Journal of Chromatography, 370 (1986) 315–326
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 18 991

PURIFICATION AND SOME PROPERTIES OF THREE SERINE CARBOXYPEPTIDASES FROM *ASPERGILLUS NIGER*

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(First received June 27th, 1986; revised manuscript received July 29th, 1986)

SUMMARY

Three enzymes exhibiting peptidyl-L-amino acid hydrolase and esterase activities have been purified by immobilized metal-ion affinity chromatography and ion-exchange chromatography. The three enzymes were entirely free of the acid protease activity that normally exists along with them in the crude culture filtrates of *Aspergillus niger*. Although all three *exo*-peptidases possessed nearly identical molecular weights (*ca.* 140 000), isoelectric points (*ca.* 5.0) and other properties, their affinities for the two substrates tested, carbobenzoxy-L-Glu-L-Tyr and benzoyl L-arginine ethyl ester, differed. All three peptidases were inhibited by phenylmethanesulphonyl fluoride, indicating that they are serine carboxypeptidases. They were also inhibited by tosyl phenylalanine chloromethyl ketone, suggesting the presence of a histidyl residue in their active sites. The differences in the number of accessible histidyl residues on the enzyme surfaces could explain the differences in their retentions on Cu^{2+}–iminodiacetate–Sepharose 6B.

INTRODUCTION

Many carboxypeptidases have already been isolated from plants[1–6] and microorganisms, mostly fungi[7–16], and Zuber and Matile[17] have termed them acid carboxypeptidases owing to their maximal activity in the acidic region of the pH scale. However, Hayashi and Bai[18] preferred to call them serine carboxypeptidases owing to the presence of a reactive serine residue in their active sites as evidenced by their inhibition in the presence of phenylmethanesulphonyl fluoride (PMSF) and diisopropyl phosphofluoridate (DFP).

Many *Aspergilli* are prolific producers of serine carboxypeptidases. In fact, these enzymes are secreted into the culture medium as proteolytic complexes and very often both *endo-* and *exo*-peptidase activities are present. Such cases have been reported by Ichishima[19] and Panneerselvam and Dhar[15,20] for *Aspergillus saitoi* and *Aspergillus fumigatus*, respectively. The initial aim of our work was to find a method that facilitates the separation of *endo-* and *exo*-peptidase activities from the crude extracts of *Aspergillus niger* culture media. The use of immobilized metal-ion affinity

0021-9673/86/$03.50

chromatography (IMAC) on Cu^{2+}–iminodiacetate (IDA)–Sepharose 6B, not only helped us to achieve this[21] but also to isolate and study three iso-carboxypeptidases, referred to as carboxypeptidases I, II and III, by using special elution conditions.

The enzymes were characterized using classical techniques such as sodium dodecyl sulphate polyacrylamide gel ectrophoresis (SDS-PAGE) after a final ion-exchange chromatographic step.

EXPERIMENTAL

Chemicals

The crude culture filtrate of *Aspergillus niger* was a generous gift from Drs. Chow Ching Cheng and J. M. Lebeault of the Division des Procédés Biotechnologiques of the Université de Technologie de Compiègne.

The carboxypeptidase substrates carbobenzoxy-L-Glu-L-Tyr (CGT) and benzoyl L-arginine ethyl ester (BAEE), iminodiacetic acid (IDA; disodium salt), phenylmethanesulphonyl fluoride (PMSF) and tosylphenylalanine chloromethyl ketone (TPCK) were obtained from Sigma (St. Louis, MO, U.S.A.), casein from Merck (Darmstadt, F.R.G.), ethylenediaminetetraacetate (EDTA; sodium salt) and $CuSO_4 \cdot 5H_2O$ from Prolabo (Paris, France) and Sephadex G-50 and Sepharose 6B from Pharmacia (Uppsala, Sweden).

Methods

Determination of protein concentration. The routine determination of protein concentration was carried out by measuring the absorption at 280 nm using a Jobin Yvon Model JY201 spectrophotometer.

Carboxypeptidase assay. The peptidyl-L-amino acid hydrolase activity of the extract was assayed using 10 m*M* CGT by high-performance liquid chromatography (HPLC) as described[22]. One unit of peptidyl-L-amino acid hydrolase activity is defined as the amount of enzyme required to release 1 μM of L-tyrosine from the substrate at 30°C and pH 2.7.

