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SEMI-PREPARATIVE-SCALE ISOLATION OF CARBOXYPEPTIDASE ISOENZYMES FROM *ASPERGILLUS NIGER* BY A SINGLE METAL CHELATE AFFINITY CHROMATOGRAPHIC STEP

I. PRELIMINARY ANALYSIS OF QUANTITATIVE ASPECTS

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SUMMARY

The use of immobilised metal-ion affinity chromatography with Cu^{2+}–iminodiacetate-Sepharose 6B as an affinity sorbent for the separation of endopeptidases and exopeptidases from the culture filtrates of *Aspergillus niger* is discussed. The performance of the affinity sorbent in relation to reproducibility of results and the effect of ballast substances on protein uptake were studied. The nature of the adsorption isotherms obtained with carboxypeptidase I and the semi-purified extract, containing acid protease, carboxypeptidases, pigments and various other contaminating substances, are compared. The partition coefficient, K_d, of the affinity system with respect to carboxypeptidase I was determined and the dissociation constant, K_L, of the carboxypeptidase I-Cu^{2+}-iminodiacetate complex was deduced.

INTRODUCTION

Serine carboxypeptidases[1] and acid carboxypeptidases[2] are proteolytic enzymes, capable of releasing carboxy-terminal amino acids from peptides, with rather low specificity. These enzymes are found in many plants and microorganisms. Unlike pancreatic carboxypeptidases A and B, serine carboxypeptidases are not inhibited by metal-chelating agents like 1,10-phenanthroline[3]. However, many acid carboxypeptidases of fungal origin are inhibited by metal ions, like Cu^{2+} (refs. 4 and 5). In a recent publication[6], we showed how this effect could be exploited for the separation of endo- and exopeptidase activities and for the purification of acid carboxypeptidases from an *Aspergillus niger* culture medium. The method used, called immobilised-metal-ion affinity chromatography (IMAC), takes advantage of the affinity of *A. niger* acid carboxypeptidases for Cu^{2+}, which is coupled to iminodiacetate (IDA), Sepharose 6B. In another recent paper[7], we demonstrated the presence of carboxy-

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peptidase isoenzymes (carboxypeptidases I, II, III) in *A. niger* culture filtrates, by using modified elution conditions, and we described the use of tandem desalting affinity chromatography on Sephadex G50 and Cu^{2+}-IDA-Sepharose 6B for the rapid isolation of the three carboxypeptidases. Characterization studies have shown that although these three enzymes have nearly identical molecular weights (*ca.* 140 000), isoelectric points (*ca.* 5.0) and other properties, their affinities for their substrate (carbobenzoxy-L-Glu-L-Tyr) differed. All three peptidases were inhibited by phenylmethanesulfonylfluoride, indicating that they are serine carboxypeptidases. They were also inhibited by tosyl phenylalanyl chloromethyl ketone, suggesting the presence of a histidyl residue in their active sites.

Although IMAC was developed more than a decade ago[8] and although various fundamental aspects of its functioning are already known[9], little work has been done on the quantitative aspects of immobilised-metal-ion protein interactions. This paper discusses certain preliminary results of a quantitative study of the behaviour of Cu^{2+}-IDA-Sepharose 6B with respect to the semi-preparative purification of proteins isolated earlier[7]. The study is interesting, because the carboxypeptidases interact with Cu^{2+}-IDA in a pseudospecific rather than a biospecific way. These enzymes are neither metalloproteins nor are they dependent on metals for their activity.

