Journal of Chromatography, 329 (1985) 165–170
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 17 720

Note

Purification of an acid protease and a serine carboxypeptidase from *Aspergillus niger* using metal-chelate affinity chromatography

SIVADASAN KRISHNAN and M. A. VIJAYALAKSHMI*
Laboratoire de Technologie des Séparations, Université de Technologie de Compiègne, B.P. 233, 60206 Compiègne Cedex (France)
(Received March 12th, 1985)

Serine carboxypeptidases, which are most active at acidic pH values have been isolated from many plants and microorganisms[1–6]. They are often called acid carboxypeptidases. These enzymes contain an active serine residue and are inhibited by diisopropyl phosphorofluoridate (DFP) and phenyl methyl sulphonyl fluoride (PMSF)[7] which react specifically with this residue. Unlike pancreatic carboxypeptidases A and B, serine carboxypeptidases are not metalloenzymes and are not inhibited by metal-chelating agents like ethylenediaminetetraacetate (EDTA) and 1,10-phenanthroline[8]. However, they are inhibited to different extents by certain metal ions[2,9]. Taking as an example the reactivity of Cu^{2+} with carboxypeptidase Y, a serine carboxypeptidase from yeast[9], we felt that a similar sort of inhibition could occur with the serine carboxypeptidase from *Aspergillus niger*. Preliminary studies revealed that such an inhibition does occur. This was exploited to develop a method of purifying carboxypeptidase on Cu^{2+}-iminodiacetic acid (IDA)-Sepharose 6B.

The affinity of a protein for an immobilized metal ion coupled to a chelating ligand which is itself fixed to an insoluble support is the feature exploited in metal-chelate affinity chromatography. Recently, this method has been utilized for the purification of a large number of proteins. Heavy metal ions like Cu^{2+} and Zn^{2+} have been involved in the purification of many biomolecules like interferons, nucleotides, aminoacylated tRNA, carboxypeptidase G2, etc.[10–12].

EXPERIMENTAL

Chemicals

The carboxypeptidase substrate CBZ-L-Glu-L-Tyr and IDA were from Sigma. Casein, which was the substrate for the acid protease, $CuSO_4 \cdot 5H_2O$ and EDTA were from Prolabo (France). Sepharose 6B was from Pharmacia, Sweden and L-tyrosine was from Merck.

The crude enzyme extract was a gift from the Division des Procédés Biotechnologiques of the Université de Technologie de Compiègne.

Determination of protein concentration

The concentration of protein was usually determined by measuring the absorption at 280 nm using a Jobin Yvon spectrophotometer Model JY 201.

0021-9673/85/$03.30

Peptidase assay

The enzyme was routinely assayed using CBZ-Glu-Tyr, 5 m*M* in 50 m*M* sodium-acetate buffer, pH 3.1 at 30°C following the method outlined by Nakadai *et al.*[2]. One unit of carboxypeptidase activity is defined as the amount of enzyme required to liberate 1 μmol of tyrosine per minute at 30°C and at pH 3.1. The specific activity is expressed as the number of units of carboxypeptidase activity per unit absorbance at 280 nm of the enzyme.

Estimation of acid protease activity

The acid protease activity was determined using a modification of methods developed by Anson, Kunitz and Hagihara[13]. Milk casein was used as the substrate. A 1-ml volume of 2% casein in 50 m*M* lactate buffer pH 2.7 and 1 ml of enzyme solution separately preincubated at 39°C in a water-bath were mixed and the reaction was allowed to proceed for 10 min. Trichloroacetic acid (TCA, 2 ml) was added to precipitate undigested casein. The mixture was then filtered and to 1 ml of the filtrate 5 ml of sodium carbonate were added followed by 1 ml of Folin–Ciocalteau reagent. The mixture was then incubated at 39°C for 20 min. The optical density of the mixture is read at 660 nm.

One unit of enzyme activity is the amount of enzyme that would liberate 1 μg of tyrosine per minute at 39°C and pH 2.7. The specific activity is defined as the number of units of protease activity per unit absorbance at 280 nm of the enzyme solution.

Preparation of affinity sorbents

Two sorbents were prepared: 1, tyrosine-Sepharose 6B and 2, IDA-Sepharose 6B. In both cases the metal-chelating agents tyrosine and IDA were immobilized on the matrix after epoxyactivation[14,15].