The esterase activity of the enzyme was assayed spectrophotometrically using the method described by Schwert and Takaneka[23] by measuring the increase in absorbance at 260 nm accompanying the hydrolysis of 10 m*M* BAEE in 50 m*M* acetate buffer (pH 5.0). One unit of enzyme activity is the amount of enzyme needed to hydrolyse 1 μM of BAEE at 30°C and pH 5.0.

Acid protease assay. The acid protease activity in each step of the purification was determined using 2% casein as described[21]. One unit of enzyme activity is the amount of enzyme required to liberate 1 μg of L-tyrosine per minute at 39°C and pH 2.7. However, it should be noted that the action of the carboxypeptidases on the peptides released from the casein by the endopeptidase action of the acid protease is not taken into account. For both carboxypeptidase and acid protease, the specific activity is defined as the number of units of activity per unit absorbance of the enzymes at 280 nm.

Preparation of affinity sorbents. The affinity sorbent, Cu^{2+}–IDA–Sepharose 6B, was prepared according to ref. 24. Iminodiacetate, the metal-chelating agent, was fixed after epoxy activation of Sepharose 6B. The IDA–Sepharose 6B obtained was equilibrated and prepared with 50 m*M* $CuSO_4$ as described elsewhere[21].

Purification procedures. Purification was effected as in ref. 21, with certain modifications.

Ammonium sulphate precipitation. The culture medium was filtered and subjected to ammonium sulphate precipitation using 90% saturation.

Desalting and affinity chromatography. Unlike in ref. 21, a single step was employed to simultaneously desalt and separate *endo-* and *exo*-peptidase activities using a Sephadex G-50 column (45 × 3.2 cm I.D.) coupled to a Cu^{2+}–IDA–Sepharose 6B column (20 × 2.5 cm I.D.) in tandem. In a typical experiment, 30 ml of the ammonium sulphate extract containing 1740 absorbance units (280 nm) were injected into the Sephadex G-50 column and then washed with 50 m*M* acetate starting buffer (pH 5.3). The flow-rate was maintained at 112 ml/h (13.9 cm/h for the desalting column and 22.8 cm/h for the affinity column) and 10-ml fractions were collected. The first peak (excluded) that appears at the outlet of the desalting column passed directly into the Cu^{2+} column. The Sephadex column was then disconnected and both columns were washed separately with the starting buffer. When the flow-through peak from the Cu^{2+}–IDA–Sepharose 6B column was complete, the starting buffer was replaced with the first elution buffer, 5 m*M* Gly–HCl (pH 3.0). After the appearance of the first eluted peak a second elution buffer, 100 m*M* Gly–HCl (pH 3.0), was used to continue the elution.

Ion-exchange chromatography. The three active peaks obtained after IMAC were subjected to anion-exchange chromatography on DE-52 cellulose (Whatman) for further purification. The gel was conditioned according to the manufacturer's instructions. The elution conditions are described in the legends to the figures.

The active fractions were pooled, concentrated and used for the characterization.

Partial characterization of the carboxypeptidases

Electrophoretic studies. The homogeneity and the molecular weights of the three enzymes were determined by SDS-PAGE (7%), conventional PAGE as described[25] and gradient gel electrophoresis using Pharmacia PAA 4/30 gradient gels. In all instances, thyroglobulin (MW 669 000), ferritin (440 000), catalase (232 000), lactate dehydrogenase (140 000) and bovine serum albumin (67 000) from the Pharmacia electrophoresis calibration kit were used as standards. The bands were revealed using Coomassie Brilliant Blue as described[25].

Isoelectric focusing was carried out in the pH range 3.5–9.5 using Ampholine Pagplate (LKB, Bromma, Sweden). Standards were from the Pharmacia calibration kit, ranging from p*I* 3.5 to 9.3. The bands were revealed by silver staining according to Merril *et al.*[26].

Kinetic and other studies. The Michaelis constants (K_m) of the enzymes for the two substrates, BAEE and CGT, were determined using Lineweaver–Burk double reciprocal plots.

The effect of inhibitors on the enzymes was studied using 2 m*M* and 100 μM solutions of PMSF and TPCK, respectively. Other characteristics such as pH and temperature optima for activity, the stability of the enzymes at different pHs and temperatures were also determined. Table III gives the reaction conditions.

