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Anticathepsin D Antibody-Sepharose Chromatography of Human Cathepsin D

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Cathepsin D was isolated from human tissues by anticathepsin D antibody-Sepharose 4B chromatography. Cathepsin D, released from the immunoaffinity column formed one precipitin line with specific antibody in immunodiffusion and in immunoelectrophoresis. The isolated proteinase is shown to be pure cathepsin D by activity and by inhibition with pepstatin.

The quantitative determination of cathepsin D in human tissue, taken by biopsy from scoliotic patients in very small amount, is described.

It was demonstrated that cathepsin D from human muscle and human gingival fluid was indistinguishable in immunodiffusion from the human liver cathepsin D.

INTRODUCTION

The method of affinity chromatography[1] is based on the highly specific interaction of proteins with corresponding ligands, and in many cases permits the isolation of a desired protein in a single step. Usually immobilized hemoglobin [2] or pepstatin[3] is used for the isolation of cathepsin D (EC 3.4.22.1). The interaction between cathepsin D and its antibody has been studied by many authors and used for various purposes.[4–6] In the present work we report a method capable of yielding a homogenous preparation of human liver cathepsin D, using immunoaffinity chromatography on CNBr activated Sepharose 4B. We also extended the method to a one step isolation of cathepsin D from human tissues obtained by biopsy from scoliostic patients.

MATERIALS AND METHODS

Isolation of cathepsin D. Cathepsin D was isolated from human liver. The purification steps included acid extraction at pH 4, ammonium sulphate precipitation (40—70%) and gel chromatography on Sephadex G-75.[7] The pooled fractions showing proteolytic activity toward hemoglobin, pH 3.5, named liver extract, were further purified on hemoglobin agarose resin.[2] This was followed by gel filtration on Sephadex G-100 at pH 8 in 0.01 M Tris HCl buffer, containing 0.01 M NaCl, and by ion exchange chromatography on DEAE-cellulose (gradient from 0.08—0.3 M NaCl) in the same buffer. Measurement of cathepsin D activity was made by the modified Anson's method [8] at pH 3.5. The specific activity was expressed by E_{750}/ml/h or in Anson Units/mg of protein.

Protein content was determined by the method of Lowry et al.[9]

Disc electrophoresis in gels containing 7% acrylamide was performed as outlined by Davis at pH 9.5.[10] The gels were stained in a 1% solution of Coomassie brilliant blue R-250 (Serva, Heidelberg, GDR).

Production of antiserum. 1 mg of purified cathepsin D, isolated from human liver, was injected into a rabbit. Procedure is based on the Weston's method[11] for production of antiserum to chicken liver cathepsin D : 1 ml of cathepsin D (0.6 mg/ml) was emulsified with an equal amount of Freund's complete adjuvant and injected subcutaneously 3 times : at day 1, 2 and 3. The rabbit was given a further 2 injections with 0.2 mg of cathepsin D every 14 days, without adjuvant. 10 days after the last injection, the rabbit was bled.

Double immunodiffusion analysis was carried out according to Ouchterlony's method[12] on 3% agar gel (Behringwerke AG, Marburg-Lahn, GDR) in 0.01 M phosphate buffer (PBS), pH 7.1, containing 0.1% sodium azide as bacteriostatic agent. After introduction of antigen (or sample containing cathepsin D) and specific anticathepsin D antibodies into the wells, the agar plates were developed for 48 hr at room temperature and than extensively rinsed with 0.15 M NaCl and distilled water. The dried plates were stained with 1% Coomassie brilliant blue in ethanol-acetic acid solution and destained with 5% acetic acid.

Immunoelectrophoresis was done by the method of Scheidegger[13] on 1.5% agar plates in 0.75 M veronal buffer of pH 8.6 at 8 °C for 130 min, with a voltage gradient of 8 V/cm. After electrophoresis, 50—100 µl of anticathepsin D antiserum were placed in the trough. Plates were developed for 24 hrs at room temperature. After that time, the plates were rinsed, dried, stained and destained as described above.

Measurement of the quantity of cathepsin D in human tissue. Samples of muscle tissue were taken from patients with idiopathic scoliosis during surgical treatment. From muscle samples we removed microscopic visible fat and connective tissue. A 10% homogenate was prepared from 100—500 mg of muscle tissue with 0.2 M sodium acetate buffer, pH 4.5 in a Sorvall micro-homogenizer and was then centrifuged at 300 000 g min. In supernatants cathepsin D was evaluated by the techniques proposed by Mancini et al.[14] 3 ml of 3% agarose (Calbiochem. Behring. Corp., La Jolla, USA) solution in 0.75 M veronal buffer, pH 8.6, containing 0.1% sodium azide, was mixed with 0.3 ml of antiserum at 45 °C. A known amount of tissue homogenate (2—10 µl) was placed in the wells. After 48 hrs of immunodiffusion, a standard curve was plotted, relating the diameter of the precipitation circle to the amount of standard antigen used.