EXPERIMENTAL

Chromatography

The affinity sorbent, Cu^{2+}-IDA-Sepharose 6B, was prepared according to Porath and Olin[10]. Tandem desalting affinity chromatography was performed on a Sephadex G50 column (45 × 3.2 cm) and a Cu^{2+} column (20 × 2.5 cm). The flow-rate was maintained at 112 ml/h (13.9 cm/h for the desalting column and 22.8 cm/h

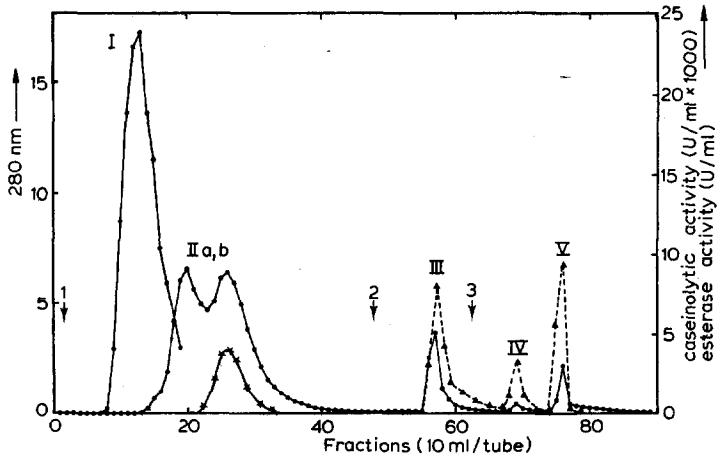

Fig. 1. Chromatography of the three carboxypeptidases and the acid protease by tandem desalting-affinity chromatography on Sephadex G50 and Cu^{2+}-IDA-Sepharose 6B. (●) Absorbance at 280 nm; (★) caseinolytic activity of acid protease; (▲) esterase activity; fractions 10 ml/tube; flow-rate 112 ml/h. 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). Peak I: excluded peak from Sephadex G50; peaks IIa,b: unretained peak from affinity column; peaks III, IV and V: eluted peaks containing the three carboxypeptidases.

for the affinity column). The capacity of the column was determined by the frontal elution technique[11] at a linear velocity of 5.4 cm/h. All elutions were carried out at room temperature (about 22°C). The elution of proteins was monitored by registering the absorbance at 280 nm.

The enzyme extract was a gift from Drs. Chow Ching Cheng and J. M. Lebeault of the Division des Procédés Biotechnologiques of the University of Compiègne. Chromatography was done after filtration on a Whatman No. 1 filter paper and precipitation with ammonium sulphate (0–90% saturation). The extract obtained after desalting on the Sephadex G50 column, was concentrated using an Amicon diafiltration cell and was used for frontal elution when studying the effect of ballast substances on the affinity column. The peak corresponding to carboxypeptidase I in Fig. 1 was concentrated by diafiltration and used for the chromatographic studies concerning carboxypeptidase I.

Assays

Carboxypeptidases were assayed with benzoyl-L-arginine ethyl ester (BAEE) according to Schwert and Takaneka[12]. Casein was used for the assay of acid protease using the method of Ichishima[13].

The partition coefficient, K_d, of carboxypeptidase I on the affinity sorbent was determined by[11]

$$K_d = \frac{\text{bound enzyme}}{\text{free enzyme}} \tag{1}$$

The dissociation constant, K_L, of the enzyme–ligand complex was determined by

$$K_L = \frac{L_0}{K_d} \tag{2}$$

where L_0 is the ligand concentration.

RESULTS

Separation of endo and exopeptidase activities

Acid protease activity was separated from that of the three carboxypeptidases as described in ref. 7. In a typical experiment, 30 ml of the ammonium sulphate extract containing 1740 absorbance units (280 nm) were injected into the Sephadex G50 column, which was subsequently washed with 50 m*M* starting buffer (acetate; pH 5.3). The flow-rates were as indicated above and 10-ml fractions were collected. The first (excluded) peak that appeared at the outlet of the desalting column was 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 unretained 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 peak, the second elution buffer (100 m*M* Gly–HCl; pH 3.0) was used to continue the elution.

