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ISOLATION OF A CARBOXYPEPTIDASE FROM MALTED BARLEY BY AFFINITY CHROMATOGRAPHY

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A carboxypeptidase was isolated from malted barley by affinity chromatography in a yield of approximately 30%. The specific activity was higher than previously obtained for similar enzymes. The enzyme was a dimer where each of the monomers consisted of two peptide chains linked by disulfide bridges. Each monomer contained a single sulfhydryl group, which was inaccessible to *p*-hydroxymercuribenzoate and ELLMAN's reagent when the enzyme was in its native state. The two peptide chains were separated and the N-terminal sequence of each of them determined. The enzyme contained 6 amino sugar residues and 8% neutral sugar. Isoelectric focusing indicated that the enzyme existed in two forms with pI of 5.65 and 5.73, respectively, but N-terminal sequence determination indicated that they had identical peptide chains. Diisopropyl phosphorofluoridate completely inhibited the enzyme while Hg^{++} only inhibited the enzyme after deprotonation of an ionizable group on the enzyme with a pK_a of approximately 6.7.

Abbreviations: BS = benzyl succinic acid; Bz = benzoyl; Caps = cyclohexylaminopropane sulfonic acid; CABS-Sepharose =[N-(ε-aminocaproyl)-*p*-aminobenzyl] succinyl-Sepharose 4B; EDTA = ethylenediamine tetraacetic acid, sodium salt; DFP = diisopropyl phosphorofluoridate; 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; PTH = phenylthiohydantoin; Tris = tris(hydroxy methyl)aminomethane; Z = carbobenzoxy.

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1. INTRODUCTION

Many plants and fungi have been demonstrated to contain carboxypeptidases which are inhibited by DFP (2, 11, 12, 20, 22, 24, 26, 27, 28, 30, 32, 38, 42, 43, 47). These enzymes were originally termed "acid carboxypeptidases" by ZUBER and MATILE (47) due to their high activity in the acidic pH range. However, HAYASHI et al. (19) reclassified this group of enzymes as "serine carboxypeptidases" (EC 3.4.16) since they do not employ the catalytic mechanism of the acidic proteinases, but rather the mechanism characteristic of the serine proteinases. This classification is also warranted by the fact that these carboxypeptidases, in contrast to the acidic proteinases, exhibit esterase and amidase activity (20).

Isolation procedures for the serine carboxypeptidases from plants are generally cumbersome and consequently these enzymes have only been characterized to a limited extent. Introduction of affinity chromatography might greatly facilitate their isolation. It was therefore of interest that a carboxypeptidase from malted barley was inhibited by benzylsuccinic acid (38). This compound has been used as ligand in affinity chromatography of the serine carboxypeptidase from baker's yeast (carboxypeptidase Y) (24) and three metallo-carboxypeptidases (3, 5, 37). In the present paper the same ligand is used for the isolation by affinity chromatography of one of the serine carboxypeptidases present in malt (32). The structural and enzymatic properties of this serine carboxypeptidase from a higher plant are compared with those of carboxypeptidase Y of fungal origin.

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 substrates were from Bachem, Switzerland. Chromatographic materials were products of Pharmacia Fine Chemicals, Sweden, and Ampholine was from LKB, Sweden. Mes, Hepes, Caps, and 2-amino-2-methyl-1,3-propanediol were purchased from Sigma, USA. [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. Carboxypeptidase Y was obtained as previously described (24).

CABS-Sepharose was prepared essentially as described by BAZZONE et al. (3). The only exception was that D,L-(*p*-aminobenzyl)succinic acid, obtained by catalytic hydrogenation of D,L-(*p*-nitrobenzyl)succinic acid, was coupled directly to the resin without prior crystallization as described in reference 24. 20 g of D,L-(*p*-nitrobenzyl)succinic acid was used for the preparation of 300 ml CABS-Sepharose.

FA-Phe-NH_2 and FA-Phe-Gly-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 4.5 ml water containing 4 mmol $NaHCO_3$ and 2 mmol L-phenylalanine amide hydrochloride or 2 mmol L-phenylalanine-glycine · H_2O. 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. The following melting points were determined FA-Phe-NH_2: 215-216 °C, FA-Phe-Gly-OH: 204-205 °C.

FA-Phe-Gly-NH_2 was prepared by enzymatic synthesis: 15 ml methanol containing 500 mg FA-Phe-OMe was slowly added to 50 ml aqueous solution of 0.5 M-Glycine amide hydrochloride, pH 9.5 containing 3 mg carboxypeptidase Y. The reaction was complete within 1 min and terminated by addition of 250 ml CH_3CN. HPLC analysis indicated that the reaction mixture consisted of 95% FA-Phe-Gly-NH_2 and 5% FA-Phe-OH. After evaporation of CH_3CN the pure compound was obtained by recrystallization from ethanol/water. Melting point: 201-202 °C.

Bz-Phe-OMe was prepared as previously described (7).

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 Z-Phe-Ala-OH spectrophotometrically at 230 nm using a Cary Model 219. The following assay (1 ml) mixture was used: 2 mM-Z-Phe-Ala-OH,

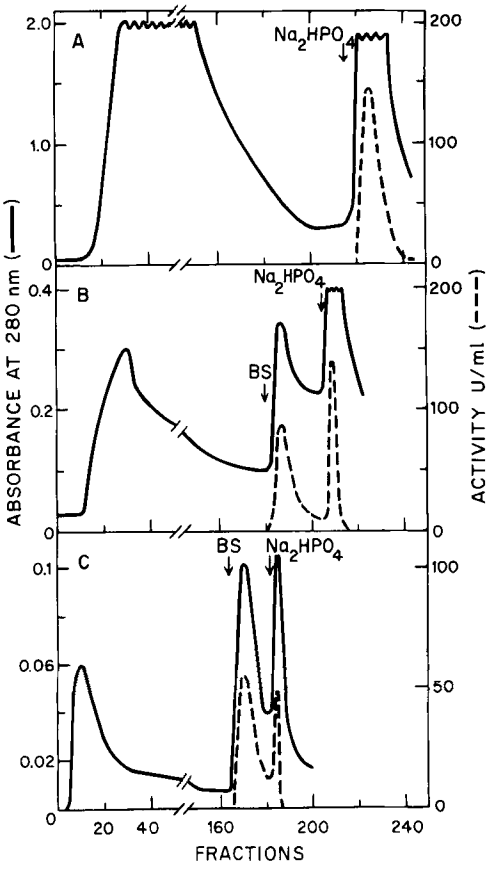

Figure 1. Purification of malt carboxypeptidase on CABS-Sepharose.