Tyrosine-Sepharose and IDA-Sepharose were packed into separate columns and washed with three column volumes of 50 m*M* copper sulphate in 50 m*M* acetate buffer pH 5.5. The columns were then washed with 50 m*M* sodium acetate buffer pH 5.5 containing 50 m*M* sodium chloride until all the excess of Cu^{2+} had been removed. A reequilibration of the columns was carried out using 50 m*M* acetate buffer pH 5.5, which is the starting buffer.

Purification procedures

Precipitation with ammonium sulphate. The culture filtrate obtained after fermentation was subjected to a fractionation using ammonium sulphate. The precipitate obtained between 70 and 80% saturation was used in further purification steps.

Dialysis. The precipitate obtained was dissolved in 102 ml of sodium acetate buffer pH 3.1 and subjected to a dialysis in the same buffer containing 5 m*M* EDTA for 48 h with three changes of the buffer.

Chromatography. The ammonium sulphate free dialysate was used for chromatography. In a typical experiment, 5 ml of the dialysed extract containing 56.5 mg of protein were injected into a column of Cu^{2+}-IDA-Sepharose 6B (19.2 × 1.25 cm) prepared as previously stated. The flow-rate was maintained at 120 ml/h and 8-ml fractions were collected. When the flow-through peak was complete the starting buffer was replaced with the elution buffer, 50 m*M* acetate pH 5.5 containing 75 m*M*

EDTA. A similar experiment was also conducted using Cu^+-tyrosine-Sepharose 6B.

The flow-through and eluted fractions were assayed for protease and carboxypeptidase activities. Then the eluted fractions and any other fractions of the flow-through peak containing Cu^{2+} were separately subjected to a diafiltration to remove the Cu^{2+}.

Diafiltration. Diafiltration was carried out using an Amicon cell with a membrane having an exclusion limit of MW 30 000. Sodium-acetate buffer pH 5.5 containing 25, 50 and 75 m*M* EDTA was used to wash the protein. Finally EDTA-free buffer was used. The protein content and protease and peptidase activities of the copper-free fractions were then redetermined.

RESULTS

Table I summarizes briefly the purification of the carboxypeptidase.

The precipitate obtained at 70–80% saturation with ammonium sulphate was the fraction that had highest specific activity and yield, and carboxypeptidase activity in the precipitate could only be assayed after the dialysis as the $(NH_4)_2SO_4$ present interferes with the ninhydrin test.

As shown in Fig. 1, the carboxypeptidase activity after chromatography is present mainly in the eluted fractions. A diafiltration is essential after chromatography in order to eliminate Cu^{2+} eluted with adsorbed proteins. The specific activity increases sharply (115-fold) after the diafiltration.

Purification of the acid protease on Cu^{2+}-IDA-Sepharose 6B

A significant drop in yield (13%) is noticed after precipitation with ammonium sulphate. However, as shown in Table II, there is a significant rise in the specific activity and extant of purification.

At the end of affinity chromatography, all the caseinolytic activity was found in the flow-through fractions (Fig. 1). Traces of Cu^{2+} were also present. So, a diafiltration was carried out, which seriously effected the protease activity. Copper interferes with the protease activity and further studies are required to interpret these data.

Affinity chromatography using Cu^+-tyrosine-Sepharose 6B

The same extract (precipitation with 70–80% ammonium sulphate, followed by dialysis) was subjected to chromatography on a column of copper coupled to

TABLE I

PURIFICATION OF THE CARBOXYPEPTIDASE ON Cu^{2+}-IDA-SEPHAROSE 6B

Fraction	*Protein (mg)*	*Activity (u)*	*Specific activity (u/mg)*	*Yield in activity (%)*	*Purification factor*
Culture filtrate	43 000	2 600 000	60.5	100	1
Precipitate with 70–80% $(NH_4)_2SO_4$, then dialysed	1570	1 611 600	1026	62	17
After affinity chromatography and diafiltration	133	925 344	6957	36	115