TABLE I

REPRODUCIBILITY OF ELUTION VOLUMES, YIELDS AND CONCENTRATION FACTORS UNDER NON-SATURATING CONDITIONS

$V_{R(I)}$	$\sigma_{(I)}$	$V_{R(II)}$	$\sigma_{(II)}$	$V_{R(III)}$	$\sigma_{(III)}$	*Yields*				*Purification*	$\sigma_{(purification)}$
						Protein	$\sigma_{(protein)}$	*Activity*	$\sigma_{(activity)}$		
105	0.18	58	0.28	115	0.63	95	0.14	79	0	26	0.12
108	1.12	56	0.01	112	3.03	96.5	0.73	85.7	4.49	26.5	0.26
103.5	0.14	56	0.01	114	1.23	96	0.48	72	4.9	22	0.84
104.3	0.01	64	5.93	119	0.22	95	0.14	76.3	0.73	23	0.36
102	0.71	56	0.01	116	0.23	94	0.01	77	0.4	22	0.84
105	0.01	58	0.29	110	0.63	90	1.44	73	3.6	23	0.36
104.5	0.01	49.5	4.62	121	1.23	90.5	1.09	89	10	31	3.72
103	0.28	59	0.73	117	0.03	90	1.44	76.6	0.58	23	0.36
106	0.18	53	1.09	123	3.02	99	2.70	78	0.1	25	0
106	0.18	53.5	0.78	120	0.63	92	0.32	82	0.9	27	0.44
103	0.28	56.5	0.01	116	0.23	94	0.01	80	0.1	25	0
104.66 ± 0.53		56.3 ± 1.12		117.5 ± 1.00		93.8 ± 0.88		79 ± 1.53		24.9 ± 0.815	

Performance of tandem Sephadex G50–Cu^{2+}-IDA-Sepharose 6B

Chromatography was carried out on a tandem system to study the performance under non-saturated conditions and the reproducibility of the elution volumes of the three peaks containing the carboxypeptidase activity (Table I). We also studied the reproducibilities of yields and purification factors. Repeated adsorption and desorption was performed in eleven experiments. Minor variations in the elution volumes were noticed ($V_{R(CPase\ I)}$ = 104.66 ml ± 0.529; $V_{R(CPase\ II)}$ = 56.3 ml ± 1.118; $V_{R(CPase\ III)}$ = 117.3 ml ± 1.004). Similarly, the yields in activity and protein remained almost constant (79% ± 1.53 and 93.8% ± 0.88, respectively), while the average purification factors varied little (24.9 fold ± 0.815). Furthermore, there was no need to renew the Cu^{2+} load on the column after each run. No removal of Cu^{2+} was noticed.

Capacity of Cu^{2+}-IDA-Sepharose 6B for carboxypeptidase I and the adsorption isotherm

Purified enzyme solutions (peak corresponding to carboxypeptidase I in Fig. 1) of increasing concentrations were used for frontal elution chromatography in order to determine the adsorption isotherm of the system and, subsequently, the capacity of the affinity adsorbent for carboxypeptidase I. The volume of the column was 2.85 ml, and the linear velocity was 5.4 cm/h. The retained protein was desorbed with 5 m*M* Gly–HCl (pH 3.0) after saturating the column with carboxypeptidase I.

The adsorption isotherm (Fig. 2a) is linear up to a particular concentration of the injected enzyme. Beyond that, it becomes convex and flattens out. The maximum capacity achieved was about 55 mg of protein per ml of sorbent, when the sample concentration was 10 mg/ml or higher. When the sample concentration was below 10 mg/ml, the adsorption is lower. Evidently, the amount of solute taken up by the

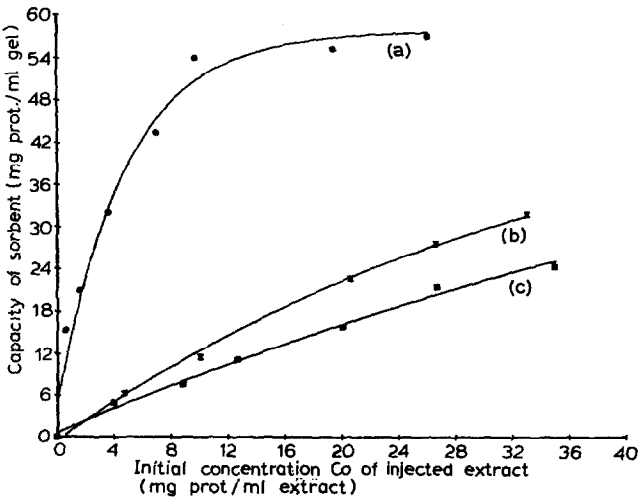

Fig. 2. Adsorption isotherms of the affinity sorbent (a) for purified carboxypeptidase I, (b) under repeated saturations and desorptions using a desalted extract containing the carboxypeptidases, acid protease and ballast substances, (c) using a fresh column for each run (same extract as for b).

