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ISOLATION OF CARBOXYPEPTIDASE III FROM MALTED BARLEY BY AFFINITY CHROMATOGRAPHY

by

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A novel serine carboxypeptidase has been isolated from malted barley by affinity chromatography. The enzyme has been termed malt carboxypeptidase III in correspondence with the nomenclature suggested by MIKOLA to distinguish it from the previously isolated carboxypeptidases I and II. Malt carboxypeptidase III is a monomer with a molecular weight around 48,000, composed of a single peptide chain, two glucosamine residues and 2% neutral sugar. The peptide chain is N-terminally blocked and it contains a single unblocked cysteinyl residue.

Like other serine carboxypeptidases malt carboxypeptidase III exhibits peptidase activity with an acidic pH optimum. However, unlike other such enzymes, the pH optimum for the esterase activity is also acidic. The kinetic analysis showed that this probably is due to deprotonation of an ionizable group with a pK_a around 7 which controls the conformation of the active site. The transition of the active acidic form into the inactive basic form is reversible but time dependent.

Malt carboxypeptidase III exhibits a preference for substrates with Phe, Met, Ile and Val in the P'_1 position and Phe, Leu, Met and Ala in the P_1 position. The enzyme is inhibited by diisopropyl phosphorofluoridate, mercuric ions and various organomercuric compounds.

1. INTRODUCTION

Resting barley grains contain large amounts of storage proteins which are degraded during germination by proteolytic enzymes induced at this stage (11). The serine carboxypeptidases play an important role in this process and MIKOLA (17, 18, 19) has presented evidence for the presence in malt of five such enzymes with complementary specificites. In this laboratory two of these have been isolated by affinity chromatography and their enzymatic properties and sequences have been determined (2, 3, 4, 6, 7, 23, 24). The isolation and characterization of CPD-M_{III} is reported here.

Abbreviations: BS = benzyl succinic acid; Bz = benzoyl; Bzl = benzyl; CABS-Sepharose = [N-(ε-aminocaproyl)-p-aminobenzyl] succinyl-Sepharose 4B; CPD-M_{III} = malt carboxypeptidase III; DFP = diisopropyl phosphorofluoridate; E = enzyme; EDTA = ethylenediamine tetraacetic acid, sodium salt; Et-Hg-Cl = ethylmercuric chloride; FA = furylacryloyl; GYBS-Sepharose = [(N-glycyl-L-tyrosine)-p-azo-benzyl] succinyl-Sepharose 4B; Me-Hg-I = methylmercuric iodide; Mes = 2-(N-morpholino)ethane sulfonic acid; Ph-Hg-Cl = phenylmercuric chloride; p-HMB = parahydroxymercuribenzoate; SDS = sodium dodecyl sulfate, Z = carbobenzoxy. Other abbreviations of amino acids, amino acid derivatives and peptides are according to the guidelines of the IUPAC-IUB Commission on Biochemical Nomenclature. The binding site notation for the enzyme is that of SCHECHTER and BERGER (21). Accordingly, the binding site for the C-terminal amino acid residue of the substrate is denoted S'_1 and those for the amino acid residues in the amino-terminal direction away from the scissile bond are denoted S_1, S_2....., S_i. The substrate positions are denoted P'_1, P_1, P_2,.... P_i in correspondence with the binding sites.

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2. MATERIALS AND METHODS

2.1. Materials

Malted Grit barley was obtained from United Breweries, Denmark, and D,L-benzylsuccinic acid from Burdick and Jackson, USA. All Z-Ala-X-OH and Bz-Y-OMe substrates were from Bachem, Switzerland. Sepharose 4B was from Pharmacia Fine Chemicals, Sweden and Me-Hg-I was from Koch-Light Laboratories, England. Mes and Hepes were 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.

The following materials were prepared as previously described: CABS-Sepharose (2), GYBS-Sepharose (16), FA-Phe-Ala-OH (5), FA-Ala-Phe-OH (26) and FA-Ala-OBzl (5).

