CROATICA CHEMICA ACTA CCACAA 49 (3) 587—592 (1977)

YU ISSN 0011-1643
CCA-1033 547.96:545.844
Original Scientific Paper

Purification of Cathepsin D by Affinity Chromatography on Pepstatin Sepharose Column

I. Kregar, I. Urh, H. Umezawa, and V. Turk*

*Department of Biochemistry, J. Stefan Institute, University of Ljubljana, 61000 Ljubljana, Yugoslavia and *Institute of Microbial Chemistry 3-14-23, Kamiosaki, Shinagawa-ku, Tokyo, Japan*

Received December 28, 1976

A method was developed for the isolation of cathepsin D by affinity chromatography on immobilized pepstatin. This inhibitor was coupled to agarose by water soluble carbodiimide. Further purification included gel filtration on Sephadex G-100. The obtained cathepsin D exists in three active forms which were resolved on DEAE cellulose. Electrophoresis in the presence of sodium dodecyl sulphate revealed that the first form consists of only one polypeptide chain having molecular weight 42 000. The second and the third form contain also polypeptides having molecular weight 27 000 and 14 000.

INTRODUCTION

The highly specific interaction between enzymes and suitable matrix-bound ligands, their substrates and inhibitors, have led to many attempts to isolate rapidly and in high state of purity various enzymes from complex mixtures, among them also proteinases[1,2].

Cathepsin D (EC 3.4.23.5), one of the major intracellular acid proteinases, was recently purified in our laboratory using affinity chromatography on hemoglobin covalently bound to agarose resin[3]. We have shown that this rapid procedure yields undegraded cathepsin D on the contrary to conventional methods giving an enzyme containing considerable amounts of smaller polypeptides. Usually an immobilized inhibitor is used for the affinity chromatography of enzymes. Synthetic octapeptide containing the D-isomer of phenylalanine bound to Sepharose 4B was used for the isolation of cathepsin D[4]. Immobilized pepstatin[5], a pentapeptide of microbial origin and an inhibitor of acid proteases, was used for the purification of renin[6–8]. Our studies presented in this paper describe a procedure for the purification of cathepsin D by affinity chromatography. In addition, the separation of multiple forms of cathepsin D is presented and their polypeptide compositions are compared.

EXPERIMENTAL

All reagents were of analytical grade. Deionized water containing 1 mmol sodium azide was used for the preparation of solutions.

Preparation of the Affinity Column

60 mg of pepstatin (Institute of Microbial Chemistry Tokyo, Japan) was dissolved in a small volume of methanol and then mixed with 30 ml of 5 mM NaOH. Pepstatin

solution was added to 12 ml of wet AH-Sepharose 4B (Pharmacia, Uppsala, Sweden) followed by the addition of 0.5 g 1-ethyl-3(3-dimethyl-aminopropyl) carbodiimide (Calbiochem, Los Angeles, U.S.A.). pH was maintained at 6.2. After 2 h another 0.5 g of carbodiimide was added, pH was controlled for one more hour and the reaction mixture was allowed to stand overnight in a cold room. The resin was washed extensively with water and buffers used later for affinity chromatography. The amount of bound pepstatin was 2 μg/g of dry resin as determined by amino acid analysis.

Proteolytic activity was measured by the modified method of Anson[9].

The protein determination was performed either by the method of Lowry et al.[10] or by recording the absorbance at 280 nm.

Polyacrylamide gel electrophoresis was carried out at pH 8.5 in the Canalco apparatus (Canalco, Rockville, U.S.A.). Gel electrophoresis in the presence of sodium dodecyl sulphate (SDS) was run following the method of Weber and Osborn.[11] The protein samples were dissolved in gel buffer (phosphate buffer, pH 7.4) containing 1% SDS and 0.5% 2-mercaptoethanol. Protein standards for molecular weight determination were products of Serva, Heidelberg, Germany.

Initial Purification Step

Calf thymus was used as a source of the enzyme. Immediately after slaughtering the organs were taken and brought to the laboratory on ice and 33% homogenate in water was prepared. Supernatant was obtained by centrifugation of homogenate at 5000 × g for 100 min at 0 °C in Sorvall RC 2B refrigerated centrifuge (Sorvall, Norwalk, U.S.A.). Proteins in the supernatant were concentrated by precipitation with 70% ammonium sulphate. After centrifugation (100 000 g min) the precipitated proteins were resuspended in a small volume of water and dialyzed overnight against 10 l of water in order to remove ammonium sulphate. Before the application to the affinity column, the supernatant was adjusted to the desired pH by the addition of one third of the volume of concentrated sodium acetate buffer (0.4 M sodium acetate in 4 M NaCl).

