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ISOLATION OF CARBOXYPEPTIDASE II FROM MALTED BARLEY BY AFFINITY CHROMATOGRAPHY

by

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A serine carboxypeptidase isolated from malted barley by affinity chromatography was termed malt carboxypeptidase II to distinguish it from another malt carboxypeptidase previously described (Carlsberg Res. Commun. 48, 217-230 (1983)), henceforth called malt carboxypeptidase I. Our nomenclature is in agreement with the nomenclature formerly suggested by MIKOLA. Malt carboxypeptidase II has a molecular weight of 110,000-120,000. It appears to be a dimer where each monomer is composed of two peptide chains linked by disulfide bridges: one monomer contains an A-chain (34,000) and a B-chain (27,000), the other an A-chain and a C-chain (24,000). The enzyme contains 28 residues of glucosamine and 15% neutral sugar. The N-terminal sequence of the A-chain was NH_2-Ala-Gly-Gly-His-Ala-Ala-Asp-Arg-Ile-Val- while the B- and C-chains appeared to be N-terminally blocked. The amino acid compositions of the B- and C-chains were identical suggesting that their different molecular weights are due to different contents of carbohydrate.

Malt carboxypeptidase II is inhibited by diisopropyl phosphorofluoridate and by Hg^{++}. It exhibits a strong preference for substrates containing Lys and Arg as C-terminal amino acid residues but it also hydrolyses substrates with hydrophobic amino acid residues in this position.

1. INTRODUCTION

MIKOLA (25) has described that malt extracts by ion exchange chromatography can be separated into five fractions exhibiting carboxypeptidase activity, indicating that malt contains five different carboxypeptidases. Several reports on the purification of carboxypeptidases from malt have appeared (3, 28, 31, 34) but only after the recent introduction of affinity chromatography has it been possible to obtain a homogeneous preparation of one of these enzymes in amounts that allowed a more detailed characterization (7).

In cases where the isolation of carboxypeptidase from malt by affinity chromatography was followed by measurements of carboxypeptidase activity towards two substrates, i.e. FA-Phe-Ala-OH and FA-Phe-Phe-OH, it was observed that two carboxypeptidases were bound to the affinity resin. The two enzymes differed by the ratios of their specific activities towards the two substrates, i.e. FA-Phe-Ala-OH/FA-Phe-Phe-OH, the enzyme previously described (7) exhibiting a ratio of 27 compared to 1.6 for the second enzyme. In the present paper this difference is utilized to monitor the purification of the second

Abbreviations: BS = benzyl succinic acid; Bz = benzoyl; CABS-Sepharose = [N-(ε-aminocaproyl)-p-aminobenzyl]succinyl-Sepharose 4B; DFP = diisopropyl phosphorofluoridate; 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) ethane sulfonic acid; p-HMB = parahydroxymercuribenzoate; SDS = sodium dodecyl sulfate; Tris = tris(hydroxy methyl)aminomethane; Z = carbobenzoxy.

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enzyme. It appears that the enzyme now isolated corresponds to malt carboxypeptidase II in the nomenclature of MIKOLA (25) whereas the one previously described corresponds to malt carboxypeptidase I.

2. MATERIALS AND METHODS

2.1. Materials

Malted Gula barley was obtained from United Breweries, Denmark, and D,L-benzylsuccinic acid from Burdick and Jackson, USA. All Z-Ala-X-OH substrates were from Bachem, Switzerland. Sepharose 4 B was from Pharmacia Fine Chemicals, Sweden, and Bio-Gel P-200 was from Bio-Rad, USA. Mes was purchased from Sigma, USA and (1,3-^{3}H) diisopropyl phosphorofluoridate was a product of Amersham International, England. All other reagents and solvents were of analytical purity and obtained from Merck, W. Germany.

CABS-Sepharose was prepared as previously described (7). FA-Phe-Ala-OH and FA-Phe-Phe-OH were prepared according to the methods outlined by BLUMBERG and VALLEE (4): 2 mmol FA-N-hydroxysuccinimide ester was dissolved in 4.5 ml dioxane and added to 4.5 ml water containing 4 mmol $NaHCO_3$ and 2 mmol L-phenylalanyl-L-alanine-hydrochloride or L-phenylalanyl-L-phenylalanine-hydrochloride. After 4 hours the dioxane was evaporated and recrystallization from ethanol/water yielded the pure compounds as judged by HPLC analysis and amino acid analysis. Melting points: FA-Phe-Ala-OH: 200-202 °C, decomp., FA-Phe-Phe-OH: 200-202 °C.