Panel A: The ammonium sulfate precipitate was dissolved in 0.05 M-NaH_2PO_4 pH 4.5 and the conductivity was adjusted to 35 · 10-3 S by addition of water. This sample was applied to the affinity column (10×14 cm = 1100 ml) which then was washed with approximately 5 l of 0.05 M-NaH_2PO_4, 0.1 M-NaCl, pH 4.5. The elution was performed with 0.05 M-Na_2HPO_4, pH 7.5. A flow rate of 800 ml · hour-1 was used.

Panel B: The eluate from Panel A was made 0.2 M-NaCl and pH was adjusted with 6 M-HCl to pH 4.5 and reapplied to the CABS-Sepharose column (5×17 cm = 325 ml). The column was washed with approximately 4.5 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, 1 mM-BS, pH 4.5 followed by 0.05 M-Na_2HPO_4, pH 7.5. A flow rate of 270 ml · hour -1 was used.

Panel C: The BS eluate from Panel B was diafiltered against 0.05 M-NaH_2PO_4, 0.1 M-NaCl, pH 4.5 as described in section *2.2.2.* to remove BS and then reapplied to a CABS-Sepharose column (5×8 cm = 156 ml). The column was washed with approximately 4.5 l 0.05 M-NaH_2PO_4, 0.1 M-NaCl, pH 4.5, eluted with 0.1 mM-BS, 0.05 M-NaH_2PO_4, 0.1 M-NaCl, pH 4.5 and subsequently with 0.05 M-NaH_2PO_4, pH 7.5. A flow rate of 270 ml · hour-1 was used. Throughout, fractions of 25-30 ml were collected.

0.05 M-Mes, 1 mM-EDTA, pH 4.75 (the established pH optimum for the purified enzyme). One unit was defined as the amount of enzyme necessary to release one micromole of alanine per min at 25 °C calculated on basis of $\Delta\varepsilon$=210.

Protein concentration was determined spectrophotometrically at 280 nm using $A_{280}^{1\%}$ = 19, 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

11 kg of malted Gula barley was ground and suspended in 110 l demineralized water. 44 g L-ascorbic acid was added and pH adjusted to 4.9 by addition of 6 M-HCl. The mixture was stirred for 2-3 hours at 18-22 °C, continuously controlling pH, and then allowed to stand for 16 hours at 2 °C to permit insoluble material to settle. The supernatant, approximately 80 l, was pumped off and concentrated by ultrafiltration with an ultrafiltration apparatus from the Danish Sugar Refineries equipped with a Model 600 membrane (cut-off: mol.wt. 20000). 230 g ammonium sulfate per liter concentrate was added (30% saturation) and the suspension allowed to stand for 2-3 hours at 20 °C. The resultant precipitate was removed by centrifugation and discarded. To the supernatant was added 130 g ammonium sulfate per liter (58% saturation). The resultant suspension was centrifuged after 16 hours at 4 °C yielding a precipitate which could be stored at 4 °C for at least 2 weeks without loss of activity. The further purification of the enzyme was performed in three affinity chromatographic steps utilizing CABS-Sepharose.

Step 1: The 58% ammonium sulfate precipitate was dissolved in approximately 800 ml 0.05 M-NaH_2PO_4, pH 4.5 and the conductivity was

adjusted to $35 \cdot 10^{-3}$ S by the addition of water. This sample was applied to the CABS-Sepharose affinity column (Figure 1A), equilibrated with 0.05 M-NaH_2PO_4, 0.1 M-NaCl, pH 4.5. All enzymatic activity was bound to the resin which was washed with approximately 5 l 0.05 M-NaH_2PO_4, 0.1 M-NaCl, pH 4.5 until A_{280} of the eluate was below 0.3. The enzyme was then eluted with 0.05 M-Na_2HPO_4, pH 7.5. The affinity resin was regenerated with 0.01 M-Tris, 3 M-NaCl, pH 8.5 and then reequilibrated with 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 the affinity column (Figure 1B) which then was washed with 0.05 M-NaH_2PO_4, 0.1 M-NaCl, pH 4.5 until A_{280} was below 0.1. Part of the bound enzyme was then eluted with the same buffer containing 1 mM of the inhibitor BS and residual enzyme bound to the column was eluted with 0.05 M-Na_2HPO_4, pH 7.5. Only the fraction eluted with BS was used in Step 3, while the fraction eluted by shifting pH to 7.5 could be reused in Step 2. The resin was regenerated as described in Step 1.

Step 3: The eluate from Step 2 was diafiltered against 0.05 M-NaH_2PO_4, 0.1 M-NaCl, pH 4.5, thereby removing BS. A Pellicon Cassette System from Millipore, equipped with a PTGC 00005 membrane (cut-off: mol.wt. 10000) was used for this purpose, and a theoretical 1000 times dilution of BS was obtained. The resultant fraction was reapplied to the CABS-Sepharose column (Figure 1C) which then was washed with 0.05 M-NaH_2PO_4, 0.1 M-NaCl, pH 4.5 until A_{280} was below 0.01. The enzyme was eluted with the same buffer containing 0.1 mM-BS and residual enzyme bound to the column was eluted with 0.05 M-NaH_2PO_4, pH 7.5. The purified enzyme was diafiltered against 0.1 M-NaCl, using the equipment previously described. The enzyme was then concentrated in an Amicon ultrafiltration cell equipped with a UM 10 membrane and kept frozen at -18 °C without loss of enzymatic activity.