TABLE I

PURIFICATION OF THE THREE SERINE CARBOXYPEPTIDASES BY TANDEM DESALTING–METAL CHELATE AFFINITY CHROMATOGRAPHY ON SEPHADEX G-50–Cu^{2+}–IDA-SEPHAROSE 6B

Extract	*Protein (mg)*	*Activity (U)*	*Specific activity (U/mg)*	*Purification factor*	*Yield (%)*
Crude extract	6930	660	0.095	1	100
0–90% $(NH_4)_2SO_4$ extract	3132	662	0.211	2.2	100
After tandem desalting–affinity chromatography	I, 173; II, 20.5; III, 80	483 99 354	2.79 4.54 4.42	29 47 46	73 15 53
After ion-exchange chromatography	I, 39; II, 11.3; III, 28	426 98 319	10.9 8.7 11.4	115 92 120	64 15 48

RESULTS

Purification of the three serine carboxypeptidases

The purification of the three serine carboxypeptidases by ammonium sulphate precipitation, tandem desalting–affinity chromatography and anion-exchange chromatography is summarized in Table I. Instead of fractional precipitation as in ref.

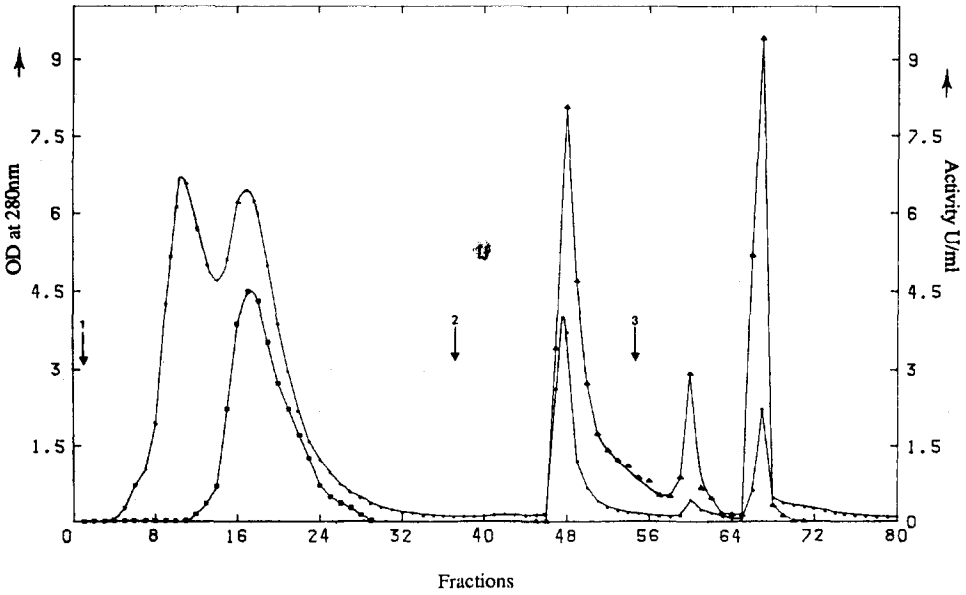

Fig. 1. Chromatography of the three carboxypeptidases and the acid protease by tandem desalting–metal chelate affinity chromatography on Sephadex G-50–Cu^{2+}–IDA–Sepharose 6B. ●, Absorption at 280 nm; ■, caseinolytic activity of the acid protease; ▲, esterase activity. Other details as in the text. Buffers: 1, 50 m*M* acetate (pH 5.3); 2, 5 m*M* Gly–HCl (pH 3.0); 3, 100 m*M* Gly–HCl (pH 3.0).