2.2. Methods

2.2.1. Routine determination of enzymatic activity and protein concentration

Carboxypeptidase activity was routinely assayed at 25 °C by following the hydrolysis of FA-Phe-Ala-OH and FA-Phe-Phe-OH spectrophotometrically at 333 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 0.1 M-Mes, 1 mM-EDTA, pH 4.75 (the established pH optimum for the hydrolysis of Z-Phe-Ala-OH by the purified enzyme), followed by 10 µl enzyme solution. One unit was defined as the amount of enzyme necessary to release one micromole of alanine or phenylalanine per min at 25 °C.

Protein concentration was determined spectrophotometrically at 280 nm using $A^{1\%}_{280} = 18.8$, determined from amino acid composition and sugar content of the pure enzyme. This value was also used at purification steps where the enzyme was not pure.

2.2.2. Purification of malt carboxypeptidase II

The enzyme was purified according to a modification of the method used in ref. 7. 11 kg of malted Gula barley was ground and suspended in 60 l demineralized water and pH adjusted to 3.7 by the slow addition of approximately 1.5 l glacial acetic acid. The mixture was stirred for 1 hour at 18-22 °C and then left for 16 hours at 2 °C to permit insoluble material to settle. The supernatant, approximately 36 l, was concentrated approximately 10 fold by ultrafiltration, and 230 g ammonium sulfate per liter concentrate was added (38% saturation). After 2-3 hours at 4 °C the precipitate was removed by centrifugation and to the supernatant was added 180 g ammonium sulfate per liter (65% saturation). After 16 hours at 4 °C the resultant suspension was centrifuged yielding a precipitate which could be stored at 4 °C for at least 2 weeks without loss of enzymatic activity. The further purification of the enzyme was performed in two affinity chromatographic steps utilizing CABS-Sepharose.

Step 1: The 65% ammonium sulfate precipitate was dissolved in approximately 600 ml H_2O and pH was adjusted to 4.5 followed by adjustment of the concentration of ammonium sulfate to approximately 0.25 M, corresponding to a conductivity of $35 \cdot 10^{-3}$S at room temperature. This sample was applied to a CABS-Sepharose affinity column (10×19 cm), equilibrated with 0.05 M-NaH_2PO_4, 0.1 M-NaCl, pH 4.5. Essentially all enzymatic activity was bound to the resin which was washed with approximately 13 l 0.05 M-NaH_2PO_4, 0.1 M-NaCl, pH 4.5 until A_{280} of the eluate was below 0.1. The enzyme was eluted with 0.1 M-Na_2HPO_4, pH 7.5. The affinity resin was regenerated with 0.01 M-Tris, 3 M-NaCl, pH

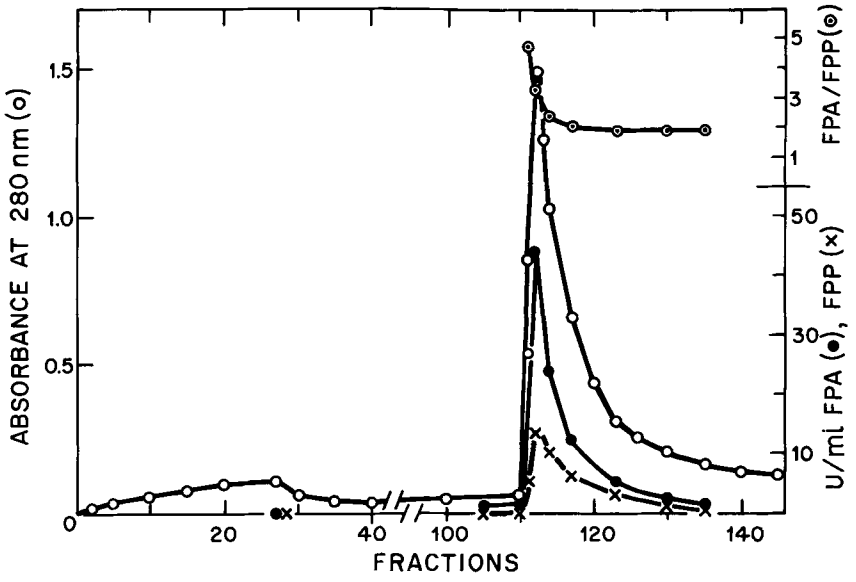

Figure 1. Purification of malt carboxypeptidases I and II on CABS-Sepharose.