2.2.3. Isoelectric focusing

Isoelectric focusing of 10 mg malt carboxypeptidase was performed in an LKB 8100 column (110 ml) using a 1.5% Ampholine gradient from pH 5 to pH 7. A sucrose gradient from zero to 50% was used to stabilize the pH gradient and the column was thermostatted at 4 °C. After 20 hours at 1600V, the content of the column was eluted in 1 ml fractions and the pH gradient determined. The absorbance at 280 nm and activity towards Z-Phe-Ala-OH was measured as described in section *2.2.1.*

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 (9). The analysis was performed at 25 °C in a sodium phosphate buffer, pH 6.9, $\mu = 0.2$, at 20,410 rev. per min. Equilibrium was reached after 6 hours. The partial specific volume, 0.721, was calculated from the amino acid composition (10) and the sugar content (17).

The molecular weight was also determined by chromatography on Sephadex G-150 Superfine (column: 1.5×90 cm), equilibrated with the buffer used in ultracentrifugation. The following "standards" were used: ribonuclease A (mol.wt. 13700), chymotrypsinogen (mol.wt. 25000), ovalbumin (mol.wt. 43000), bovine serum albumin (mol.wt. 67000) and aldolase (mol.wt. 158000).

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 (45) and by electrophoresis on Pharmacia gradient gels PAA 4/30, as recommended by the manufacturer, using 0.04 M-Tris, 0.02 M-sodium acetate, 0.02 M-EDTA, 0.2% SDS, pH 7.4 as electrophoresis buffer. The size of the peptide chains were estimated by polyacrylamide gel electrophoresis after reduction with dithiothreitol. All samples were boiled and run both before and 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, 72 and 96 hours (33). The evaporated hydrolysates were analyzed on a

Durrum D-500 amino acid analyzer. Tryptophan contents were estimated by the method of GOODWIN and MORTON (18). Half-cystine was determined as cysteic acid after performic acid oxidation (21). Free thiol groups were measured by means of ELLMAN's reagent in 5 M-guanidine hydrochloride at pH 8.0 (15). An approximate estimation of the carbohydrate content was obtained by means of the phenol-sulfuric acid method of DUBOIS (13), using glucose as standard. The content of hexosamine was determined on the amino acid analyzer after hydrolysis for 3, 6 and 9 hours in 6 M-HCl at 110 °C in vacuo. Glucosamine hydrochloride was used as a standard.

2.2.6. Separation of the A- and B-chain and determination of N-terminal sequences

Reduction and alkylation of malt carboxypeptidase was performed by a procedure analogous to the procedure of FRIEDMAN et al. (16). 6.5 mg malt carboxypeptidase was dissolved in 2 ml 0.1 M-Tris, 8 M-urea, 2 mM-EDTA, pH 7.5. The solution was bubbled with nitrogen for 5 min and 15 µl 2-mercaptoethanol was added. After 16 hours at 20 °C 20 µl 2-vinylpyridine was added. After bubbling with nitrogen for 2 min the reaction mixture was left at 20 °C for 2 hours. 0.5 ml glacial acetic acid was added and the alkylated peptides were separated from low molecular weight substances by chromatography on Sephadex G-25 equilibrated with 10% acetic acid and lyophilized. The peptide chains were separated from each other by chromatography on Sephadex G-100 (1.5×90 cm), equilibrated with 10% acetic acid.

N-terminal amino acid sequences were determined using a Beckman 890C liquid phase sequencer as previously described by JOHANSEN et al. (25). Identification of PTH-amino acids was made by HPLC as described by SVENDSEN et al. (41). Additional information was obtained by back hydrolysis to the free amino acids as described by MENDEZ and LAI (31).

2.2.7. Stability of malt carboxypeptidase

The stability of malt carboxypeptidase was investigated by incubating the enzyme (0.024 mg · ml^{-1}) at 25 °C in the following buffers containing 1 mM-EDTA: 0.1 M-Mes, pH 4.5 and 6.0; 0.1 M-Hepes, pH 7.5, 8.0 and 8.5. The activity (see section *2.2.1.*) was followed as a function of time. The incubation at pH 6.0 was also performed at 50 °C, 55 °C and 60 °C to investigate the heat stability of the enzyme.

2.2.8. Comparison of enzymatic properties of carboxypeptidase Y and malt carboxypeptidase

The pH profile for the hydrolysis of FA-Phe-OMe, FA-Phe-Gly-OH, FA-Phe-NH_2 and FA-Phe-Gly-NH_2 by malt carboxypeptidase and carboxypeptidase Y was investigated using 0.2 mM substrate. 25 µl of an 8 mM substrate solution in methanol was added to 965 µl buffer. After addition of 10 µl enzyme solution the decrease in the absorbance at 337 nm was recorded on a Cary 219 spectrophotometer. The following buffer systems were used: 50 mM-Mes, pH 4.0 - 6.5; 50 mM-Hepes, pH 7.0 - 8.0; 50 mM-2-amino-2-methyl-1,3-propanediol, pH 8.5 - 9.5; 50 mM-Caps, pH 10.0 - 10.5. The results were plotted as initial rates of hydrolysis.

A comparison of the specificity of malt carboxypeptidase and carboxypeptidase Y was investigated by determining 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 buffer. 10 µl enzyme solution was added and a decrease in the absorbance at 230 nm was recorded on a Cary 219 spectrophotometer. The reaction was performed in 0.1 M-Mes, 1 mM-EDTA, pH 4.7 for malt carboxypeptidase and pH 6.5 for carboxypeptidase Y. These pH values were the established pH optima for their hydrolysis of Z-Phe-Ala-OH (see section *2.2.1.* and ref. 24).