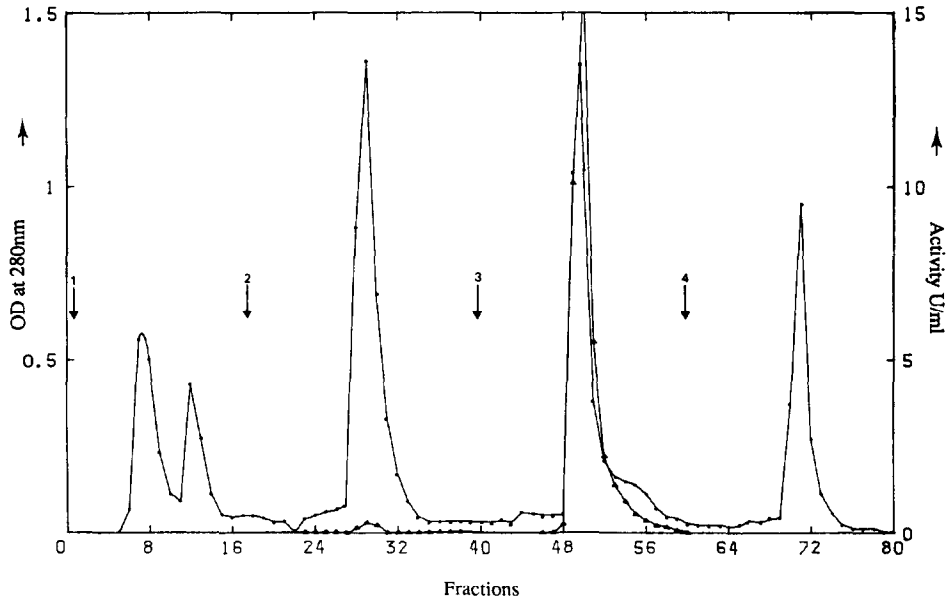

Fig. 2. Ion-exchange chromatography of carboxypeptidase I on DE-52 cellulose. Column: 16 × 2.5 cm I.D. ●, Absorption at 280 nm; ▲, esterase activity. Buffers: 1, 50 m*M* citrate (pH 5.9) (starting); 2, starting + 100 m*M* NaCl; 3, starting + 200 m*M* NaCl; 4, starting + 500 m*M* NaCl. Flow-rate, 90 ml/h (18.3 cm/h); fraction volume, 6 ml per tube.

21, a total precipitation using 90% saturation in ammonium sulphate was used. The desalting step was carried out simultaneously with the affinity step using a tandem system. Fig. 1 shows the efficiency of the affinity sorbent Cu^{2+}–IDA–Sepharose 6B for separating *endo-* and *exo*-peptidases. A stepwise gradient using Gly–HCl (pH 3.0) eluted the three enzymes in three peaks. Anion-exchange chromatography on DE-52 cellulose further purified the three carboxypeptidases (Figs. 2–4). Only a partial purification of the acid protease, by weak affinity or size-exclusion effects, was achieved (Table II and Fig. 1). The purities of the carboxypeptidases increased considerably after tandem desalting–affinity chromatography: carboxypeptidase I was 29-fold pure and II and III were each (46-fold pure). Pure extracts were obtained after the ion-exchange step. The protease obtained after IMAC, however, was only 7-fold pure. In all instances, high yields, sometimes exceeding 100%, were achieved.

Characteristics of carboxypeptidases I, II and III

Molecular weight and homogeneity. The three carboxypeptidases were found to be homogeneous electrophoretically in both the presence and the absence of SDS. The samples used were those obtained after anion-exchange chromatography. Fig. 5 shows a plot of log(molecular weight) *versus* distance from the cathode made after gradient-gel electrophoresis for the three enzymes. As is evident, all three enzymes seem to have molecular weights neighbouring that of lactate dehydrogenase (140 000). The molecular weights of the three enzymes were calculated to be carboxypeptidase I 141 000, carboxypeptidase II 135 000 and carboxypeptidase III 132 000 (Table III).

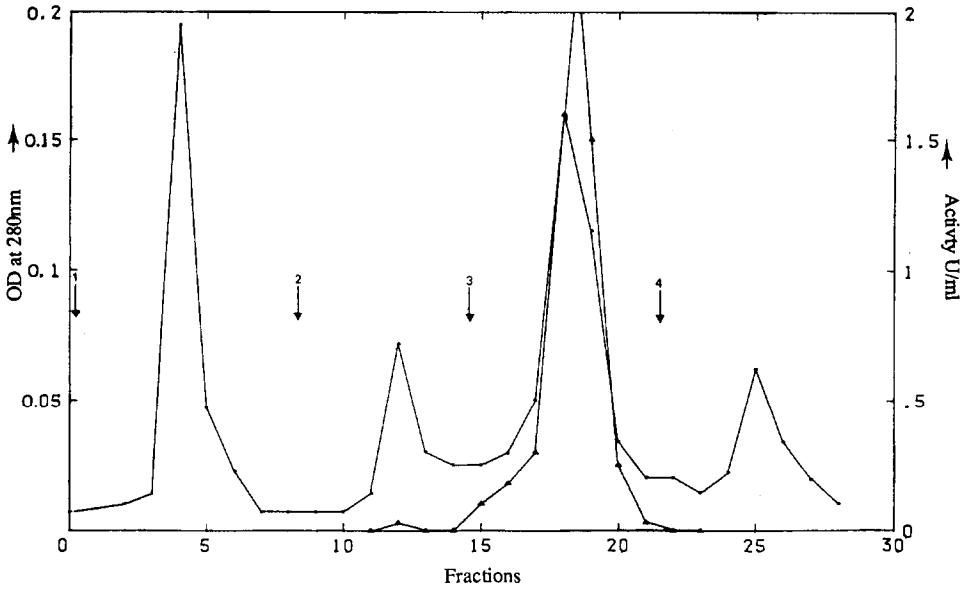

Fig. 3. Ion-exchange chromatography of carboxypeptidase II on DE-52 cellulose. Column: 7.7 × 1 cm I.D. Flow-rate, 14.1 ml/h; fraction volume, 1.7 ml per tube. Other details as in Fig. 2.