Preparation of immunoglobulins. IgG were purified from anticathepsin D antiserum by gel filtration on Sephadex G-150 followed by precipitation with 50% saturated ammonium sulphate at 4 °C, by the procedure described by Flodin and Killander.[15]

Preparation of immunoaffinity column. An antibody-Sepharose 4B column was prepared as suggested by Musi et al.[16] for rat liver cathepsin D, and by the procedure of the manufacturer (Pharmacia, Uppsala, Sweden). Anticathepsin D antibody (20 mg IgG) was coupled to 4 g CNBr activated Sepharose 4B in 0.2 M sodium bicarbonate buffer at pH 8, containing 0.5 M NaCl. The antibody--Sepharose conjugate was washed extensively with borate buffer, pH 8, containing 0.5 M NaCl, and acetate buffer, pH 4, and finally with 0.15 M phosphate buffer, pH 8, containing 0.05 M NaCl.

Purification of cathepsin D.

The extract of human liver, previously dialyzed against 0.15 M phosphate buffer, pH 8, containing 0.05 M NaCl, was applied to the antibody-Sepharose chromatography column. After sample binding (approx. 1.3 mg of protein in 1

ml), the column was washed with starting buffer (phosphate, pH 8) to remove unbound proteins. The protein which showed proteolytic activity against hemoglobin, pH 3.5, was released with glycine-HCl buffer pH 2.2. Each tube contained a few drops of 0.5 M NaOH to bring the pH to 6. 2ml fractions were collected.

A supernatant of human muscle homogenate (1 ml) previously dialyzed against 0.15 M phosphate buffer, pH 8, containing 0.05 M NaCl, was applied to the immunoaffinity column. After binding the muscle cathepsin D and washing off unbound proteins, the active enzyme was released with glycine-HCl buffer, pH 2.2.

RESULTS

The elution profile and purification yields of human liver cathepsin D are given in Figure 1 and Table I, respectively. From Figure 1, it is evident that by one step separation using affinity chromatography on antibody-Sepharose, the isolation of enzyme protein was achieved in fractions 10—15. As can be seen from Table I, the yield of eluted protein was 6.5% and that of released enzyme activity was 10%. The characteristic property of the eluted proteniase was also tested by pepstatin inhibition.[15] Pepstatin, a naturally occuring tightly-binding inhibitor of aspartic proteinase cathepsin D, completely (100%) inhibited the eluted proteinase.

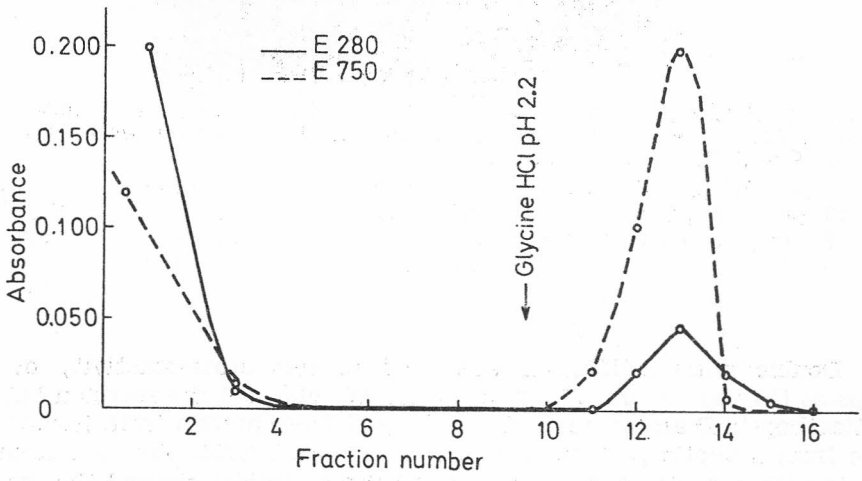

Figure 1. Elution pattern of protein, released from anticathepsin D antibody-Sepharose 4B chromatography column.

TABLE I
The Yield of Cathepsin D, Purified from Human Tissues.

Purification step	Protein mg	Yield %	Activity A.U.	Yield %
Human liver extract	1.300	100	4.370	100
Antibody-Sepharose	0.085	6.5	0.473	10
Human muscle supernatant	0.460—2.000	100	2.000—6.000	100
Antibody-Sepharose	0.007—0.030	~15	0.400—1.200	~20

The immunological identification and homogeneity of eluted cathepsin D (from fractions 10—15 in Figure 1) were confirmed by the double immunodiffusion, as shown in Figure 2.