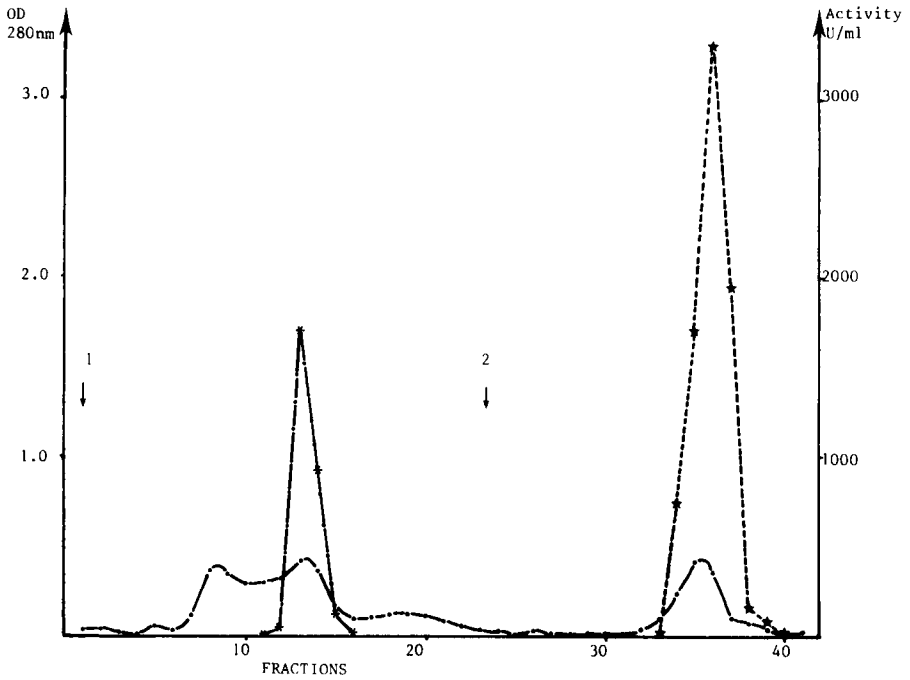

Fig. 1. Chromatography of the carboxypeptidase and protease on Cu^{2+} coupled to IDA-Sepharose 6B. ●—●, Absorption at 280 nm; ✱—·—✱, caseinolytic activity of the acid protease; ★---★, carboxypeptidase activity. Other details as in the text. Buffers: 1, 50 m*M* acetate, pH 5.5; 2, 50 m*M* acetate, pH 5.5 + 75 m*M* EDTA.

tyrosine-Sepharose 6B. The eluted fractions were dialysed for 48 h against acetate buffer pH 3.1 containing 10 m*M* EDTA in order to determine the actual protein content and enzyme activity. Fig. 2 shows the profile of protein content, and the protease and carboxypeptidase activities of each fraction. Both the protease and carboxypeptidase were found to be present in the eluted fractions.

TABLE II

PURIFICATION OF THE ACID PROTEASE ON Cu^{2+}-IDA-SEPHAROSE 6B

Fraction	*Protein (mg)*	*Activity (u)*	*Specific activity (u/mg)*	*Yield in activity (%)*	*Purification factor*
Culture filtrate	43 000	5 100 000	118.6	100	1.0
Precipitate with 70–80% $(NH_4)_2SO_4$, then dialysed	1570	642 000	409.6	12.6	3.5
After affinity chromatography	141	231 795	1641	5	14

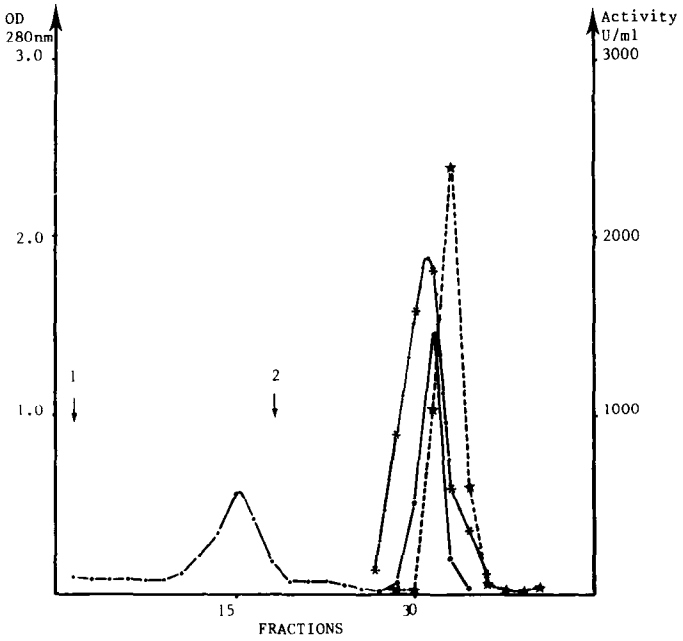

Fig. 2. Chromatography of the carboxypeptidase and protease on Cu^{+}-tyrosine-Sepharose 6B. Details as in Fig. 1.