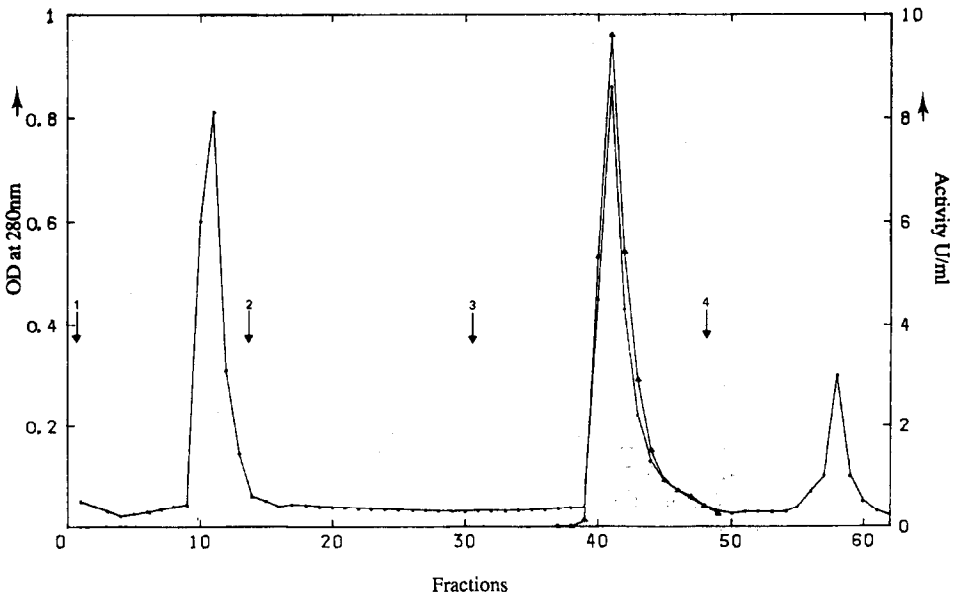

Fig. 4. Ion-exchange chromatography of carboxypeptidase III on DE-52 cellulose. Fraction volume, 4 ml per tube. Other details as in Fig. 2.

TABLE II

PURIFICATION OF THE ACID PROTEASE BY TANDEM DESALTING–WEAK AFFINITY CHROMATOGRAPHY ON SEPHADEX G-50–Cu^{2+}–IDA-SEPHAROSE 6B

Extract	*Protein (mg)*	*Activity (U/ml)*	*Specific activity (U/mg)*	*Purification factor*	*Yield (%)*
Crude extract	6930	620 840	90	1	100
0–90% $(NH_4)_2SO_4$ extract	3132	545 076	174	1.9	88
After tandem desalting–weak affinity chromatography	864	516 366	597	6.6	83

Isoelectric points. Isoelectric focusing enabled us to determine approximately the isoelectric points of the three serine enzymes. Fig. 6 shows a plot of pH *versus* distance from the cathode for the calibration proteins. The p*I* values of the three enzymes fit very close to one another on the curve. They all possess isoelectric points neighbouring pH 5.0 (Table III).

Kinetic studies. The Michaelis constants (K_m) and V_{max} values of the enzymes I, II and III determined using Lineweaver–Burk double-reciprocal plots are represented in Table IV together with the conditions of the reactions. The enzymes are distinct from one another in their affinities for the two substrates tested, namely BAEE and CGT (data given for CGT only).