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-Ala-Phe-OH spectrophotometrically at 337 nm using a Cary Model 219. The following assay mixture was used: 25 µl 8 mM substrate dissolved in methanol was added to 965 µl 0.1 M-Mes, 1 mM-EDTA, pH 4.7 (the established pH optimum of the purified enzyme), followed by 10 µl enzyme solution. When the pH of the enzyme solution exceeded 5.5 it was necessary to add the 10 µl enzyme solution to the assay buffer and wait at least three minutes for the enzyme to equilibrate before addition of the 25 µl substrate. 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 $A_{280}(1\%) = 20$, 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 CPD-M_{III}

25 kg of malted Grit barley was ground and suspended in 200 l demineralized water and pH was adjusted to 3.7 by slow addition of approximately 4 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 140 l, was concentrated approximately 15 fold by ultrafiltration, and 230 g ammonium sulfate per liter concentrate was added (38% saturation). After 16 hours at 4 °C the precipitate was removed by centrifugation and to the supernatant was added 190 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 three steps using ion exchange and affinity chromatography.

Step 1: The 65% ammonium sulfate precipitate was dissolved in 6 l water, concentrated to 1 l using ultrafiltration equipment from Millipore and then dialysed against 20 mM-NaH_2PO_4, pH 6.7. This sample was applied to a DE-52 cellulose column (10×34 cm) and washed with 20 mM-NaH_2PO_4, pH 6.7 as described by MIKOLA (17, 18, 19). CPD-M_{III} eluted in the breakthrough peak whereas essentially all of carboxypeptidases I and II were bound to the column.

Step 2: The breakthrough peak from Step 1 was adjusted to 0.05 M-NaH_2PO_4, 0.2 M-NaCl, pH 4.4 by addition of solid NaH_2PO_4 and NaCl, followed by adjustment of pH by addition of 1 M-HCl. This sample was applied to a GYBS-Sepharose column (5×10 cm), equilibrated with 0.05 M-NaH_2PO_4, 0.1 M-NaCl, pH 4.4. The column was washed with approximately 4 l 0.05 M-NaH_2PO_4, 0.5 M-NaCl, pH 4.4 until A_{280} of the eluate was below 0.05. The enzyme was eluted with 0.05 M-NaH_2PO_4, 0.1 M-NaCl, pH 6.4.

Step 3: The eluate from Step 2 was adjusted to pH 4.4 by addition of 1 M-HCl and applied to a GYBS-Sepharose column (2.6×3.5 cm), equilibrated with 0.05 M-NaH_2PO_4, 0.1 M-NaCl, pH 4.4. The column was washed with approximately 1.5 l of the same buffer until A_{280} was below 0.01. The elution was accomplished with 5 mM-BS, 0.05 NaH_2PO_4, 0.1 M-NaCl, pH 4.4. The active fractions with A_{280} above 0.04 were

concentrated by ultrafiltration using equipment and a YM 10 membrane from Amicon. After dialysis against 0.02 M-sodium acetate, 0.2 M-NaCl, pH 4.3 the enzyme was frozen at -18 °C.

2.2.3. Homogeneity and molecular weight

The homogeneity and molecular weight of denatured CPD-M_{III}, with and without reduction with dithiothreitol, was determined by SDS gel electrophoresis in 1 mm thick 7.5% slab gels with the SDS-phosphate continuous buffer system (25). A Pharmacia gel electrophoresis apparatus GE-2/4 LS was used. The molecular weight of the native enzyme was determined by HPLC gel filtration, using a Protein Pak 300 SW column, equilibrated with 50 mM-NaH_2PO_4, pH 7, and other equipment from Waters Associates.

2.2.4. Chemical composition

Samples were hydrolyzed in 6 M-HCl at 110 °C in vacuo for 24, 48 and 72 hours. The evaporated hydrolysates were analyzed on a Durrum D-500 amino acid analyzer. Tryptophan contents were estimated by the method of GOODWIN and MORTON (12). Half-cystine was determined as cysteic acid after performic acid oxidation (14). Free thiol groups were determined by reaction with p-HMB in 10 mM-NaH_2PO_4 pH 7.0 (20).

An approximate estimation of the carbohydrate content was obtained by means of the phenol-sulfuric acid method of DUBOIS et al. (10), 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 standard. N-terminal amino acid sequence determination was attempted using a Beckman 890 C liquid phase sequencer.

2.2.5. Stability of CPD-M_{III}

The stability of CPD-M_{III} was investigated by incubating the enzyme (0.04 mg · ml^{-1}) at 25 °C in the following buffers containing 0.1 M-NaCl and 1 mM-EDTA: 0.05 M-sodium formate, pH 2.5 and 3.0; 0.05 M-sodium acetate, pH 3.6 and 4.5; 0.05 M-Mes, pH 5.0 and 6.7; 0.05 M-Hepes, pH 7.5, 8.0 and 8.6. The activity towards FA-Ala-Phe-OH (see section *2.2.1*) was followed as a function of time.