DISCUSSION

This work shows that, using the method described, protease and carboxypeptidase from the culture filtrates of *Aspergillus niger* could be separated. The chromatographic step is a very simple one where the carboxypeptidase adsorbed onto the Cu^{2+}-IDA-Sepharose 6B is eluted using 75 m*M* EDTA, while the protease is present in the flow-through fractions. A diafiltration is then carried out in order to eliminate Cu^{2+} found together with the eluted protein. As a result, a 115-fold purification of carboxypeptidase with 36% yield could be obtained. The protease was purified 14-fold with a 5% yield in activity.

While chromatography on a column with Cu^{2+} coupled to IDA-Sepharose separates the protease and carboxypeptidase in the flow-through and eluted fractions respectively, a similar separation is not obtained using copper coupled to tyrosine-Sepharose. Both the enzymes are present in the eluted fractions. Copper coupled to tyrosine is monovalent (Cu^{+}). Thus, the protease behaves in one manner towards Cu^{2+} and in another towards Cu^{+}. Also, the presence of the tyrosine could increase the affinity of the protease for the copper(I)–tyrosine complex owing to the hydrophobicity and to charge-transfer interactions.

However, chromatography on Cu^{2+}-IDA-Sepharose carried out at 4°C also yielded a profile with both protease and carboxypeptidase in the eluted fractions.

Previous studies have shown that Cu^{2+} is effectively a competitive inhibitor of the carboxypeptidase[16]. This explains the retention of the enzyme on Cu^{2+}-IDA-Sepharose. Hayashi *et al.*[9] reported that while carboxypeptidase Y is strongly in-

hibited by Cu^{2+}, there is less inhibition by Cu^{+}. In our case, however, the carboxypeptidase exhibits the same behaviour towards the Cu^{2+}–IDA and Cu^{+}–Tyr complexes during chromatography.

Further work to characterize the purified enzyme and to localize the ligand–enzyme interactions is nearing completion[17]. Preliminary work has indicated that, like all serine carboxypeptidases, our enzyme also possesses a histidine residue in its active site. The purified enzyme was electrophoretically homogeneous.

REFERENCES

1 H. Zuber and Ph. Matik, *Z. Naturforsch., B: Anorg. Chem., Org. Chem.*, 23b (1968) 663–665.
2 T. Nakadai, S. Nasuro and N. Iguchi, *Agric. Biol. Chem.*, 36 (1972) 1343–1352.
3 E. Ichishima, *Biochim. Biophys. Acta*, 258 (1972) 274–288.
4 J. T. Johansen, K. Breddam and M. Ottesen, *Carlsberg Res. Commun.*, 41 (1976) 1–14.
5 I. Kunagai, M. Yamasaki and N. Ui, *Biochim. Biophys. Acya*, 659 (1981) 3234–343.
6 E. Ichishima, K. Yoehimura and K. Tomoda, *Phytochemistry*, 22 (1983) 825–829.
7 R. Hayashi, Y. Bai and T. Hata, *J. Biol. Chem.*, 250 (1975) 5221–5226.
8 L. M. Peterson, M. Sokolowsky and B. L. Vallee, *Biochemistry*, 15 (1976) 2501–2508.
9 R. Hayashi, Y. Bai and T. Hata, *J. Biochem. (Tokyo)*, 77 (1975) 1313–1318.
10 J. Porath, J. Carlsson, I. Olsson and G. Belfrage, *Nature (London)*, 258 (1975) 598–599.
11 B. Lonnerdal and C. L. Keen, *J. Appl. Biochem.*, 4 (1982) 203–208.
12 D. A. P. Small, T. Atkinson and C. R. Lowe, in I. M. Chaiken, M. Wilchek and I. Parikh (Editors), *Affinity Chromatography and Biological Recognition*, Academic Press, Orlando, 1983, pp. 267–268.
13 B. Hagihara, *Annu. Rep. Sci. Works, Fac. Sci., Osaka Univ.*, 2 (1954) 35.
14 M. A. Vijayalakshmi and J. Porath, *J. Chromatogr.*, 177 (1979) 201.
15 P. Hubert and J. Porath, *J. Chromatogr.*, 198 (1980) 247–255.
16 S. Krishnan and M. A. Vijayalakshmi, *Proceedings of the First National Congress on "Purification Technologies of Proteins", Paris, October 1st–3rd, 1984.*
17 S. Krishnan and M. A. Vijayalakshmi, 1984, in preparation.