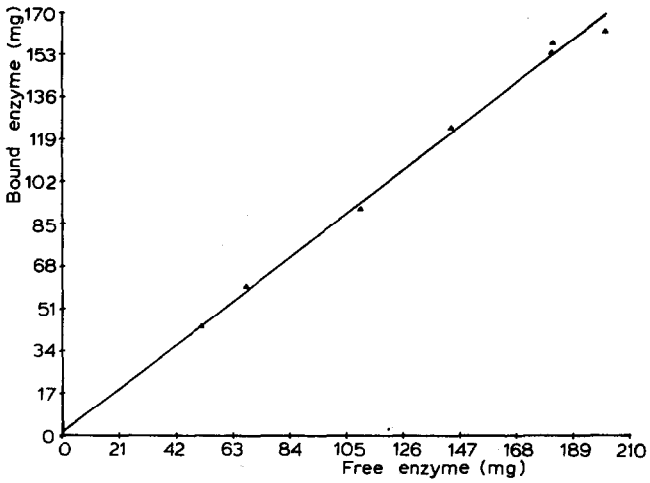

Fig. 3. Stability of the partition coefficient of the affinity system with carboxypeptidase I as solute.

stationary phase is dependent on the concentration of the protein in the sample. This indicates that adsorption is determined by the partition coefficient of the protein.

At the end of each elution, the amount of protein transferred to the affinity sorbent for a given concentration of solute in the mobile phase was calculated. The linearity of the graph in Fig. 3 indicates the stability of the partition coefficient, K_d, of carboxypeptidase I. The slope of the graph yields a partition coefficient of 0.83. The correlation coefficient is 0.998.

Dissociation constant of the enzyme–ligand complex

With the partition coefficient, K_d, determined above, the dissociation constant, K_L, of the carboxypeptidase I-Cu^{2+}-IDA complex was calculated from eqn. 2. A value of $5.14 \cdot 10^{-5}$ M was obtained, indicating the fairly high affinity of carboxypeptidase I for the adsorbent.

Influence of ballast proteins on the capacity of the affinity adsorbent

Two series of experiments were carried out to study the effect of ballast substances in the extract on the capacity of the affinity column for the carboxypeptidases. The column volume was 11 ml, and the linear velocity was 5.4 cm/h. The extract contained acid protease, carboxypeptidase, and other proteins and pigments.

In the first series of experiments, increasing concentrations of the extract were injected on a given affinity column, until saturation occurred. The protein was then desorbed from the saturated column, and the capacity of the gel for the carboxypeptidases was determined. The adsorption isotherm (Fig. 2b) obtained was convex, and the slope of the curve near the origin was lower than in Fig. 2a.

In a second set of experiments, a column with fresh gel was used for each chromatographic run, and increasing concentrations of the extract were injected. The capacity of the affinity adsorbent was calculated at the end of each elution. The slope of the isotherm (Fig. 2c) is higher than in the isotherm obtained for the former series of elutions. The difference in the capacities of the gels determined from the two iso-

therms (Fig. 2b and c) indicates the effect of the accumulation of impurities when the same affinity column is used for repeated saturations.

DISCUSSION

The IMAC-based purification method described here is a convenient method for separating endo- and exopeptidase activities. Furthermore, under appropriate conditions, carboxypeptidase isoenzymes can be eluted individually.

Under non-saturated conditions, tandem desalting IMA chromatography on Sephadex G50 and Cu^{2+}-IDA-Sepharose 6B is highly reproducible. No significant variations in elution volumes, yields and purification factors were observed, even after 11 cycles. However, pigments found in the crude extract bind irreversibly to Cu^{2+}-IDA, and their accumulation begins to be visible at the inlet of the column. Upon saturation, these ballast substances hinder the correct functioning of the system by lowering the capacity of the affinity sorbent. This is in accordance with previous observations, even in the case of biospecific affinity sorbents[14].