2.2.9. Inhibition studies

Modification of malt carboxypeptidase with DFP was performed in the following manner: 25 µl[^{3}H]DFP (1.2 mM in propylene glycol) was added to 1 ml 10 mM-Na_2HPO_4, pH 7.0, containing 0.25 mg enzyme, i.e. the concentration of enzyme was 5 µM and the concentration of reagent was 30 µM. The enzymatic activity, using the as-

say 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 10 mM-NaH_2PO_4, 0.1 M-NaCl, pH 6.0. The A- and B-chains were separated as described in section *2.2.6.*

The effect of Hg^{++} on the activity of malt carboxypeptidase was investigated by incubating the enzyme (0.05 mg · ml^{-1} = 1.0 μM) with 4.1 μM-$HgCl_2$ 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, 4.1 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.

3. RESULTS

3.1. Purification of malt carboxypeptidase

The crude preparation of malt carboxypeptidase, obtained as an ammonium sulfate precipitate, was purified by a three step affinity chromatographic procedure utilizing CABS-Sepharose. The result of the purification is listed in Table I. The overall yield of this chromatographic procedure was 31%, but recycling the phosphate eluates obtained in step 2 and 3 might increase the yield significantly.

The isolated enzyme was homogeneous by polyacrylamide gel electrophoresis at pH 4.3 according to the procedure of REISFELD et al. (39). The homogeneity was confirmed by the results of the N-terminal sequence determination (see section 3.2.).

3.2. Physical chemical characterization of malt carboxypeptidase

Incubation of malt carboxypeptidase at 25 °C in various buffers indicated that the enzyme was stable for 4 hours at pH values below 7.5 (Figure 2). At higher pH, the enzyme lost its activity relatively fast. The enzyme remained stable at pH 6.0 and 50 °C (Figure 3) while an increase in the temperature to 55 °C and 60 °C resulted in a rapid loss of activity.

The molecular weight was determined by sedimentation equilibrium centrifugation. The linear plot of the logaritm of the fringe displacement versus the square of the radial distance indicated that the enzyme was homogeneous and had a molecular weight of 97000.

Gelfiltration on Sephadex G-150 equilibrated with the same buffer as used in the sedimentation equilibrium experiment similarly indicated a molecular weight of approximately 100000.

SDS gel electrophoresis of malt carboxypeptidase in the presence of dithiothreitol revealed two bands with mobilities corresponding to mo-

Table I.
Purification of malt carboxypeptidase.

	ml	A_{280}	U/ml	U_{Total}	U/mg
Extract	80000	19	0.9	72000	0.09
After ultrafiltration	4200	75	15	63000	0.38
Supernatant after 38% ammonium sulfate	4400	48	12	53000	0.48
Dissolved ammonium sulfate precipitate	1600	41	32	51000	1.5
Step 1, phosphate eluate	590	3.7	78	46000	40
Step 2, BS eluate	550	0.276	47	26000	320
after diafiltration	480	0.247	58	28000	450
Step 3, BS eluate	400	0.075	40	16000	1000
after diafiltration	400	0.070	40	16000	1080

The extraction procedure was performed with 11 kg malt. In step 2 and 3 of the affinity chromatographic procedure elution with 0.05 M-Na_2HPO_4, pH 7.5 was performed after the elution with BS. This resulted in a fraction in step 2, containing 12000 U with a specific activity of 21 U/mg and in a fraction in step 3 containing 5000 U with a specific activity of 180 U/mg. The combined phosphate eluates, 17000 U, were recycled.

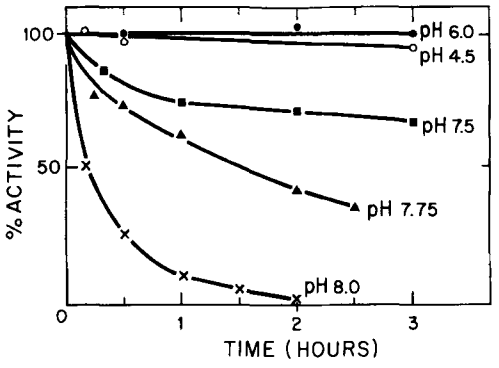

Figure 2. pH stability of malt carboxypeptidase.

The enzyme was incubated in buffers with different pH (see section *2.2.7.*). The enzymatic activity was followed with time (see section *2.2.1.*).

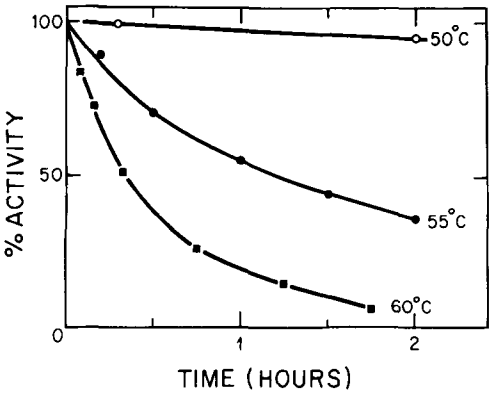

Figure 3. Temperature stability of malt carboxypeptidase.

Incubations were performed at the indicated temperatures in 0.05 M-Mes, 1 mM-EDTA, pH 6.0 using an enzyme concentration of 0.024 mg · ml^{-1}. The peptidase activity towards Z-Phe-Ala-OH was measured (see section *2.2.1.*).

lecular weights of 32000 and 19000, respectively. In the absence of dithiothreitol one major band (mol.wt. = 49000) and two minor bands (mol.wt. = 32000 and 19000) appeared. Gradient gel electrophoresis in the presence of SDS exhibited only a single band with a mobility corresponding to a molecular weight of 46000. These results indicated that the enzyme is a dimer where each monomer is composed of two chains with molecular weights 32000 (A-chain) and 19000 (B-chain), respectively, linked with disulfide bridges. The molecular weight of the monomer is therefore estimated to be 51000 and this number is used in all further characterization.