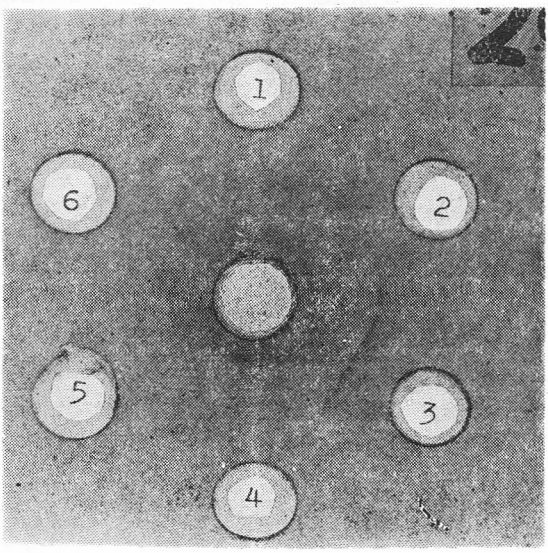

Figure 2. Double immunodiffusion of human liver cathepsin D eluted from antibody-Sepharose 4B. 10 μl of anticathepsin D antiserum were applied in the midle well. The wells contained:
1) 10 μl (13.2 μg) of human liver homogenate, 2) 10 μl (6 μg) of antigen, 3) 10 μl (12.8 μg) of isolated cathepsin D (fractions 10-15 in Figure 1), 4) — 6) 10 μl of concentrated eluate, pooled from fractions 1-4, 5-9 and 16-19 in Figure 1.

Double immunodiffusion was used to test cross-reactivity of cathepsins D from different human tissues. We checked the reaction between anticathepsin D antiserum and cathepsin D from human liver, human muscle from scoliotic patients and human gingival fluid, obtained from patients with periodontitis (Figure 3). All three samples showed the reaction of »complete identity« with the antibody directed to liver cathepsin D.

The elution patterns of human muscle homogenates on immobilized human liver anticathepsin D antibody-Sepharose were identical with those of human liver extract. 7 — 30 μg of cathepsin D were released from the antibody-Sepharose chromatography column. The yield of eluted enzyme was about 15% (Table I) and that of the activity about 20%.

Besides polyacrylamide gel electrophoresis (PAGE) shown in Figure 4, and double immunodiffusion (Figure 3), the purity of isolated muscle cathepsin D was also checked by immunoelectrophoresis. The precipitin line developed with anticathepsin D antiserum and isolated muscle cathepsin D is shown in Figure 5; only one precipitin line developed, confirming the identity and purity of the isolated enzyme.

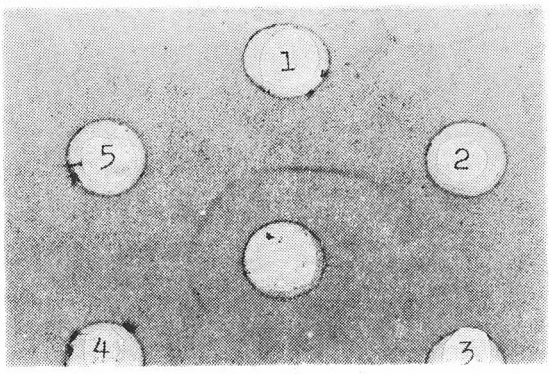

Figure 3. Double immunodiffusion of cathepsin D from different human samples. In the middle well: 10 μl of anticathepsin D antiserum. In the outer wells: 1) 10 μl (12.8 μg) of human muscle cathepsin D, 2) - 4) 2 μl of human gingival fluid, obtained from different patients, 5) 10 μl (13.2 μg) of human liver cathepsin D.

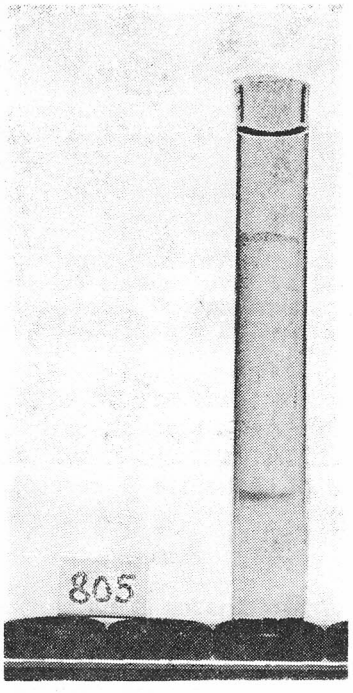

Figure 4. Gel electropherogram of isolated human muscle cathepsin D.