The eluate from step 1 was adjusted to 0.2 M-NaCl and pH 4.5 and reapplied to the CABS-Sepharose column (5 x 14 cm = 275 ml). The column was washed with approximately 2 l 0.05 M-NaH_2PO_4, 0.1 M-NaCl, pH 4.5 and elution was performed with 0.05 M-NaH_2PO_4, 0.1 M-NaCl, 5 mM-BS, pH 4.5. A flow rate of 270 ml · hour^{-1} was used. Fraction size: 27 ml. FPA = FA-Phe-Ala-OH, FPP = FA-Phe-Phe-OH.

8.5, followed by 0.05 M-NaH_2PO_4, 0.1 M-NaCl, pH 4.5.

Step 2: The eluate from Step 1 was adjusted to 0.2 M-NaCl by addition of solid NaCl and pH was lowered to 4.5 by addition of 1 M-HCl. This sample was reapplied to an affinity column (Figure 1) which was washed with 0.05 M-NaH_2PO_4, 0.1 M-NaCl, pH 4.5 until A_{280} was below 0.06. The enzyme was eluted with the same buffer containing 5 mM of the inhibitor BS. The resin was regenerated as described in Step 1.

Step 3: The active fractions from Step 2 with A_{280} > 0.25 were pooled and dialysed against 30-50 volumes water for 3 to 5 days. During this time crystallisation of malt carboxypeptidase II took place. The crystals were removed by centrifugation at 18,000 g for 1 hr 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-sodium acetate, 0.2 M-Na_2SO_4, pH 4.0 by stirring the suspension overnight at 4 °C.

2.2.3. Homogeneity of the isolated enzyme

The homogeneity of malt carboxypeptidase II was tested by polyacrylamide gel electrophoresis at pH 4.3 according to the procedure of REISFELD (29) and by SDS gel electrophoresis (see section *2.2.4*). An additional test of purity was obtained by ion exchange chromatography on DEAE-Sepharose, equilibrated with 15 mM-sodium phosphate, pH 6.5. The column was eluted with a NaCl gradient from 0 to 0.4 M and various fractions were subjected to SDS gel electrophoresis.

2.2.4. Molecular weight determination

The molecular weight was determined in a Spinco Model E analytical ultracentrifuge using the Yphantis meniscus depletion method as modified by CHERVENKA (8) in a 0.01 M-sodium acetate buffer, pH 4.0, μ = 0.2, at 13,410 rev. per min. Equilibrium was reached after 16 hours. The partial specific volume, 0.710, was

calculated from the amino acid composition (9) and the sugar content (14).

The subunit size was estimated by SDS-polyacrylamide gel electrophoresis in 1 mm thick 7.5% gel slabs with the SDS-phosphate continuous buffer system (33). 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. Chemical composition

Samples were hydrolyzed in 6 M-HCl at 110 °C in vacuo for 24, 48 and 72 hours (26). The evaporated hydrolysates were analyzed on a Durrum D-500 amino acid analyzer. Tryptophan contents were estimated by the method of GOODWIN and MORTON (15). Half-cystine was determined as cysteic acid after performic acid oxidation (17). Free thiol groups were measured by means of Ellman's reagent in 5 M-guanidine hydrochloride at pH 8.0 (12). An approximate estimation of the carbohydrate content was obtained by means of the phenol-sulfuric acid method of DUBOIS (11), using glucose as standard. The content of hexosamine was determined on the amino acid analyzer after hydrolysis for 5, 8 and 17 hours in 6 M-HCl at 110 °C in vacuo. Glucosamine hydrochloride was used as a standard.

Reduction and alkylation of malt carboxypeptidase II with 2-vinylpyridine was performed as described for malt carboxypeptidase I (7). The peptide chains were separated from each other by chromatography on Bio-Gel P-200 (2.5 × 98 cm), equilibrated with 10% acetic acid. N-terminal amino acid sequences were determined using a Beckman 890C liquid phase sequencer as described for malt carboxypeptidase I (7).

2.2.6. Stability of malt carboxypeptidase

The stability of malt carboxypeptidase was investigated by incubating the enzyme (0.1 mg · ml^{-1}) at 25 °C in the following buffers containing 0.2 M-Na_2SO_4 and 1 mM-EDTA: 0.1 M-Mes, pH 4.5 and 6.0; 0.1 M-Hepes, pH 7.5 and 8.5; 0.1 M-2-amino-2-methyl-1,3-propanediol, pH 8.7 and 9.0. The activity (see section *2.2.1*) was followed as a function of time. The incubation at pH 6.0 was also performed at 61 °C and 70 °C to investigate the heat stability of the enzyme.