The disulfide bridges connecting the A- and B-chains of malt carboxypeptidase were cleaved by reduction and pyridylethylation of the enzyme (see section *2.2.6.*). SDS gel electrophoresis in the absence of reducing agents confirmed that the reaction had gone to completion since the band with mobility corresponding to a molecular weight of approximately 50000 had disappeared in favor of two bands corresponding to molecular weights of 32000 and 19000. The two chains were separated by chromatography on Sephadex G-100, the A-chain eluting slightly ahead of the B-chain (Figure 4). The fractions were pooled as indicated in the figure and subjected to N-terminal sequence determination (Table II). Each fraction contained only a single sequence and the question mark in position 4 of the B-chain probably represents a glycosylated amino acid residue. N-terminal sequence determination of the non-reduced enzyme resulted in equal amounts of the two sequences listed in Table II. No traces of other sequences were seen suggesting that the isolated enzyme was homogeneous.

The amino acid compositions of malt carboxypeptidase and its A- and B-chains are listed in

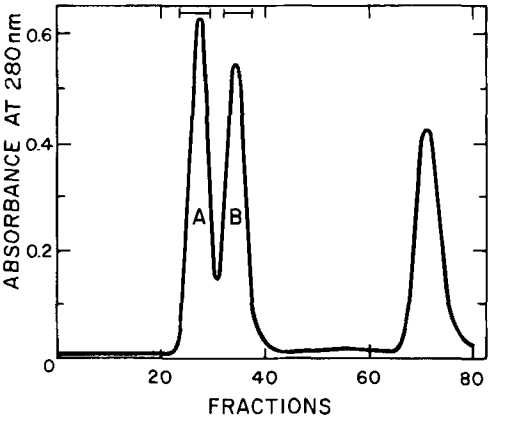

Figure 4. Separation of the A- and B-chain in malt carboxypeptidase after reduction and alkylation (see section *2.2.6.*).

The lyophilized reaction mixture was dissolved in 2 ml 10% acetic acid and applied to the Sephadex G-100 column (1.5×90 cm), equilibrated with 10% acetic acid. Fractions of 1.9 ml were collected using a flow rate of 20 ml · hour^{-1}. The third peak eluted consists of small molecular weight compounds, e.g. reagent.

Table II.
Amino terminal sequence of the separated peptide chains of malt carboxypeptidase.

A-chain (mol.wt. 32000):	Ala-Pro-Gln-Gly-Ala-Glu-Val-Thr-Gly-
B-chain (mol.wt. 19000):	Gly-Val-Pro-?-Met-Ser-Asp-Glu-Val-

Table III.
Amino acid composition of malt carboxypeptidase.

Amino acid	Residues per 51000	A-chain Residues per 32000	B-chain Residues per 19000
Asp	38,1	27.4	11.5
Thr[a]	23.1	13.0	10.5
Ser[a]	38.4	24.8	14.3
Glu	27,8	17.4	10.4
Pro	23.2	15.2	6.9
Gly	47.5	31.9	16.4
Ala	32.4	15.7	16.7
Val[b]	28.8	20.2	11.7
Met	5.5	2.7	3.1
Ile[b]	19.0	13.0	7.2
Leu	31.8	22.5	10.9
Tyr	27.6	17.9	9.6
Phe	20.5	14.3	6.9
His	16.1	9.3	6.4
Lys	18.6	12.3	6.4
Arg	6.1	2.8	3.7
Cys[c]	5.5	3.2	1.9
Trp[d]	~10	~4	~3
Glucosamin[a]	6	4	3
Neutral sugar[e]	8%	nd	nd

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 3, 6 and 8 hours of hydrolysis.

a) Extrapolated to zero time.

b) Value after 96 hours of hydrolysis.

c) Determined as cysteic acid after performic acid oxidaton.

d) Determined spectrophotometrically by the method of GOODWIN and MORTON (18).

e) Determined by the method of DUBOIS et al. (13). nd = Not determined for the individual chains due to high background sugar in the eluate from Sephadex G-100.

Table III. It is seen that the sum of the compositions of the A- and B-chain is in fair agreement with the composition of the whole enzyme. The enzyme also contains 6 residues of glucosamine and 8% neutral sugar. Treatment of the enzyme with ELLMAN's reagent in the presence of 5 M-guanidine hydrochloride resulted in an instantaneous modification of 1.0 sulfhydryl group per monomer, suggesting that each monomer contained two disulfide bridges and one free sulfhydryl group. This sulfhydryl group was apparently not accessible when the enzyme was in its native state since it did not react with *p*-HMB at pH 7.0. However, treatment with ELLMAN's reagent at pH 8.0 resulted in a slow reaction with approximately one sulfhydryl group, the rate being parallel with the rate of denaturation at this pH (see Figure 2), suggesting that the reaction is conditioned by a prior unfolding of the enzyme.

Isoelectric focusing of the enzyme preparation revealed two overlapping peaks, corresponding to isoelectric points of pH 5.65 and 5.73, respectively (Figure 5). Both components were enzymatically active towards Z-Phe-Ala-OH, although the more acidic component appeared to have slightly lower specific activity than the other component. N-terminal sequence determination of aliquots with pI of approximately 5.55 and 5.85, respectively, yielded in both cases results identical to the two sequences listed in Table II, suggesting that the apparent heterogeneity observed by isoelectric focusing was not due to different amino acid sequences in the two isolated enzyme forms.