RESULTS AND DISCUSSION

Preliminary experiments were done in order to establish the most suitable pH for binding of cathepsin D to the pepstatin Sepharose column. It was found that at pH 3.5 the enzyme was bound almost completely, however, 35% of its total activity was lost after acidification. At pH 5.0 20% of the enzyme totally applied did not bind, but the loss of activity due to the acidification was less than 5%. In further experiments pH 4.0 was used. The adsorbed enzyme was eluted with 0.1 *M* tris buffer pH 8.6—1 *M* NaCl.

A typical affinity chromatography separation is shown in Figure 1. Active material eluted in the second peak was not completely pure as found by gel electrophoresis. The gel pattern of this fraction was essentially the same as when obtained by affinity chromatography on immobilized hemoglobin[3]. Further separation was achieved by gel chromatography on Sephadex G-100 (Figure 2). The first protein peak was devoid of proteolytic activity against hemoglobin as substrate at pH 3.5. The nature of this inactive protein is unknown.

The active peak which was completely separated from the inactive material contained three principal protein bands as shown by gel electrophoresis. These proteins, representing the multiple forms of cathepsin D were resolved on DEAE cellulose using 0.025 M Tris buffer, pH 8.0 with a linear gradient of NaCl (Figure 3). It is evident that the first form was completely separated from the second and the third one. Gel electrophoresis at pH 8.5 proved the successful separation of multiple forms (Figure 4). These separated multiple forms showed only minor differences in the specific activity. Electrophoresis of active forms in the presence of sodium dodecyl sulphate showed that the first form was a

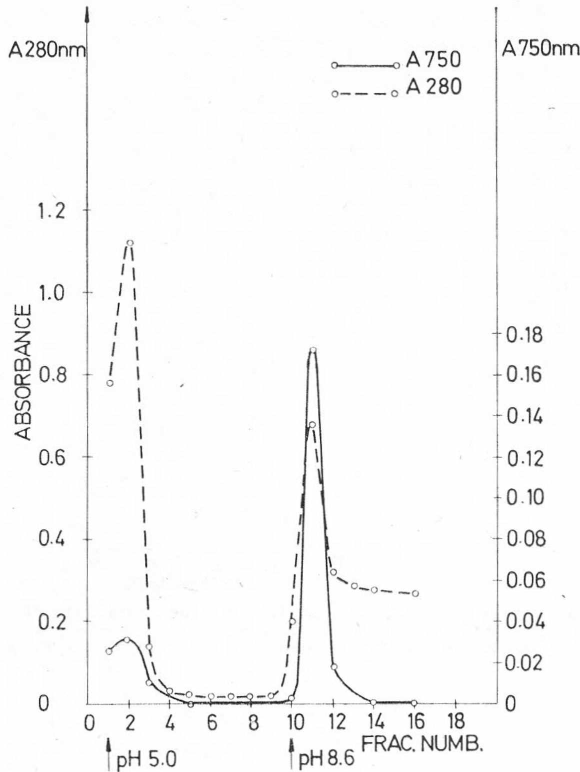

Figure 1. Pepstatin-Sepharose chromatography of 0-70% ammonium sulphate precipitated fraction from thymus. ——— proteolytic activity on hemoglobin at pH 3.5; — — — A_{280}, Dimensions of the column were 6.5 × 1 cm, flow rate was 15 ml/h.

single polypeptide of an apparent molecular weight of 42 000, whereas the second and the third form contained also polypeptides of 27 000 and 14 000. The presence of polypeptides lower than 42 000 can be explained by in vivo or in vitro degradation of the enzyme[3,12] The results agree completely with our previous findings as well as with the data reported by other authors who found multiple forms of cathepsin D using different isolation techniques[12–14]. It is therefore surprising that Kazakova and Orekhovich were able to purify cathepsin D using pepstatin-Sepharose column without noticing multiple forms[15]. They claim their enzyme to be electrophoretically homogeneous but they do not mention its polypeptide composition.