Inhibition of the three carboxypeptidases by PMSF was also studied to determine conclusively the existence of a serine residue in their active sites. The effect of

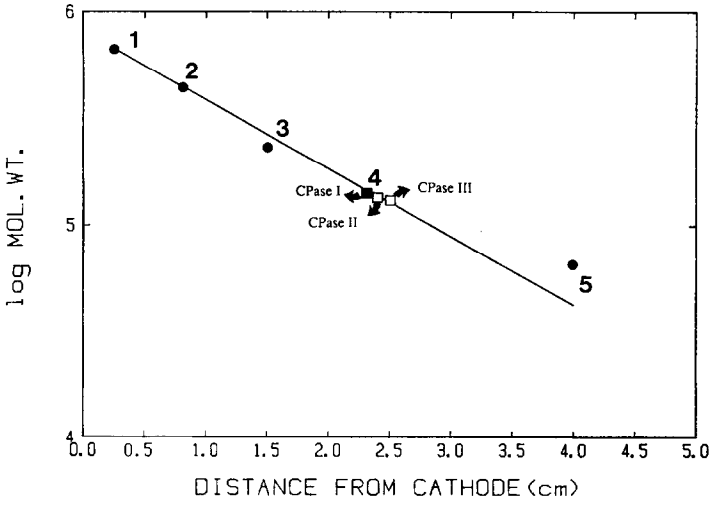

Fig. 5. Log(molecular weight) *vs.* distance from the cathode for the three carboxypeptidases and the calibration proteins. 1, Thyroglobulin (MW 669 000); 2, ferritin (440 000); 3, catalase (232 000); 4, lactate dehydrogenase (140 000); 5, bovine serum albumin (67 000).

TABLE III

GENERAL SUMMARY OF SOME OF THE PROPERTIES OF THE THREE CARBOXYPEPTIDASES

Enzyme concentrations used: I, $1.435 \cdot 10^{-3}$ mg; II, $2.5 \cdot 10^{-3}$ mg; III, $5.07 \cdot 10^{-3}$ mg.

Property	*Method*	*I*	*II*	*III*	*Conditions*
Homogeneity and molecular weight	SDS-PAGE (7%)	Homogeneous	Homogeneous	Homogeneous	As in ref. 25
	Gradient-gel electrophoresis	141 000	135 000	131 000	As recommended by Pharmacia
Isoelectric point	Isoelectric focusing	5.1	4.9	4.9	As recommended by Pharmacia in their IEF p*I* calibration kit instruction manual
Effect of inhibitors:					
2 m*M* PMSF	Kinetically	47% residual activity after 2 h	56% residual activity after 2 h	71% residual activity after 17.2 h	Assayed for esterase activity Acetate buffer 50 m*M* (pH 5.0) at 30°C
100 μM TPCK	Kinetically	88% residual activity after 7 h	No residual activity after 7 h	79% residual activity after 7 h	
Optimum temperature (°C)	CGTase assay	50	50	50	At pH 2.7
	Esterase assay	50	50	50	At pH 5.0
Optimum pH	CGTase assay	3.4	3.4	3.4	At 30°C
	Esterase assay	5.0	5.0	5.0	At 30°C

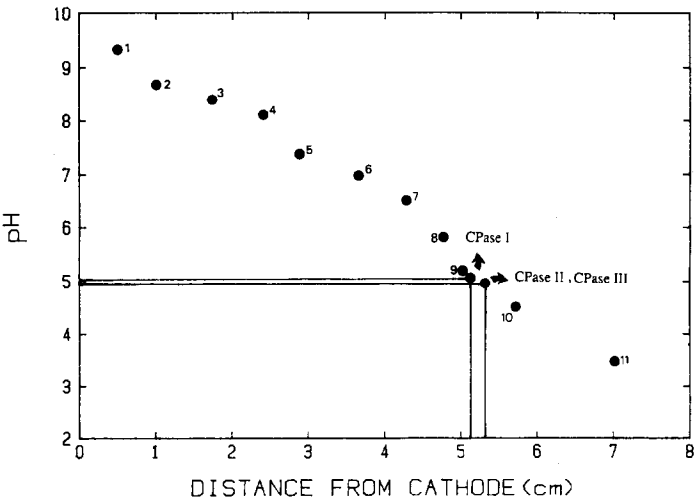

Fig. 6. pH *vs.* distance from the cathode for the three carboxypeptidases and the calibration proteins. 1, Trypsinogen (pH 9.3); 2, lentil lectin (8.65); 3, lentil lectin (8.45); 4, lentil lectin (8.15); 5, myoglobin (7.35); 6, myoglobin (6.85); 7, human carbonic anhydrase (6.55); 8, bovine carbonic anhydrase β (5.85); 9, β-lactoglobulin A (5.2); 10, soyabean trypsin inhibitor (4.55); 11, amyloglucosidase (3.5).