2.2.6. Inhibition studies

Modification of CPD-M_{III} with DFP was performed in the following manner: 5 µl(^{3}H) DFP (1.2 mM in propylene glycol) was added to 240 µl 50 mM-Na_2HPO_4, 0.2 M-NaCl, pH 7.0, containing 0.20 mg enzyme · ml^{-1}, i.e. the concentration of enzyme was 4.2 µM and the concentration of reagent was 24 µM. The enzymatic activity, using the assay described in section *2.2.1*, was followed as a function of time.

The influence of mercurials on the activity of CPD-M_{III} was studied in the following way: 50 µl enzyme (0.43 mg/ml) was added to 50 µl 0.2 M-Mes, pH 6.0 followed by 5 µl of either $HgCl_2$, Ph-Hg-Cl, Et-Hg-Cl or Me-Hg-I (0.025-0.2 mM). The solutions of Ph-Hg-Cl, Et-Hg-Cl and Me-Hg-I were diluted in water from a 10 mM solution in dimethylformamide. After incubation for 15 minutes the enzyme was assayed in the following manner: 10 µl enzyme was added to 965 µl 0.1 M-Mes, pH 6.0, and after incubation for 5 minutes, 25 µl 8 mM substrate (FA-Ala-OBzl) in methanol was added.

2.2.7. Enzymatic properties of CPD-M_{III}

The specificity of CPD-M_{III} was investigated by determination of k_{cat}/K_m values for the hydrolysis of a series of Z-Ala-X-OH and Bz-Y-OMe substrates (X and Y = amino acid residues). 10 µl enzyme solution was added to 965 µl 0.1 M-Mes, 0.1 M-NaCl, 1 mM-EDTA, pH 4.7 for the Z-Ala-X-OH substrates and 0.1 M-Mes, 0.1 M-NaCl, 1 mM-EDTA, pH 5.8 for the Bz-Y-OMe substrates. 25 µl of a 20 mM substrate solution in methanol or water was added and, after mixing, the hydrolysis was followed spectrophotometrically to completion on a Cary 219 spectrophotometer at 25 °C as a decrease in absorbance at 230 nm for the Z-Ala-X-OH substrates and as an increase in absorbance at 254 nm for the Bz-Y-OMe substrates. The k_{cat}/K_m values were determined from the integrated form of the Michaelis-Menten equation (22).

Table I. Purification of CPD-M_{III}

			FA-Ala-Phe-OH			FA-Phe-Ala-OH			FA-Ala-Phe-OH
	ml	mg/ml	U/ml	U_{TOTAL}	U/mg	U/ml	U_{TOTAL}	U/mg	FA-Phe-Ala-OH
Concentrated extract	7500	42	2.8	21000	0.033	7.7	58000	0.092	0.36
Sample applied to DE 52	2500	23	6.3	16000	0.13	14	35000	0.30	0.45
Breakthrough fraction from DE 52	1800	8	0.57	1000	0.035	0.47	850	0.029	1.2
Eluate from first affinity column	250	0.17	2.0	500	12	0.61	150	3.6	3.3
Concentrated and dialysed eluate from second affinity column	9	0.27	13	117	48	2.3	21	8.5	5.7

The k_{cat} and K_m values for the hydrolysis of FA-Ala-Phe-OH and FA-Ala-OBzl were determined, from Lineweaver-Burk plots, as a function of pH using the following buffers containing 0.2 M-NaCl and 1 mM-EDTA: 0.1 M-sodium formate, pH 3.5-4.5; 0.1 M-Mes, pH 4.5-6.9; 0.1 M-Hepes, pH 7-7.5. The rates of hydrolysis were determined spectrophotometrically at 329-337 nm at 25 °C.