2.2.7. Inhibition studies

Modification of malt carboxypeptidase with DFP was performed in the following manner: 100 µl (^{3}H) DFP (1.2 mM in propylene glycol) was added to 5.25 ml 50 mM-Na_2HPO_4, 0.2 M-NaCl, pH 7.0, containing 0.33 mg enzyme ml^{-1}, i.e. the concentration of enzyme was 5.4 µM and the concentration of reagent was 22 µM. The enzymatic activity, using the assay described in section *2.2.1*, was followed as a function of time, and after 4 hours of incubation, the reaction mixture was dialyzed against water. The A- and B-chains were separated as described in section *2.2.5*.

The effect of Hg^{++} on the activity of malt carboxypeptidase was investigated by incubating the enzyme (0.2 mg · ml^{-1} = 3.3 µM) with varying concentrations of $HgCl_2$ in 0.1 M-Hepes, pH 7.5. The esterase activity of the enzyme was measured with time by adding 10 µl of this mixture to the following solution: 0.3 mM-Bz-Phe-OMe, 10 µM-$HgCl_2$, 2.5% methanol (v/v), 0.1 M-Hepes, pH 7.5. The rate of hydrolysis was measured by the increase in absorbance at 254 nm, using a Cary 219 spectrophotometer.

2.2.8. Enzymatic properties of malt carboxypeptidase II

The pH optimum for the hydrolysis of Z-Phe-Ala-OH was determined at 2 mM substrate in 0.1 M-Mes, 1 mM-EDTA and the pH optimum for the hydrolysis of Bz-Phe-OMe was determined at 0.3 mM substrate in 0.1 M-Hepes, 1 mM-EDTA. The specificity of malt carboxypeptidase II was investigated by determination of k_{cat}/K_m values for the hydrolysis of a series of Z-Ala-X-OH substrates (X = amino acid residue). The hydrolysis was followed to completion and the k_{cat}/K_m values were determined from the integrated form of the Michaelis-Menten equation. 25 µl of a 20 mM substrate solution in methanol was added to 965 µl 0.1 M-Mes, 1 mM-EDTA, pH 4.75 and 10 µl enzyme was added.

Table I.
Purification of malt carboxypeptidase II

			FA-Phe-Ala-OH			FA-Phe-Phe-OH			Ala/Phe
	ml	A_{280}	U/ml	U_{Total}	U/mg	U/ml	U_{Total}	U/mg	
Extract	36000	12	0.57	20500	0.088	0.24	8600	0.038	2.4
Supernatant after 38% ammonium sulfate	4400	37	4.1	18000	0.21	1.6	7000	0.081	2.6
Dissolved ammonium sulfate precipitate	1000	25	14	14000	1.03	6.0	6000	0.45	2.3
Step 1, phosphate eluate	850	0.89	13	11100	28.2	5.4	4600	11.5	2.4
Step 2, BS eluate	385	0.62	16.7	6430	50.6	6.1	2350	11.4	2.7
After crystallisation:									
Supernatant	410	0.23	9.2	3800	75.2	1.16	476	9.4	7.9
Dissolved crystals	103	1.54	29.3	3000	35.7	18.6	1920	22.7	1.6
Malt carboxypeptidase I					217			8.0	27

3. RESULTS

3.1. Purification of malt carboxypeptidase II

Malt carboxypeptidases I and II were purified from the crude ammonium sulfate precipitate by a two step affinity chromatographic procedure utilizing CABS-Sepharose. The elution in step 2 was accomplished by the addition of the inhibitor BS to the washing buffer. In the early fractions of the elution FA-Phe-Ala-OH/FA-Phe-Phe-OH was significantly higher than in the later fractions indicating that malt carboxypeptidase I was displaced from the column before malt carboxypeptidase II (Figure 1). From the combined fractions obtained in step 2 malt carboxypeptidase II could be crystallised (Table I). The crystals obtained in this fashion are shown in Figure 2. The overall yield of this procedure was 25% in terms of malt carboxypeptidase II.

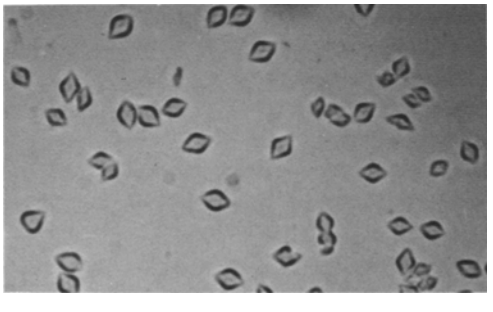

Figure 2. Crystals of malt carboxypeptidase II.