Affinity chromatography on immobilized pepstatin proved to be a very successful method for the preparation of cathepsin D. The final yield of all three forms was approximately 15% with a purification of up to 1000 fold. The purified enzyme was identical regarding the molecular weight and polypeptide composition with cathepsin D isolated on hemoglobin Sepharose column[3]. Pepstatin interacts with cathepsin D strongly within pH range 3—6; at pH 8 the enzyme inhibitor complex is already completely dissociated. The procedure is efficient and rapid and avoids many of the difficulties previously encountered

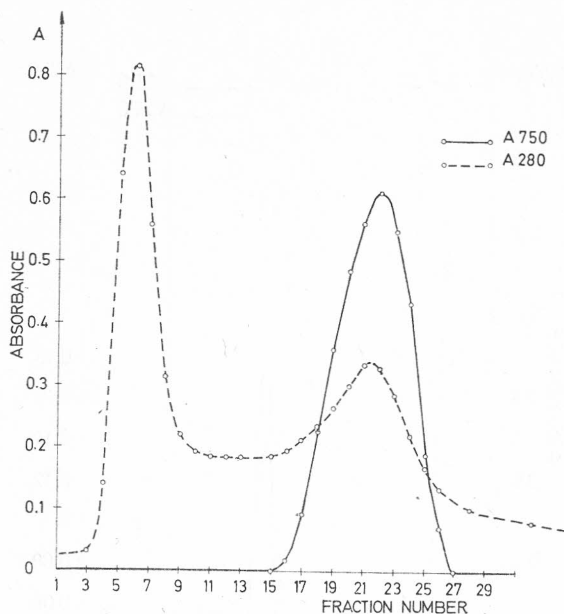

Figure 2. Sephadex G-100 chromatography of the enzyme from the affinity chromatography column. ——— proteolytic activity on hemoglobin at pH 3.5; — — — protein measured as A_{280}. Dimensions of the column were 100 × 2 cm, flow rate was 15 ml/h.

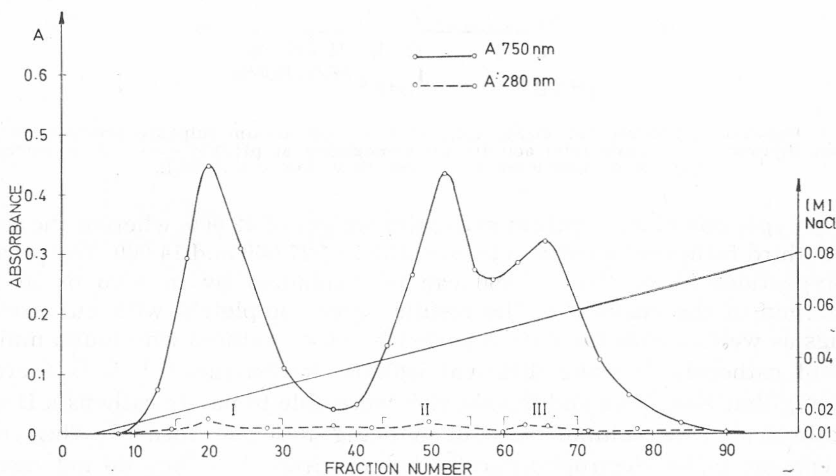

Figure 3. Separation of active fractions from Sephadex G-100 on DEAE cellulose column. Dimensions of the column were 30 × 2 cm. 0.025 M Tris buffer in 0.01 M NaCl was used for the the elution and later a linear gradient toward 0.08 M NaCl was applied. Flow rate vas 33 ml/h.

in the purification of cathepsin D[16]. It may be expected that this procedure would have an important application in the purification of cathepsin D from different sources.

Acknowledgements. We thank Mrs. M. Pregelj, Mrs. A. Burkeljc and Mr. K. Lindič for their excellent technical assistance. This work was supported by a grant from the Research Council of Slovenia and in part by a grant from the National Science Foundation, U.S.A., grant no. GF-31389.

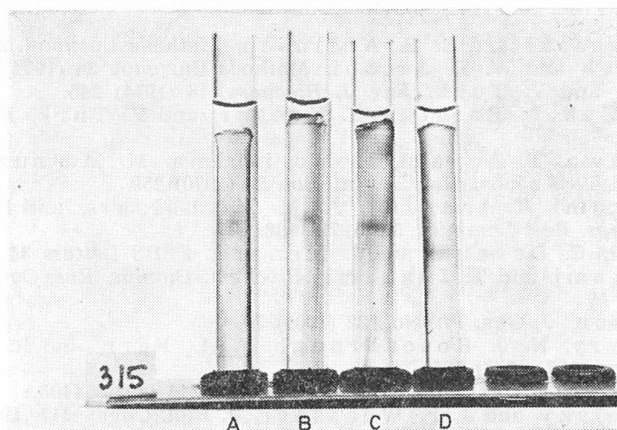

Figure 4. Polyacrylamide gel electrophoresis at pH 8.5. Gel A shows the enzyme fraction from Sephadex G-100, gels B, C and D show the separated multiple forms of cathepsin D (I, II, III).