3.3. Enzymatic properties of malt carboxypeptidase

The enzymatic characteristics of carboxypeptidase Y and malt carboxypeptidase were compared by studying the pH profiles of their peptidase, esterase, amidase and peptidyl amino acid amide hydrolase activities, using as substrates FA-Phe-Gly-OH, FA-Phe-OMe, FA-Phe-NH_2 and FA-Phe-Gly-NH_2 (6), respectively. Due to its lack of stability at higher pH, malt carboxypeptidase could only be studied in the pH range 4-7.5 (see section 3.2.), while carboxypeptidase

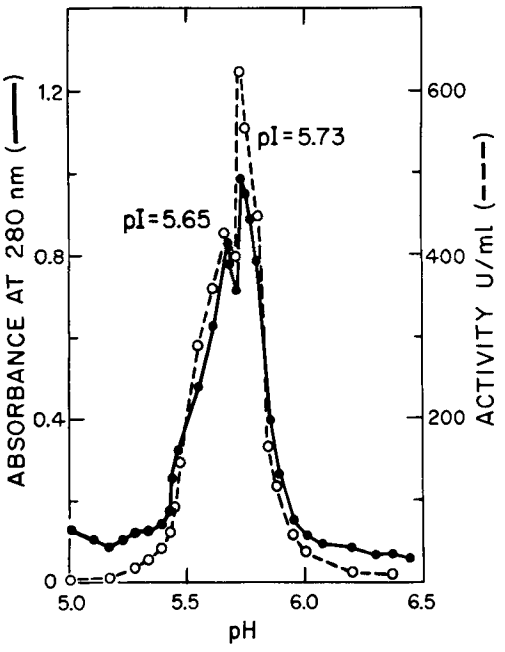

Figure 5. Isoelectric focusing of malt carboxypeptidase in an Ampholine gradient pH 5-7.
A_{280} (●); enzymatic activity (see section *2.2.1.*) (O).

Y could be studied in the pH range 4-10 (8). The stability of both enzymes within these ranges were sufficient for measurements of enzymatic activity, which in no cases lasted more than 10 min. All rates of hydrolysis, performed at a substrate concentration of 0.2 mM, were found to follow first order kinetics, and the pH profiles therefore reflected k_{cat}/K_m. It was observed that the peptidase (Figure 6A), peptidyl amino acid amide hydrolase (Figure 6C) and amidase (Figure 6D) activities of malt carboxypeptidase have pH optima which are significantly lower than the corresponding optima for carboxypeptidase Y. The pH profiles for the hydrolysis of FA-Phe-OMe, on the other hand, are very similar for the two enzymes (Figure 6B).

The specificity constants, k_{cat}/K_m, for the hydrolysis of N-blocked dipeptides with the general formula Z-Ala-X-OH (X = amino acid residue) indicated that both carboxypeptidase Y and malt carboxypeptidase hydrolyzed substrates with C-terminal hydrophobic amino acid residues much faster than the corresponding substrates with hydrophilic amino acids (Table IV). However, carboxypeptidase Y hydrolyzed substrates with C-terminal Arg and Lys whereas malt carboxypeptidase did not. On the other hand C-terminal glycine was released much faster by malt carboxypeptidase than by carboxypeptidase Y.

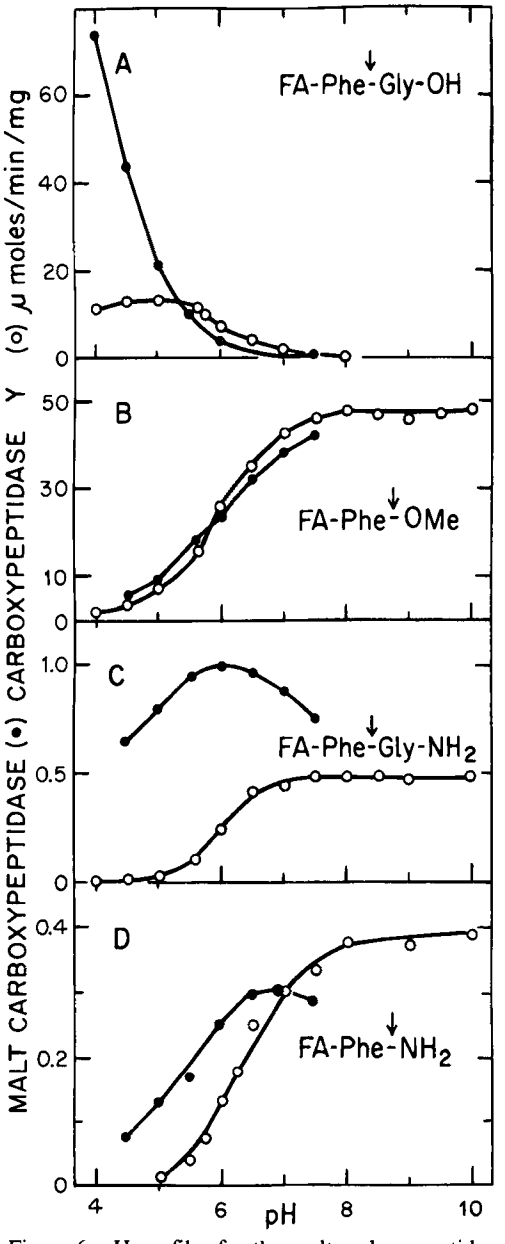

Figure 6. pH profiles for the malt carboxypeptidase (●) and carboxypeptidase Y (O) catalyzed hydrolysis of FA-Phe-Gly-OH (A), FA-Phe-OMe (B), FA-Phe-Gly-NH_2 (C) and FA-Phe-NH_2 (D).
The ordinate indicates activities in μmoles/min/mg.

The kinetic parameters of the malt carboxypeptidase catalyzed hydrolysis of Z-Phe-Ala-OH were determined at pH 5.2 in 50 mM-Mes, i.e. conditions identical to those employed by RAY (38) and VISURI et al. (43). From a Lineweaver-Burk plot, which did not deviate from linearity, a K_m of 1.9 mM and V_{max} of 1400 μmoles/min/mg was obtained. Using the molecular weight of the monomer, 51000, k_{cat} was estimated to be 71000 min^{-1}.

3.4. Inhibition studies

In an experiment using [^{3}H]DFP it was observed that the enzyme lost its activity towards Z-Phe-Ala-OH in an apparent first order reaction ($t_{1/2}$ = 35 min). After 4 hours there were less than 3% of the original peptidase activity left. Separation of the chains as described in section *2.2.6.* indicated that the radioactivity was only incorporated in the A-chain, suggesting that the molecule may contain two active sites, one in each subunit.