The isolated enzyme was homogeneous by polyacrylamide gel electrophoresis and it eluted at approximately 0.15 M-NaCl from a DEAE-Sepharose column as a symmetrical peak with constant specific activity. Fractions obtained from different locations in the peak all appeared to contain the same three peptide chains as determined by SDS gel electrophoresis in the presence of dithiothreitol.

3.2. Physical chemical characterization of malt carboxypeptidase

Malt carboxypeptidase was stable at 25 °C for 4 hours at pH values between 4.5 and 8.7, while at pH 9.0 the enzyme lost its activity with a $t_{1/2}$ of 45 min. At pH 6.0 and 61 °C it remained stable but at 70 °C $t_{1/2}$ was 10 min.

Sedimentation equilibrium centrifugation indicated that the enzyme was homogeneous and had a molecular weight of 113,000. SDS gel electrophoresis of malt carboxypeptidase II in the presence of dithiothreitol revealed a major band with a mobility corresponding to a molecular weight of 36,000 (A-chain) and in addition, two minor bands corresponding to molecular weights of 28,000 (B-chain) and 25,000 (C-chain). The disulfide bridges connecting the peptide-chains of malt carboxypeptidase II were also cleaved by reduction and pyridylethylation of the enzyme (see section *2.2.5*). The resulting peptide mixture was separated by chromatogra-

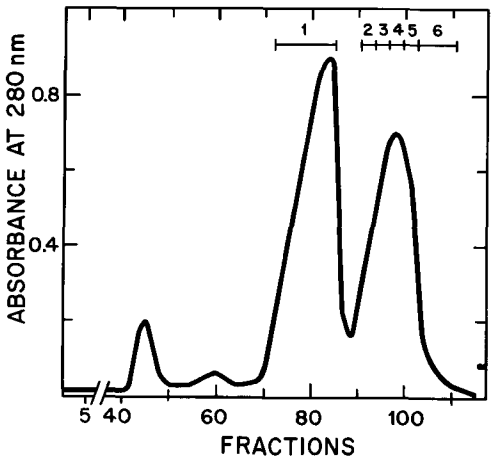

Figure 3. Separation of the A- and B-chain in malt carboxypeptidase after reduction and alkylation (see section *2.2.6*).

The lyophilized reaction mixture was dissolved in 5 ml 10% acetic acid and applied to the Bio-Gel P-200 column (2.5 x 98 cm), equilibrated with 10% acetic acid. Fractions of 3 ml were collected using a flow rate of 4 ml · hour^{-1}.

phy on Bio-Gel P-200 (Figure 3) and the fractions indicated in the figure were subjected to SDS gel electrophoresis. The A-chain eluted in the first large peak while the B- and C-chains eluted in the second large peak such that fractions 2 and 3 contained the B-chain only, fractions 4 and 5 a mixture of B- and C-chains and fraction 6 only the C-chain. Since the second peak contains approximately equimolar amounts of B- and C-chains and since the total amount of these two chains corresponds to the amounts of the A-chain eluting in the first peak it is reasonable to assume the composition of malt carboxypeptidase II to be A_2BC. The SDS gels of the separated A-, B- and C-chains indicated molecular weights of 34,000, 27,000 and 24,000, respectively.

When SDS gel electrophoresis was performed without reduction of the enzyme two bands were observed corresponding to molecular weights of 59,000 and 57,000. These results, combined with 113,000 as the molecular weight of the native enzyme indicates that the enzyme is a dimer consisting of two monomers. Each of these is presumably composed of two peptide chains linked by disulfide bridges with the one monomer containing an A-chain (34,000) and a B-chain (27,000), while the other contains an A-chain and a C-chain (24,000). The mean molecular weight of the monomer is therefore estimated to be approximately 60,000 and this number is used in all further calculations.

The isolated A-, B- and C-chains were subjected to N-terminal sequence determination. It was found that the preparation of A-chain contained only the following sequence:

NH_2-Ala-Gly-Gly-His-Ala-Ala-Asp-Arg-Ile-Val-

The preparations of B- and C-chains did not react with Edman's reagent, suggesting that they are N-terminally blocked. N-terminal sequence analysis of the native enzyme resulted in only the N-terminal sequence of the A-chain with no traces of other sequences, confirming that the isolated enzyme was homogeneous.

The amino acid compositions of malt carboxypeptidase II and its constituent peptide chains are listed in Table II. It is seen that the sum of the amino acids of two A-chains, one B-chain and one C-chain is in fair agreement with the composition of the whole enzyme. The enzyme also contains 28 residues of glucosamine and 15% neutral sugar. No free sulfhydryl could be detected from reactions with Ellman's reagent in the presence of 5 M-guanidine hydrochloride or p-HMB.