TABLE IV

KINETIC CONSTANTS OF THE THREE ENZYMES DETERMINED FOR CGT

Enzyme	K_m *(M)*	V_{max} *(min^{-1})*	*Conditions*
I	$1.9 \cdot 10^{-3}$	10 000	50 m*M* lactate
II	$4.9 \cdot 10^{-3}$	5128	buffer (pH 2.7) at
III	$6.1 \cdot 10^{-3}$	5000	30°C

the inhibitor on the enzymes is shown in the Fig. 7 whereas carboxypeptidases I and II were strongly inhibited by PMSF, only limited inhibition of the third enzyme was observed. At each time interval inhibitor-free standards were used to compare with the inhibited enzyme sample so as not to neglect any possible thermal inactivation that could have occurred, especially with carboxypeptidase III, which was incubated in the presence of the inhibitor for 17 h at 30°C. The effect of another inhibitor TPCK was also studied (Table III).

Other parameters such as the effect of temperature and pH on enzyme activity and the stabilities of the enzymes at different pHs and temperatures are also represented in Table III, the enzymes exhibited maximal activity on both BAEE and CGT at 50°C. Maximal esterase activity was at pH 5.0 and peptidyl-*L*-amino acid hydrolase activity at pH 3.4. The enzymes remained stable after incubation for 2 h at pH 7.0 and 30°C; at pH 5.0 and 60°C the residual activity was zero. There was, relatively little activity loss at pH 5.0 and 50°C after 2 h.

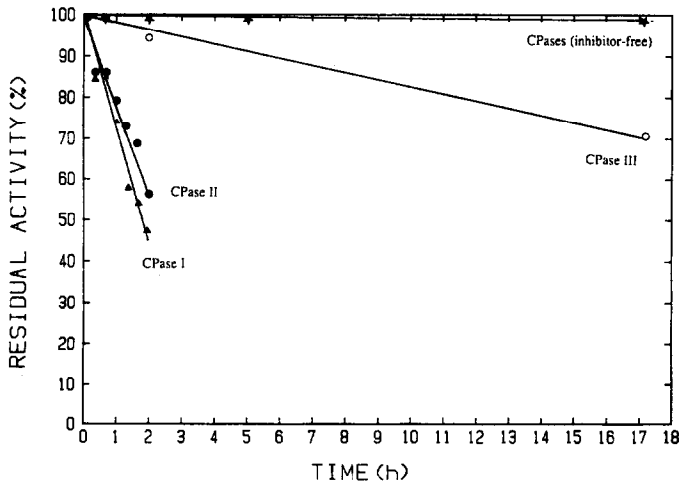

Fig. 7. Time-course inhibition of the three carboxypeptidases in the presence of 2 m*M* PMSF. ▲, I; ●, II; O, III; ★, inhibitor-free samples.

DISCUSSION

Purification

The purification method outlined here consisting of ammonium sulphate precipitation and tandem desalting–IMAC has permitted us not only to separate the *endo*-peptidase activity from the *exo*-peptidase activity but also, under appropriate conditions, to elute individually the three carboxypeptidases in the medium from the affinity column. The three enzymes were found to be isoenzymes. This is an important advantage of the method, as the enzymes could be eluted individually even though they possessed similar physico-chemical properties. Anion-exchange chromatography was used to procure pure extracts of the enzymes. As a result, carboxypeptidase I was purified 115-fold with a 64% yield, carboxypeptidase II was purified 92-fold with a 15% yield and the carboxypeptidase III specific activity was increased 120-fold with a 48% yield. In the best instances the overall yield was around 125%. Yields exceeding 100% are not rare as the removal of inhibitors during the purification could reveal some of the masked activity. The purification was 7-fold and the yield 80% for the partially purified acid protease. It should be noted that the purity of the enzymes after chromatography is dependent on the efficiency of the pre-treatment applied. Unlike in ref. 21, here the carboxypeptidases were not electrophoretically homogeneous after IMAC even though high yields were obtained and so an additional ion-exchange step was necessary.

The choice of elution conditions in affinity chromatography is of critical importance. Sulkowsky[27], based on the work of Porath and Olin[28] and others, suggested three ways of eluting proteins adsorbed in metal columns. The first is by the protonation of electron-donor groupings on the surface of the protein to reverse its coordination to IDA–M^{2+}; ligand exchange[29] is the second method and the third is chelate annihilation, normally the last resort as it involves a complete removal of metal and protein using a chelating agent stronger than IDA. The last method was our first elution procedure[21] and, although it proved effective, reloading the column with Cu^{2+} and re-equilibration became expensive and tiresome, especially for larger columns. The use of a stepwise gradient of Gly–HCl solved this problem and increased the overall performance of the entire procedure.