Table IV.
Hydrolysis of Z-Ala-X-OH substrates (X = amino acid residue) with malt carboxypeptidase and carboxypeptidase Y.

Substrate	k_{cat}/K_m ($min^{-1} \cdot mM^{-1}$)	
	Malt carboxypeptidase	Carboxypeptidase Y
Z-Ala-Gly-OH	480	<30
Z-Ala-Ala-OH	22000	13000
Z-Ala-Val-OH	35000	15000
Z-Ala-Ile-OH	22000	32000
Z-Ala-Met-OH	18000	56000
Z-Ala-Phe-OH	5700	19000
Z-Ala-Pro-OH	2600	370
Z-Ala-Asp-OH	370	<30
Z-Ala-Asn-OH	270	520
Z-Ala-Lys-OH	<30	520
Z-Ala-Arg-OH	<30	2000
Z-Ala-His-OH	120	190
Z-Ala-Ser-OH	1080	1700

Assay conditions: 0.5 mM substrate, 2.5% CH_3OH, 0.1 M-Mes, 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.

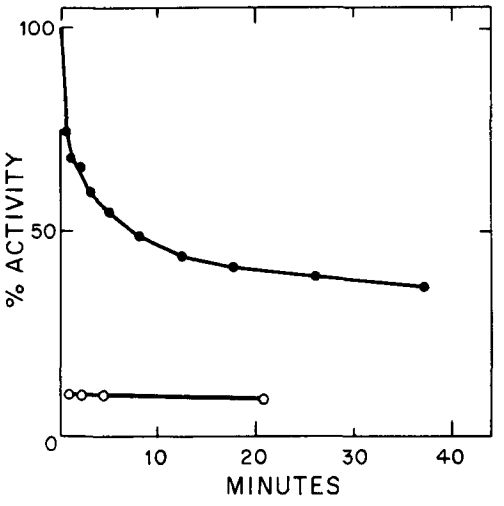

Figure 7. Reactivation of Hg^{++} inactivated malt carboxypeptidase by assay in the absence of added $HgCl_2$.

The enzyme was incubated at a concentration of 0.05 mg · ml^{-1} (1.0 μM) in 0.05 M-Hepes, pH 7.5 with 4.1 μM-$HgCl_2$. 10 μl aliquots were assayed against 0.3 mM-Bz-Phe-OMe, 0.05 M-Hepes, pH 7.5 (●). For comparison, the enzyme was also assayed in the presence of 4.1 μM-$HgCl_2$ (O). Activities are given as percentage of the activity of the enzyme before addition of $HgCl_2$ as measured by the assay containing no $HgCl_2$.

Treatment of malt carboxypeptidase with a fourfold excess of $HgCl_2$ at pH 7.5 resulted in an instantaneous loss of approximately 90% of its esterase activity when measured in the same buffer and in the presence of the same concentration of $HgCl_2$ as during the incubation (see section *2.2.10.*). No further loss of activity was observed within 20 min (Figure 7). Varying the concentration of Hg^{++}, a dissociation constant of the enzyme-Hg^{++} complex of 6.3 · 10^{-7} M at pH 7.5 was determined (Figure 8A). Using a fixed concentration of 4.1 μM $HgCl_2$, the inhibition was found to be dependent on the deprotonation of an ionizable group with an apparent pK_a of 6.7 (Figure 8B). Addition of both 100 μM KI and 4.1 μM-$HgCl_2$ to the cuvette at pH 7.5 prior to the addition of the enzyme apparently prevented the formation of the enzyme-Hg^{++} complex since no inactivation was observed. This suggested that Hg^{++} binds less tightly to the enzyme than to I^-. However, if the sequence was changed such that

the enzyme was mixed with $HgCl_2$ before KI was added, the enzyme exhibited 65% of the activity of the control, suggesting that the enzyme-Hg^{++} complex cannot be fully dissociated by I^- or, alternatively, that the enzyme had been partially denatured by the treatment. In another series of experiments, the enzyme (0.05 mg · ml^{-1}) was reacted with Hg^{++} (4.1 µM) at pH 7.5 in a test tube while its esterase activity was assayed after 100 fold dilution in the same buffer but without $HgCl_2$ added which should be sufficient to almost dissociate the enzyme-Hg^{++} complex since K_d is $6.3 \cdot 10^{-7}$ M. It was found that with increased reaction period, an increasing proportion of the enzyme was irreversibly inactivated (Figure 7), and addition of 100 µM-KI or 1 mM-mercaptoethanol, which had no adverse effect on the unmodified enzyme, was not capable of reactivating the enzyme.

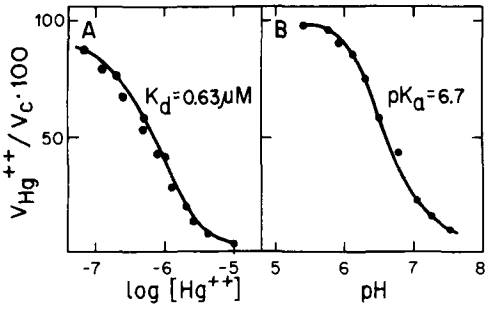

Figure 8A. Influence of the concentration of Hg^{++} in the assay mixture on the activity of malt carboxypeptidase.

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 8 µl of a solution of $HgCl_2$ and 10 µl enzyme (0.05 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.

Figure 8B. Influence of pH on the inhibition of malt carboxypeptidase by Hg^{++}.

The enzyme was assayed at various pH values, using 0.1 M-Hepes or Mes buffers, with no $HgCl_2$ added to the assay mixture (V_c) or with 4.1 µM-$HgCl_2$ added ($V_{Hg^{++}}$). Assay conditions were otherwise as described in Figure 8A.