3. RESULTS

3.1. Purification of CPD-M_{III}

No specific assay for CPD-M_{III} has been found; all substrates of this enzyme are also hydrolyzed by carboxypeptidases I and II. However, CPD-M_{III} is characterized by a FA-Ala-Phe-OH/FA-Phe-Ala-OH ratio of 5.7 whereas it is only 0.07 for carboxypeptidase I and 0.3 for carboxypeptidase II. This ratio was consequently utilized to monitor the purification of CPD-M_{III}. The ammonium sulfate precipitate is characterised by a FA-Ala-Phe-OH/FA-Phe-Ala-OH of 0.5 (Table I), but the breakthrough fraction from the ion exchange chromatography on DE-Cellulose of the dialysed crude ammonium sulfate precipitate is characterized by a ratio of 1.2, indicating that this chromatographic step to some extent has separated CPD-M_{III} from carboxypeptidases I and II in accordance with the observations by MIKOLA (17, 18, 19). A further increase in this ratio was obtained by the two step affinity chromatographic procedure utilizing GYBS-Sepharose (Table I). The elution in the first step was accomplished by a pH change and in the second step by including the inhibitor BS in the washing buffer.

3.2. Physical-chemical characterization of CPD-M_{III}

SDS gel electrophoresis of CPD-M_{III} in the presence and in the absence of dithiothreitol revealed a single band with a mobility corresponding to a molecular weight of 48,000, indicating that the enzyme is a single peptide chain. The HPLC gel filtration also indicated that the enzyme had a molecular weight of approximately 48,000. The enzyme did not react with Edman's reagent, suggesting that it is N-terminally blocked. The amino acid composition of CPD-M_{III} is listed in Table II. It contains a single free sulfhydryl group, as determined with p-HMB, as well as two residues of glucosamine and 2% neutral sugar.

CPD-M_{III} was stable at 25 °C for four hours at pH values between 3.0 and 7.5. At pH 2.5, 8.0 and 8.6 the enzyme lost its activity with $t_{1/2}$ values of 4, 31 and 3 minutes, respectively.

Table II. Amino acid composition of CPD-M_{III}

Amino acid	Residues per 48,000
Asp	52.0
Thr[a)]	19.2
Ser[a)]	27.4
Glu	37.6
Pro	17.7
Gly	39.4
Ala	33.0
Val[b)]	25.8
Met	12.0
Ile[b)]	22.9
Leu	33.4
Phe	19.6
Tyr	29.4
His	12.7
Lys	23.9
Arg	17.1
Cys[c)]	9
Trp[d)]	8
Glucosamine[a)]	2
Neutral sugar[e)]	2%

The experimental values were based on the average of two amino acid analysis after hydrolysis in 6 M-HCl for 24, 48, and 72 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 (12). [e)] Determination by the method of DUBOIS et al. (10).

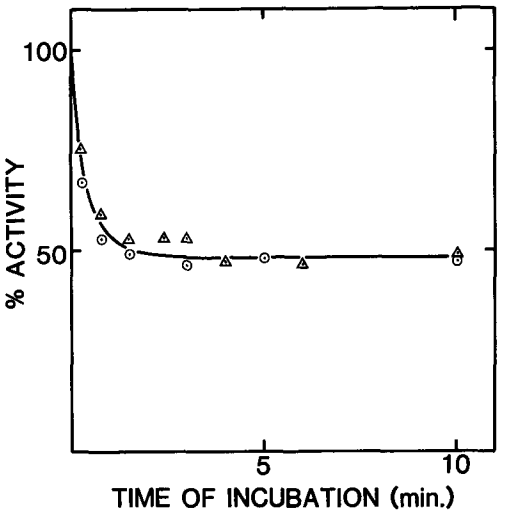

Figure 1. Time dependent change in activity of CPD-M_{III} after shift in pH. The assays were performed under the following conditions: 10 µl CPD-M_{III} (in 0.1 M-Mes 0.2 M-NaCl, 1 mM-EDTA, pH 4.5) was added to a cuvette containing 965 µl 0.1 M-Mes, 0.2 M-NaCl, 1 mM-EDTA, pH 6.5. At the time indicated 25 µl 8 mM FA-Ala-Phe-OH (⊙) or 25 µl 0.8 mM FA-Ala-OBzl (△) was added and the rate of hydrolysis determined spectrophotometrically (see section 2.2).