Ligand exchange, using imidazole, was also an effective elution method but the purifications and yields obtained from run to run were irreproducible and haphazard[30]. This method of elution, however, provided some information on the possible mechanism of interaction between Cu^{2+} and the proteins purified. Analytical chromatography using disposable columns (Bio-Rad Labs.) with imidazole-saturated Cu^{2+}–IDA–Sepharose 6B resulted in the absence of retention of the carboxypeptidases, strongly suggesting the role of histidyl residues on the protein surfaces in the interaction with Cu^{2+}. Our hypothesis was eventually confirmed by Sulkowsky[27]. Moreover, he correlated the intensity of retention of a protein on a Cu^{2+}–IDA column directly with the number of histidyl residues on the protein surfaces. Therefore, logically the number of accessible histidines on the three enzymes would decrease in the order carboxypeptidase I < II < III. Earlier we reported[21] that preliminary studies showed that Cu^{2+} competitively inhibited carboxypeptidase in solution. In the present study, however, we have shown that immobilised Cu^{2+}, although not conclusively site-directed, interacts, to different extents, with the solute depending on the accessibility of the reactive groups on its surfaces.

Properties

From the properties studied, it is evident that the three acid carboxypeptidases closely resemble those of the other *Aspergilli.* The molecular weights of carboxypeptidases I, II and III are comparable to the enzymes isolated from *Aspergillus saitoi*[13], *Aspergillus niger* var. *macrosporus*[16], carboxypeptidase I from *Aspergillus oryzae*[9] and carboxypeptidase O from *Aspergillus oryzae*[31]. These enzymes often exist in dimeric and trimeric forms. The isoelectric points of these enzymes also seem to be very close to one another. Isoelectric focusing in the pH range 3.5–9.5 revealed that all three enzymes possess p*I* values neighbouring pH 5.0.

Unlike many reported carboxypeptidases, those from *Aspergilli* exhibit maximal peptidyl-L-amino acid hydrolase activity below pH 5.0[9–13,15,32]. The three carboxypeptidases reported here also maximally hydrolyse CGT at pH 3.4. The esterase activity, however, is maximal at pH 5.0. The temperature optima also resemble those of other *Aspergillus* carboxypeptidases. Maximal esterase and peptidyl-L-amino acid hydrolase are seen at 50°C at their respective optimum pHs. The three carboxypeptidases are relatively thermostable as there is little activity loss even after incubation for 2 h at 50°C. However, there is drastic loss of activity at 60°C.

The action of inhibitors such as PMSF and DFP confirms that the carboxypeptidases from *Aspergilli* belong to the class of serine carboxypeptidase[9,32,33] like carboxypeptidase Y from yeast[34] and carboxypeptidase from *Penicillium janthinellum*[8]. In our case, whereas carboxypeptidases I and II are strongly inhibited by PMSF, carboxypeptidase III is more resistant to PMSF action. Inhibition of all three enzymes by TPCK indicates the role of a histidyl residue in their active sites[18].

What most clearly distinguishes the three enzymes from one another, however, is their affinities for the two substrates tested. In effect, although the enzymes possess very similar molecular weights and isoelectric points, conformational differences could result in differences in their substrate specificities. Thus, the K_m values of carboxypeptidases I, II and III for CGT were $1.9 \cdot 10^{-3}$ *M*, $4.9 \cdot 10^{-3}$ *M* and $6.1 \cdot 10^{-3}$ *M*, respectively, and similar differences were observed for BAEE. Indeed, in cases such as ours where the enzymes exhibit almost identical physico-chemical properties, a detailed study of their substrate specificities, as in refs. 9–12 and 35, could be the only means of distinguishing one isoenzyme from another.

Hence IMAC using Cu^{2+}–IDA–Sepharose 6B has not only permitted the complete separation of *endo-* and *exo*-peptidase activities but equally the separation of three serine carboxypeptidases exhibiting different affinities for the Cu^{2+} gel. This allows us to postulate that the technique would find wider application in the purification of proteolytic enzymes and in studying the heterogeneity of proteolytic complexes. The properties of the three enzymes are so similar that we consider them to be isoenzymes. Only their substrate specificities could differ. From this study and other references, it is evident that microbial forms secrete proteolytic "cocktails" for specific purposes of protein degradation.

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