4. DISCUSSION

Malt carboxypeptidase was extracted from ground malt using a previously published procedure (43) with the exception that acetate buffer was avoided. This was motivated by an observation of acetate being an inhibitor of malt carboxypeptidase which interferes with the ability of the enzyme to bind to the affinity resin. The ammonium sulfate fractionation of the crude extract increased the capacity of the CABS-Sepharose resin, and since the ammonium sulfate precipitate could be stored at 4 °C for at least two weeks without loss of activity, the purification could conveniently be stopped at this stage. The binding of malt carboxypeptidase to CABS-Sepharose required that the conductivity of the sample was at least 10^{-2} S. For the affinity chromatographic procedure to proceed in high yield, it was essential that the capacity of the resin was fully utilized, but not exceeded. In step 1, 2 and 3 the capacity was estimated to be 50, 150 and 170 U/ml resin, respectively. However, even under favorable conditions, complete elution of the enzyme required rather large volumes of elution buffer. Compared to previously published procedures for the isolation of carboxypeptidases from malt (2, 32, 38, 43), the present procedure is both faster and affords a higher yield. The enzyme appeared homogeneous by all criteria employed, except isoelectric focusing where at least two species were indicated. However, the N-terminal amino acid sequences were apparently identical regardless of pI, suggesting they were derived from a single protein molecule which existed in more than one ionic form. It has previously been shown that carboxypeptidase Y exists in multiple forms due to variations in the sugar content which affect both enzymatic activity and pI (24, 29), and it is conceivable that this also is the case for malt carboxypeptidase. Isolation of such enzymes which exhibit charge microheterogeneity is difficult by traditional means employing ion exchange chromatography, while the use of affinity chromatography, where the interaction between enzyme and chromatographic support largely depends on the properties of the active site, is especially advantageous in these cases.

It has been suggested that malted barley contains several different carboxypeptidases (2, 32,

38, 43, 46). The enzyme described in the present paper is probably identical to the enzyme which was partially purified by RAY (38), since it has practically identical K_m for Z-Phe-Ala-OH hydrolysis, pH optimum and isoelectric points. However, the amino acid compositions are different, probably due to the presence of impurities in RAY's enzyme preparation. His enzyme preparation had also lower specific activity towards Z-Phe-Ala-OH than the enzyme described in the present paper. The present enzyme is different with respect to the K_m value for the hydrolysis of Z-Phe-Ala-OH at pH 5.2 from the malt carboxypeptidases described by YABUUCHI et al. (46), who found two enzymes with K_m values 10 mM and 0.75 mM, respectively, and the malt carboxypeptidase of VISURI et al. (43) which had a K_m value of 6.7 mM. On the other hand the V_{max} obtained for the present enzyme (1400 μmoles/min/mg) agrees with the V_{max} described by VISURI et al. (1300 μmoles/min/mg). The observed differences could possibly be attributed to the use of different varieties of barley for the preparation of carboxypeptidases.

Molecular weights of malt carboxypeptidases in the range of 85-95000 have previously been reported (38, 43). This agrees reasonably well with the molecular weight obtained here, 102000, and it is interesting that all serine carboxypeptidases isolated from higher plants have molecular weights in this range (11, 12, 23, 26, 27, 28, 30, 42). The enzyme isolated from wheat bran contained two peptide chains with molecular weights of 35000 and 25000, respectively (42), and the enzyme isolated from rice bran contained two peptide chains with molecular weights 35000 and 21000, respectively (12). The results obtained with malt carboxypeptidase are very similar, i.e. 32000 and 19000, respectively, suggesting that all of these carboxypeptidases are dimers with the monomer composed of two peptide chains linked by disulfide bridges. This structure may well be shared by other serine carboxypeptidases characterized by molecular weights of approximately 100000 which have not been investigated in similar details (11, 14, 28, 30, 34, 35). In contrast to this, fungal enzymes like carboxypeptidase Y, penicillocarboxypeptidase S-1 and S-2, and carboxypeptidase III from Aspergillus oryzae are monomers with a single peptide chain and molecular weights from 45000 to 65000 (22, 24, 36) and thus clearly distinct from the carboxypeptidases found in higher plants.

It is characteristic for the fungal serine carboxypeptidases that they are inhibited by both Hg^{++} and *p*-HMB in stoichiometric amounts. The serine carboxypeptidases isolated from higher plants generally are not inhibited by *p*-HMB (11, 12, 26, 28, 30, 42), but some of these enzymes have been reported to be inhibited by Hg^{++} (26, 28, 30). Since malt carboxypeptidase was shown to possess a single sulfhydryl group per monomer which was inaccessible to *p*-HMB but reacted with Hg^{++}, this reaction was investigated in details. It was found that deprotonation of an ionizable group with a pK_a of 6.7 determines the reaction with Hg^{++}, which renders the enzyme inactive. Since the reaction is pH dependent and not stoichiometric it is different from the reaction between the free sulfhydryl group in carboxypeptidase Y and Hg^{++} (1,8). It is possible that the site of reaction is not the sulfhydryl group of malt carboxypeptidase but another residue at the active site which is capable of binding this metal, e.g. His, Glu or Asp (44). The formation of such a mercury complex may denature the enzyme and result in a secondary reaction of Hg^{++} with the sulfhydryl group, which has become accessible due to a conformational change induced by the initial formation of the enzyme-Hg^{++} complex. The differences between the reactions of Hg^{++} with carboxypeptidase Y and malt carboxypeptidase thus reflect differences in the accessibility of their free sulfhydryl group.

In spite of the structural differences between malt carboxypeptidase and carboxypeptidase Y, the catalytic properties of the two enzymes are similar. In addition to their carboxypeptidase activity they both exhibit esterase, amidase and peptidyl amino acid amide hydrolase activities. However, the pH profiles for each activity is slightly different between the two enzymes with the exception of esterase activity. This indicates that the ionizable group(s) on which the activities depend have different pK_a in the two enzymes.

At present it is not known whether malt carboxypeptidase is related to other serine carboxypeptidases in any other way than by its catalytic mechanism. Further studies will be required to

establish whether these enzymes of widely different source are genetically related or alternatively, whether their similarity in catalytic mechanisms is the result of convergent evolution.

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