3.3. Enzymatic properties of CPD-M_{III}

The routine assay of CPD-M_{III} towards FA-Ala-Phe-OH, performed at pH 4.7 (see section 2.2), exhibited abnormal kinetics when the enzyme added was preincubated at pH values above 5.5: a concave progression curve was observed during the first few minutes, indicating that the enzyme was activated during the assay. Conversely, when the enzyme was preincubated at pH 4.7 no such activation was observed. This suggested that the enzyme at pH values above pH 5.5 exists in a lesser active form and that the transition to the more active form at lower pH values is time dependent. To test this hypothesis the reverse transition was performed: the enzyme, preincubated at pH 4.5, was added to a pH 6.5 assay buffer and after various times of incubation the substrate, FA-Ala-OBzl or FA-Ala-Phe-OH, was added (see Figure 1). During the first 2 minutes of incubation the initial rate of hydrolysis of both substrates decreased to approximately 50%. Longer incubation times had no further effects. All assays were consequently performed after preincubation for three minutes to allow the enzyme to enter the form characteristic of the conditions of the assay.

The pH dependence of the peptidase and esterase activities of CPD-M_{III} was investigated with the substrates FA-Ala-Phe-OH and FA-Ala-OBzl, respectively (Figure 2). For both substrates k_{cat} increased with pH and reached a maximum at pH 5.5 – 6.0 and again declined above pH 6. The pH dependence of K_m for the two substrates differed slightly: K_m for the hydrolysis of FA-Ala-Phe-OH increased slightly in the pH range 3.3 – 5.5 and sharply above pH 5.5, whereas K_m for the hydrolysis of FA-Ala-OBzl was constant in

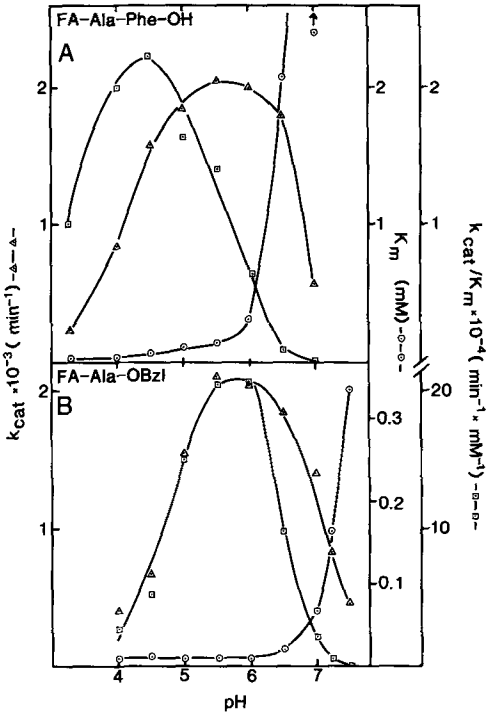

Figure 2. Influence of pH on the kinetic parameters for the CPD-M_{III} catalyzed hydrolysis of FA-Ala-Phe-OH (A) and FA-Ala-OBzl (B). The assays were performed in 0.1 M buffer, 0.1 M-NaCl, 1 mM-EDTA. The mixture contained 2.5% CH_3OH. The buffers employed are listed in section 2.2.

Table III. Hydrolysis of Z-Ala-X-OH and Bz-Y-OMe substrates (X and Y = amino acid residue) with serine carboxypeptidases

Substrate	k_{cat}/K_m (min^{-1} · mM^{-1}) CPD-M_I	CPD-M_{II}	CPD-M_{III}	CPD-Y
Z-Ala-Gly-OH	480	11	11	<30
Z-Ala-Ala-OH	22000	560	2100	13000
Z-Ala-Val-OH	35000	1800	17000	15000
Z-Ala-Ile-OH	22000	3300	27000	32000
Z-Ala-Met-OH	18000	3200	43000	56000
Z-Ala-Phe-OH	5700	2100	94000	19000
Z-Ala-Pro-OH	2600	6	120	370
Z-Ala-Asp-OH	370	71	<1	<30
Z-Ala-Asn-OH	270	62	<1	520
Z-Ala-Lys-OH	<5	25000	7	520
Z-Ala-Arg-OH	<5	18000	9	2000
Z-Ala-His-OH	120	1100	1	190
Z-Ala-Ser-OH	1080	150	5	1700
Bz-Gly-OMe	<5	<5	<1	3
Bz-Ala-OMe	370	19	200	490
Bz-Val-OMe	22	7	35	180
Bz-Ile-OMe	17	8	34	260
Bz-Leu-OMe	420	310	820	5600
Bz-Met-OMe	1200	160	750	2800
Bz-Phe-OMe	14600	5700	1000	51000
Bz-Asp-OMe	<5	<5	2	<1
Bz-Lys-OMe	18300	2200	3	1
Bz-Arg-OMe	25300	950	18	12
Bz-His-OMe	5600	76	23	13
Bz-Thr-OMe	69	<5	5	18
Bz-Pro-OMe	<5	<5	<1	<1