3.3. Enzymatic properties of malt carboxypeptidase II

The pH optimum for the hydrolysis of the peptide substrate Z-Phe-Ala-OH and the ester substrate Bz-Phe-OMe was 4.7 and 8.0, respectively. Consequently, the specificity of the enzyme with respect to the C-terminal amino acid residue of peptide substrates was determined at pH 4.7. The highest k_{cat}/K_m values were obtained for substrates containing C-terminal amino acid residues with positively charged side-chains, i.e. Arg and Lys, medium values for those with hydrophobic side-chains, i.e. Ile, Met, Phe, Val, and Ala, and low values for those with Gly, Pro, Asn, Asp and Ser (Table III).

The kinetic parameters of malt carboxypeptidase II catalyzed hydrolysis of Z-Phe-Ala-OH

Table II.
Amino acid composition of malt carboxypeptidase II.

Amino acid	Residues per 119,000	A-chain Residues per 34,000	B-chain Residues per 27,000	C-chain Residues per 24,000	A_2BC
Asp	89.9	29.7	15.3	14.7	89.4
Thr[a]	58.4	10.6	19.5	19.2	59.9
Ser[a]	66.9	19.8	9.5	9.3	58.4
Glu	63.8	19.6	13.5	13.2	65.9
Pro	48.9	14.5	11.1	11.1	51.2
Gly	76.9	24.5	15.6	15.3	79.9
Ala	82.1	26.3	16.7	16.6	85.9
Val[b]	60.0	19.6	11.8	11.6	62.6
Met	12.9	3.7	3.1	3.1	13.6
Ile[b]	30.0	8.8	6.6	6.8	31.0
Leu	71.3	20.7	16.7	16.3	74.4
Tyr	56.5	18.1	11.5	11.2	58.9
Phe	33.1	13.4	3.9	3.8	34.5
His	28.1	8.9	5.8	5.7	29.3
Lys	10.7	3.3	2.1	2.0	10.7
Arg	47.4	11.8	12.3	11.8	47.7
Cys	9.7[e]	4.1[f]	2.5[f]	2.6[f]	13.3
Trp[d]	22.8	5.4	6.2	6.1	23.1
Glucosamin[a]	28.4	5.6	8.9	6.0	26.1
Neutral sugar[c]	15%	12%	18%	12%	

The experimental values were based on the average of two amino acid analysis after hydrolysis in 6 M-HCl for 24, 48, 72 and 96 hours. The content of glucosamine was based on analysis after 5, 8 and 17 hours of hydrolysis.
[a] Extrapolated to zero time. [b] Value after 72 hours of hydrolysis. [c] Determined as cysteic acid after performic acid oxidation. [d] Determined spectrophotometrically by the method of GOODWIN and MORTON (15). [e] Determined by the method of DUBOIS et al. (11). [f] determined as 2-pyridylethylcysteine.

were determined at pH 5.2 in 50 mM-Mes, i.e. the conditions previously used to characterize purified or partially purified malt carboxypeptidases (28, 31, 34). From a linear Lineweaver-Burk plot a K_m of 0.53 mM and V_{max} of 132 $\mu moles \cdot min^{-1}mg^{-1}$ was obtained. Using the molecular weight of the monomer, 60,000, k_{cat} was estimated to be 7,900 min^{-1}.

3.4. Inhibition studies

After 4 hours incubation with (^{3}H) DFP the activity of malt carboxypeptidase II towards FA-Phe-Ala-OH had decreased to less than 2% of the control. Separation of the chains as described in section *2.2.5* indicated that radioactivity had only been incorporated into the A-chain, suggesting that the molecule may contain two active sites, one in each subunit.

The effect of Hg^{++} was measured by the esterase activity of malt carboxypeptidase II since its peptidase activity in the basic pH range is negligible. Treatment with a threefold excess of $HgCl_2$ at pH 7.5 resulted in an instantaneous loss of approximately 90% of its activity in the presence of $HgCl_2$ and no further loss of activity was observed within 20 min. The influence of varying the concentration of Hg^{++} on the activity of malt carboxypeptidase II indicated an apparent dissociation constant of the enzyme-Hg^{++} complex of $1.3 \cdot 10^{-6}$ M at pH 7.5 (Figure 4). Using a fixed concentration of 5.0 μM-$HgCl_2$, the inhibition was found to be dependent on the deprotonation of an ionizable group with an apparent

Table III.
Hydrolysis of Z-Ala-X-OH substrates (X = amino acid residue) with malt carboxypeptidases I and II