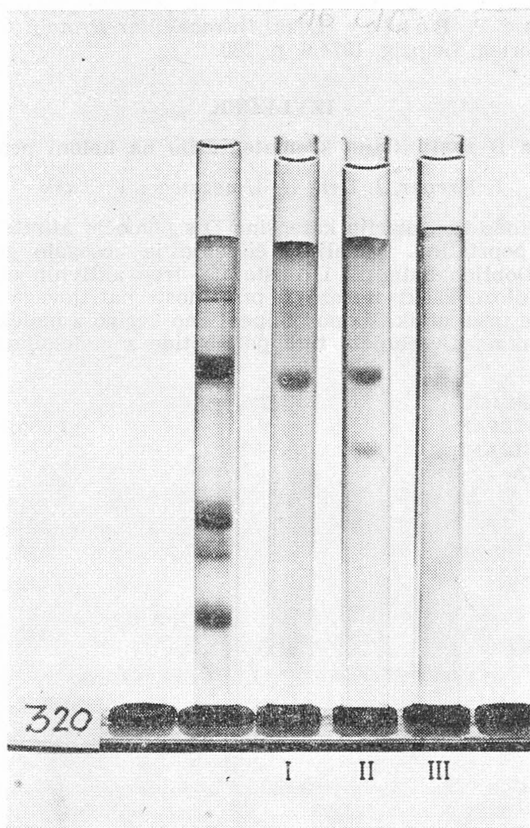

Figure 5. Dodecyl sulphate-polyacrylamide gel electrophoresis of separated multiple forms of cathepsin D (I, II, III). The standard proteins (left gel) have molecular weights of 45 000 (ovalbumin), 25 000 (chymotrypsinogen A) and 14 200 (lysozyme).

REFERENCES

1. P. Cuatrecasas and C. B. Anfinsen, *Methods Enzymol.* **22** (1972) 345.
2. M. Wilchek and W. B. Jacobi, *Methods Enzymol.* **34** (1974) 3.
3. R. Smith and V. Turk, *Eur. J. Biochem.* **48** (1974) 245.
4. F. Gubenšek, L. Barstow, I. Kregar, and V. Turk, *FEBS Letters* **71** (1976) 42.
5. H. Umezawa, T. Aoyagi, H. Morishima, M. Matsuzaki, M. Hamada, and T. Takeuchi, *J. Antibiot.* **23** (1970) 259.
6. K. Murakami, T. Inagami, A. M. Michelakis, and S. Coen, *Biochem. Biophys. Res. Commun.* **54** (1973) 482.
7. P. Corvol, C. Devaux, and J. Menard, *FEBS Letters* **34** (1973) 189.
8. K. Murakami and T. Inagami, *Biochem. Biophis. Res. Commun.* **62** (1975) 757.
9. M. L. Anson, *J. Gen. Physiol.* **22** (1939) 79.
10. O. H. Lowry, N. J. Rosenbrough, A. L. Farr, and R. J. Randall, *J. Biol. Chem.* **193** (1951) 265.
11. K. Weber and M. Osborn, *J. Biol. Chem.* **244** (1969) 4406.
12. A. I. Sapolsky and J. F. Woessner, *J. Biol. Chem.* **247** (1972) 2069.
13. A. J. Barrett, *Biochem. J.* **117** (1970) 601.
14. J. B. Ferguson, J. R. Andrews, I. M. Voynick, and J. S. Fruton, *J. Biol. Chem.* **248** (1973) 6701.
15. O. V. Kazakova and V. N. Orekhovich, *Biokhimiya* **40** (1975) 969.
16. V. Turk, I. Kregar, F. Gubenšek, R. Smith, and S. Lapanje, in: H. Hanson and P. Bohley (Eds.), *Intracellular Protein Catabolism,* J. Ambrosius Barth Verlag, Leipzig, 1974/6, p. 260.

IZVLEČEK

Čiščenje katepsina D z afinitetno kromatografijo na koloni pepstatin-sepharoze

I. Kregar, I. Urh, H. Umezawa i V. Turk

Razvili smo metodo za izolacijo katepsina D s pomočjo afinitetne kromatografije na imobiliziranem pepstatinu. Nadaljnje čiščenje je obsegalo gelsko filtracijo na Sephadexu G-100. Dobljen katepsin D obstaja v treh aktivnih oblikah, katere smo ločili na DEAE celulozi. Elektroforeza v prisotnosti natrijevega dodecil sulfata je pokazala, da vsebuje prva oblika samo polipeptidno verigo z molekularno težo 42 000. Druga in tretja oblika pa vsebujeta tudi polipeptide z molekularnimi težami 27 000 in 24 000.

ODDELEK ZA BIOKEMIJO
INSTITUT JOŽEF STEFAN
UNIVERZA V LJUBLJANI
61000 LJUBLJANA

Prispjelo 28. prosinca 1976.