Assay conditions: 0.5 mM substrate, 2.5% CH_3OH, 0.1 M-Mes, 1 mM-EDTA pH 4.7 for Z-substrates and pH 5.8 for Bz-substrates. The rate of hydrolysis was measured spectrophotometrically at 230 nm for Z-substrates and 254 nm for Bz-substrates. The reaction was fol- lowed to more than 95% completion. From the progres- sion curves k_{cat}/K_m values were determined utilizing the integrated form of the Michaelis-Menten equation. The values for malt carboxypeptidase III are based on a molecular weight of 48.000. The values for carboxypeptidases I, II and Y are from refs. 1, 2, 3, 6 and 7.

the pH range 4 – 6 and increased sharply above pH 6.5. This different effect of pH on K_m with the two substrates explain why k_{cat}/K_m for the hydrolysis of FA-Ala-Phe-OH is maximal at pH 4.5 while for FA-Ala-OBzl it is maximal at pH 5.8. High K_m values precluded determinations at pH values above 7.0 for FA-Ala-Phe-OH and 7.5 for FA-Ala-OBzl.

The substrate preference of CPD-M_{III} with respect to the P_1' position of peptide substrates was determined at pH 4.7, using a series of Z-Ala-X-OH substrates and that with respect to the P_1 position of ester substrates was determined using a series of Bz-Y-OMe substrates at pH 5.8. The results are listed in Table III and compared with the corresponding data for malt carboxypeptidases I and II and carboxypeptidase Y. Listing the results with CPD-M_{III} in decreasing order of k_{cat}/K_m the following influence of residue X was observed: Phe > Met > Ile > Val >> Ala >> Pro >> Gly, Ser, Arg, Lys > Asn, Asp, His and of residue Y: Phe, Leu, Met > Ala > Val, Ile, His, Arg > Thr, Lys, Asp > Gly, Pro.

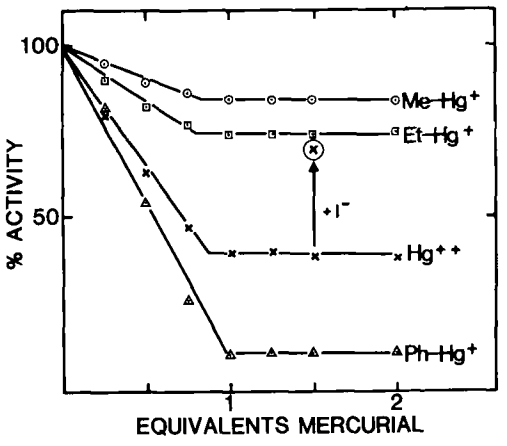

Figure 3. Inhibition of CPD-M_{III} with mercurials. The enzyme was incubated 15 minutes at 25 °C with the amount of mercurial indicated and then assayed with FA-Ala-OBzl as substrate (see section 2.2).

The activity of CPD-M_{III} towards FA-Ala-Phe-OH was completely abolished after 3 hours reaction with DFP. CPD-M_{III} was inhibited by various mercurials (Figure 3). In all cases the activity towards FA-Ala-OBzl decreased linearly with increasing concentrations of mercurials until maximal inactivation was reached at approximately equimolar concentrations. The residual activity with Me-Hg^+, Et-Hg^+, Hg^{++} and Ph-Hg^+ as reagents was 74%, 64%, 39% and 11% of the control, respectively. Inclusion of 10^{-4} M-KI in the assay medium increased the level of residual activity of the Hg^{++} modified enzyme from 33% to 70%, but had no influence on the activities of the enzymes modified with Me-Hg^+, Et-Hg^+ and Ph-Hg^+.

4. DISCUSSION

The purification procedure for CPD-M_{III} resembles the earlier described procedures for malt carboxypeptidases I and II (2, 6). It was found convenient, although not strictly necessary, to separate CPD-M_{III} from the bulk of carboxypeptidases I and II by ion exchange chromatography as previously described (17, 18, 19) before the affinity chromatographic purification. CPD-M_{III} exhibited very little affinity for the CABS-Sepharose resin, previously utilized for isolation of carboxypeptidases I and II (2, 6). However, it exhibited high affinity for GYBS-Sepharose, in contrast to carboxypeptidase I, permitting the isolation of CPD-M_{III} from the break-through fraction of the ion exchange column. Both GYBS-Sepharose and CABS-Sepharose contain the BS ligand but on different spacer arms.