Substrate	k_{cat}/K_m ($min^{-1} \cdot mM^{-1}$)	
	Malt carboxypeptidase II	Malt carboxypeptidase I
Z-Ala-Gly-OH	11	480
Z-Ala-Ala-OH	560	22000
Z-Ala-Val-OH	1800	35000
Z-Ala-Ile-OH	3300	22000
Z-Ala-Met-OH	3200	18000
Z-Ala-Phe-OH	2100	5700
Z-Ala-Pro-OH	6	2600
Z-Ala-Asp-OH	71	370
Z-Ala-Asn-OH	62	270
Z-Ala-Lys-OH	25000	< 30
Z-Ala-Arg-OH	18000	< 30
Z-Ala-His-OH	1100	120
Z-Ala-Ser-OH	150	1080

Assay conditions: 0.5 mM substrate, 2.5% CH_3OH, 0.1 M-Mes, 1 mM-EDTA pH 4.7. The rate of hydrolysis was measured spectrophotometrically at 230 nm 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 were based on the molecular weights of the monomers 51,000 and 60,000 for malt carboxypeptidases I (7) and II, respectively.

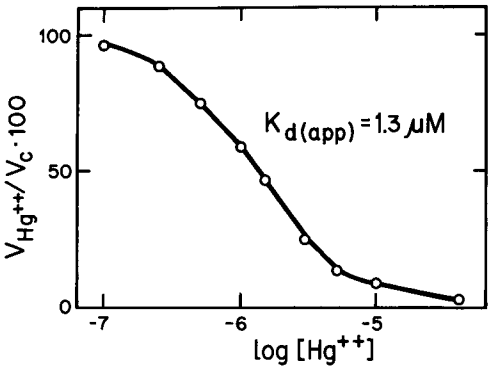

Figure 4. Influence of the concentration of Hg^{++} in the assay mixture on the activity of malt carboxypeptidase.

The enzyme-Hg^{++} complex was formed in the cuvette in the following way: To 960 µl 0.1 M-Hepes, pH 7.5 was added 10 µl of a solution of $HgCl_2$ and 10 µl enzyme (0.20 mg · ml^{-1}). After mixing, 25 µl 12 mM-Bz-Phe-OMe was quickly added. V_{Hg}++ represents the activity of the enzyme towards Bz-Phe-OMe at given concentration of Hg^{++} and V_c represents the activity without $HgCl_2$ added.

pK_a of 6.4 (data not shown). Addition of both 100 µM-KI and 5.0 µM-$HgCl_2$ to the cuvette at pH 7.5 prior to the addition of the enzyme prevented the inactivation, indicating that no enzyme-Hg^{++} complex was formed. This suggested that Hg^{++} binds less tightly to the enzyme than to I^-. In another series of experiments, the enzyme (0.20 mg · ml^{-1}) was reacted with Hg^{++} (10 µM) at pH 7.5 for varying length of time while its esterase activity was assayed after 100 fold dilution in the same buffer but without $HgCl_2$ added. This dilution should be sufficient to dissociate the enzyme-Hg^{++} complex since K_d is $1.3 \cdot 10^{-6}$ M. Nevertheless, with time the enzyme could not be fully reactivated: after 1 hour of reaction it was found that only 45% of the activity of the enzyme could be regenerated, suggesting that the residual enzyme was irreversibly inactivated. Similarly, addition of 100 µM-KI which had no adverse effect on the unmodified enzyme, was not capable of reactivating the mercury inactivated enzyme.

4. DISCUSSION

Malt carboxypeptidase II is only slightly soluble in water (see below) and hence, extraction in water at pH 4.9, as previously described for malt carboxypeptidase I (7), was not optimal for this enzyme. However, when the extraction was performed in 2% acetic acid at pH 3.7 the amount of malt carboxypeptidase II solubilised was increased five fold, whereas the amount of carboxypeptidase I remained constant.

The ammonium sulfate fractionation of the crude extract was required to protect the CABS-Sepharose resin against contaminations and to increase its capacity. The ammonium sulfate precipitate could be stored at 4 °C for at least two weeks without loss of activity such that the purification conveniently could be stopped at this stage. Binding of malt carboxypeptidase to CABS-Sepharose required a salt concentration above 0.1 M and to obtain a high yield it was essential that the capacity of the resin was fully utilized, but not exceeded. This capacity was

approximately 10 and 40 U/ml (Substrate: FA-Phe-Ala-OH) in steps 1 and 2, respectively.

