-chain CPD-W II	96		
B-chain CPD-M I	30	30	
CPD-Y (421 residues)	21	21	24

CPD-M I = malt carboxypeptidase I
CPD-M II = malt carboxypeptidase II
CPD-W II = wheat carboxypeptidase II
CPD-Y = carboxypeptidase Y

enzymes. However, Asp^{94} may be the most probable candidate because it is situated in a region, which is rather well conserved.

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