CPD-M_{III} is inhibited by DFP and thus, belongs to the class of serine carboxypeptidases like the two other carboxypeptidases from malt (2, 6). However, CPD-M_{III} is a monomeric enzyme, consisting of a single peptide chain while malt carboxypeptidases I and II are dimeric with each monomer consisting of two peptide chains, linked by disulfide bridges (2, 6, 23, 24). Furthermore, CPD-M_{III} contains far less carbohydrate than the other two carboxypeptidases.

The esterase and peptidase activities of CPD-M_{III} are both dependent on the deprotonation of a group with a pK_a around 4.5 and this value is normal within the group of serine carboxypeptidases. However, in the basic pH range the enzyme differs from the other enzymes within the group. CPD-M_{III} exists in an active acidic form and an inactive basic form, controlled by the ionic state of a group with a pK_a around 7. The transition between the two forms is time dependent, suggesting the involvement of a conformational change. The serine carboxypeptidases previously described also hydrolyse peptides with low rates at basic pH due to poor binding of such substrates (8). This has been attributed to deprotonation of a positively charged group which at acidic pH functions as binding site for the C-terminal carboxylate group of peptide substrates. However, these enzymes retain the activity towards substrates with blocked C-terminus, i.e. peptide esters and peptide amides, and thus, these enzymes do not enter an inactive conformation at basic pH (8).

In chymotrypsin the activity varies similarly to that of CPD-M_{III}: the positively charged α-ammonium group of Ile-16 forms an ion pair with the carboxylate group of Asp-194, maintaining the enzyme in an active conformation (13). At pH > 8.5 the ammonium group is deprotonated and the enzyme instantaneously enters an inactive conformation.

The specificities of malt carboxypeptidases I, II and III supplement each other. With respect to the C-terminal position carboxypeptidase I exhibits a preference for amino acid residues with uncharged aliphatic side-chains (2), carboxypeptidase II prefers amino acid residues with positively charged side-chains (6), and CPD-M_{III} prefers amino acid residues with aromatic side-chains. With respect to the penultimate position, carboxypeptidases I and II exhibit preferences for amino acid residues with positively charged and aromatic side-chains (3, 7) while CPD-M_{III} prefers amino acid residues with aromatic and uncharged aliphatic side-chains without branched β-carbon. The specificity of CPD-M_{III} is similar to that of carboxypeptidase Y with the exception that carboxypeptidase Y exhibits a much more pronounced preference for aromatic residues at the penultimate position (1) than does CPD-M_{III}.

CPD-M_{III} contains an unblocked -SH group which may be modified with one equivalent of Hg^{++}, Ph-Hg^{+}, Me-Hg^{+}, or Et-Hg^{+} with the result that the enzyme is partially inactivated. Malt carboxypeptidase II contains no unblocked -SH group (6) and the one in carboxypeptidase I is not reactive towards mercurials (2). However, the -SH group of CPD-M_{III} exhibits a reactivity similar to that of carboxypeptidase Y (Cys-341) (1) and that of the carboxypeptidase from Penicillium janthinellum (15). As in the case of carboxypeptidase Y the extent of inactivation of CPD-M_{III} correlates with the bulkiness of the mercurial but in contrast to carboxypeptidase Y no correlation with the bulkiness of the substrate has been observed (results not shown). Thus, it is not possible to assign the unblocked cysteinyl residue of CPD-M_{III} to the substrate binding site. The influence of I^- on the activity of the enzyme modified with Hg^{++} is consistent with iodide forming a complex with the enzyme-bound mercuric ion according to the following equilibrium: E-Hg^{+} + $I^- \rightleftharpoons$ E-Hg-I. Similar effects of I^- and other halides were also observed in the case of Hg^{++} modified carboxypeptidase Y (1).

Chemical modifications of Cys 341 of carboxypeptidase Y (1, 9) and its exchange with other amino acid residues by site-directed mutagenesis (26) reduce the activity to approximately 20%. These results, combined with those on CPD-M_{III}, suggest that the -SH group located at the active site of some serine carboxypeptidases has a function although it is not essential to catalysis.

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