After the final crystallisation step some residual malt carboxypeptidase II is still present in the supernatant. This could be recycled after separation from the malt carboxypeptidase I which also is present in the supernatant (see below) and this increased the yield of malt carboxypeptidase II significantly. Alternatively, a larger proportion of the malt carboxypeptidase II present in the eluate from step 2 could be crystallised if the eluate was concentrated 3 – 4 fold by ultrafiltration prior to the dialysis against water. Unfortunately, some enzyme was lost in this procedure because it adhered to the ultrafiltration membrane.

Malt carboxypeptidase II could also be purified by a simpler procedure where the initial ammonium sulfate precipitate was dissolved in a minimum volume of water, adjusted to pH 5.4 and dialysed against water. The enzyme crystallised in a yield of approximately 40% and after affinity chromatography as described for step 2 (see section *2.2.2*) the specific activity was identical to that of the dissolved crystals after two affinity steps (Table I). The overall yield of this procedure is also approximately 25% and it is simpler than the procedure shown in Table I since it facilitates handling of larger amounts of ammonium sulfate precipitate. On the other hand, the procedure shown in Table I allows the additional isolation of malt carboxypeptidase I from the supernatant after crystallization of malt carboxypeptidase II, by an affinity step using CABS-Sepharose as previously described (7).

MIKOLA (25) has previously described that malt extracts by ion exchange chromatography can be separated into five fractions with carboxypeptidase activity. This suggested the existence of five different enzymes which were termed carboxypeptidases I to V. Carboxypeptidase I was purified by VISURI et al. (31) and carboxypeptidase II has been partially purified by YABUUCHI et al. (34). From the ionic strengths at which the enzymes elute from a DEAE Sepharose column (see section *2.2.3*) it is suggested that the enzyme previously isolated by our affinity chromatographic procedure (7) corresponds to malt carboxypeptidase I in MIKOLA's nomenclature whereas the enzyme described in the present paper corresponds to malt carboxypeptidase II. The latter assignment is supported by a K_m value for the hydrolysis of Z-Phe-Ala-OH at pH 5.2 of 0.53 mM which is in fair agreement with the 0.75 mM obtained by YABUUCHI et al. (34).

While serine carboxypeptidases from fungi consist of a single peptide chain (16, 18) most of the corresponding enzymes from higher plants appear to be dimers composed of monomers each of which contains two peptide chains linked by disulfide bridges (7, 10, 30). Thus, the monomer of malt carboxypeptidase I consists of an A-chain with molecular weight 32,000 and a B-chain with molecular weight 19,000. Malt carboxypeptidase II appears to be composed of two different monomers, one containing two peptide chains with molecular weights 34,000 (A-chain) and 27,000 (B-chain), respectively, the other containing two peptide chains with molecular weights 34,000 (A-chain) and 24,000 (C-chain), respectively. It is probable that the B- and C-chains contain the same peptide portion since their amino acid compositions are identical and they both are N-terminally blocked. The B-chain contains more glucosamine and neutral sugar than the C-chain and this accounts for their difference in molecular weights.

The specificities of malt carboxypeptidases I (7) and II towards the C-terminal amino acid residue of peptide substrates supplement each other: whereas both enzymes release hydrophobic amino acid residues only malt carboxypeptidase II releases Arg and Lys and only malt carboxypeptidase I releases Pro. This has some resemblance with the pancreatic metallo carboxypeptidases where carboxypeptidase A exhibits a strong preference for hydrophobic amino acid residues and does not release Arg and Lys while carboxypeptidase B primarily releases Arg and Lys and only very slowly hydrophobic amino acids (27). However, carboxypeptidase B (1) releases hydrophobic amino acids at very low rates compared to malt carboxypeptidase II and the analogy is thus not perfect. The specificity of malt carboxypeptidase II is more similar to that of the metallo carboxypeptidase from S. griseus which releases both basic and hydropho-

bic amino acids at high rates (5).

The serine carboxypeptidases from fungi are inhibited by low concentrations of both Hg^{++} and p-HMB due to modification of a sulfhydryl group (2, 6, 18). However, the corresponding enzymes from higher plants are generally inhibited only by Hg^{++} (7, 21, 22, 23) and in the case of malt carboxypeptidase I (7) it has been demonstrated that this inhibition presumably is not due to modification of its sulfhydryl group since it requires high concentrations of Hg^{++} and is dependent on deprotonation of an ionizable group with a pK_a of 6.7. The influence of Hg^{++} on malt carboxypeptidase II which contains no sulfhydryl group is essentially identical to that on malt carboxypeptidase I, suggesting that the site of reaction in both enzymes is another residue capable of binding this metal, e.g. His, Glu or Asp (32).

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