The adsorption isotherm of carboxypeptidase I on the affinity system is linear when the initial concentration of the injected extract is low. At higher concentrations, the isotherm becomes convex and reaches its maximum capacity of about 55 mg/ml.

The partition coefficient of the system remains constant up to the saturation point of the affinity sorbent. The partition coefficient, K_d, of 0.83, determined from the slope of the graph, is not high for an affinity system. Nevertheless, the dissociation constant, K_L, of $5.14 \cdot 10^{-5}$ M, calculated using the K_d value of 0.83, indicates that the affinity of carboxypeptidase I for the sorbent is reasonably high. An indepth study by other chromatographic methods is underway to elucidate this phenomenon.

Kinetic studies have shown that Cu^{2+} competitively inhibits carboxypeptidase ($K_i = 15.8 \cdot 10^{-3}$ M)[15]. This study shows that the affinity of the carboxypeptidase is many times higher for immobilised Cu^{2+}. However, the action of immobilised Cu^{2+} is not directed towards active sites, since elution of the enzyme by using its substrate was not possible. This could also mean that the interaction of Cu^{2+} with the carboxypeptidases is dependent on the coordination geometry and on the conformation of Cu^{2+} in its free and its immobilised form.

The influence of ballast substances on the column performance is clearly shown in the two adsorption isotherms (Fig. 2b and c). The accumulation of pigments and other substances in the column drastically affects the capacity and causes fouling. Clearly, the carboxypeptidases are not the only components of the extract which have an affinity for Cu^{2+}.

The slopes of the adsorption isotherms are also indicative of the influence of ballast and other substances on the interaction between the ligand and the proteins of interest. When a crude mixture containing the three carboxypeptidases, the acid protease and other pigments and proteins is injected, the adsorption isotherms (Fig. 2b and c) are not very steep, indicating little adsorption of the solute from the mobile onto the stationary phase. This could be due to competition among the solutes (carboxypeptidases, acid protease and other proteins and non-proteins) for adsorption sites. In effect, carboxypeptidases II and III have a higher affinity for Cu^{2+}, since these enzymes are eluted after carboxypeptidase I. Furthermore, the acid protease can play the role of a competing ligand since it shows some affinity for immobilised

Cu^{2+}, as evident from its relative elution volume (V_R/V_t) of 1.06. Thus, solute–solute interactions could cause a decrease in adsorption. In contrast, when only carboxypeptidase I is injected, it is readily adsorbed, without interference by other substances in the sample. This is shown by the slope of the isotherm (Fig. 2a) near the origin. We have also found that up to 45% of the total amount of protein injected, is adsorbed on the stationary phase. In contrast, the gradient of the adsorption isotherms when a crude mixture is injected is much smaller, indicating little adsorption. In effect, only 15–20% of the total protein injected is adsorbed on the affinity gel. Of the 15–20% adsorbed, about 65% is carboxypeptidase I.

The shape of the adsorption isotherm also provides a way for predicting band shape of the solute on the sorbent[11,16]. Convex profiles indicate bands with sharp fronts and diffuse tails within the column. This phenomenon can also be observed in the elution profile of the carboxypeptidases (Fig. 1), which exhibits tailing. Such distortions are typical symptoms of diffusional constraints and slow restoration of the equilibrium between the mobile and the stationary phase[17,18]. The restoration of equilibrium is directly dependent on the flow-rate.

The present study is useful for understanding the behaviour of an affinity sorbent towards different solutes. This is even more significant in our case, which is a typical example of group-specific rather than biospecific adsorption. Moreover, the enzyme systems involved are closely related to one another. According to Sulkowski[9], differences in the number of accessible histidyl residues on the proteins' surfaces could account for the differences in the retention of the three carboxypeptidases on Cu^{2+}-IDA-Sepharose 6B. Such differences could cause differences in mass transfer when these proteins are chromatographed individually or together.

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