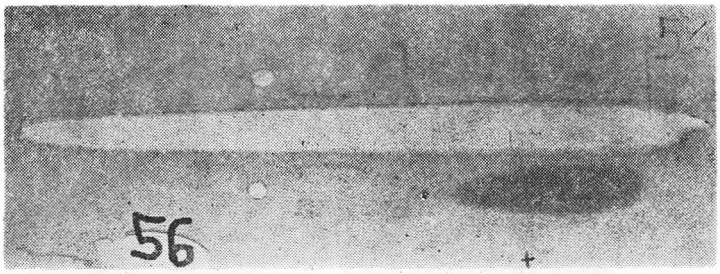

Figure 5. Immunoelectrophoresis of cathepsin D from human muscle. Well (5 μl) contained isolated muscle cathepsin D; the trough contained 50 μl of anti-liver cathepsin D antiserum.

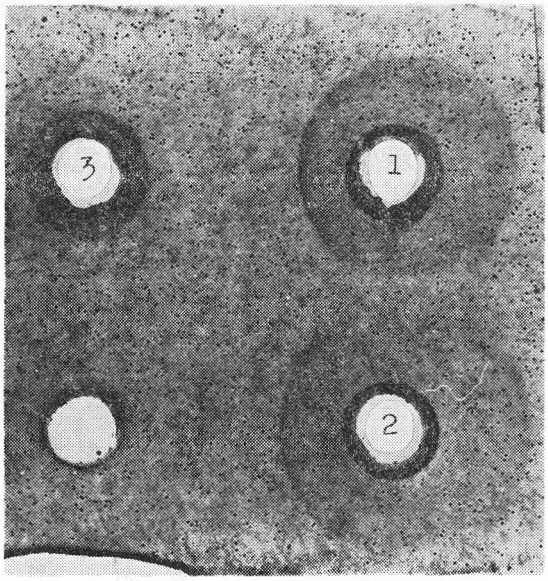

Figure 6. Quantitation of cathepsin D in supernatant of human muscle homogenates. Well 1) contained 10 μl of muscle supernatant, obtained from the first patient, and well 2) contained 10 μl of muscle supernatant from the second patient. Well 3) was a control (liver, 10 μl of cathepsin D).

From the Figure 6 it is evident that in muscle homogenates obtained from two patients with idiopathic scoliosis by biopsy, in quantities of less than 0.5 ml, containing 2.1 mg/ml and 2.8 mg/ml of protein, there were 0.69 μg/μl and 0.85 μg/μl of cathepsin D, respectively.

DISCUSSION

The determination of proteinases in human tissue in amounts of less than 500 mg represents a difficult technical problem. For quantitative measurements of cathepsin D a single radial immunodiffusion in agar gel, containing specific antibody can be used (Figure 6). The immunoprecipitate diameter correlates with quantity of the cathepsin D.

When only a small amount of material is available and it is therefore not possible to isolate cathepsin D by standard procedures[2,3,6] the antibody-Sepharose chromatography can be used. As shown in this report (Figure 1—5) the one-step separation and quantitative estimation of human cathepsin D with the described technique is very convenient. Liver cathepsin D which was released from antibody-Sepharose was identical to cathepsin D, isolated by affinity chromatography on hemoglobin resin[2] with regard to its molecular weight (unpublished results) and immunoprecipitation (Figure 2).

The use of anticathepsin D antibody-Sepharose chromatography is highly recommended, where specific and rapid extraction of the enzyme from small amounts of human tissue is desirable. It enables quantitative determination of cathepsin D under physiological and pathological conditions. The antibody-Sepharose can be used many times. In our case we carried out more than 10 experiments over a period of six months without any appreciable loss of binding capacity.

Using specific antiserum prepared against chicken liver cathepsin D, Weston[11] showed that cathepsin D from different chicken organs is identical as far as their cross-reactivity with chicken liver cathepsin D is concerned. Similarly, it was demonstrated[4] that cathepsin D from human rheumatoid synovium was indistinguishable in immunodiffusion from the human liver cathepsin D.

On the basis of these experiments it is improbable that specific antisera raised against human liver cathepsin D would fail to react with that from human muscle. The binding and elution profile of muscle cathepsin D from antibody-Sepharose were immunologically identical with those of human liver cathepsin D. Cathepsin D from human muscle, human liver and human gingival fluid showed the reaction of »complete identity« confirming that cathepsin D is species (but not organ) specific.

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REFERENCES

1. P. Cuatrecasas, M. Wilchek, and C.B. Anfinsen, *Proc. Nat. Acad. Sci. USA* **61** (1968) 636.
2. R. Smith and V. Turk, *Eur. J. Biochem.* **48** (1974) 245.
3. I. Kregar, I. Urh, H. Umezawa, and V. Turk, *Croat. Chem. Acta* **49** (1977) 587.
4. J.T. Dingle, A.J. Barrett, and P.D. Weston, *Biochem. J.* **123** (1971) 1.
5. A.H Ericson and G. Blobel, *J. Biol. Chem.* 254 (1974) 11771.
6. J. Tang, *Moll. Cell. Biochem.* 26 (1979) 93.
7. T. Zvonar, I. Kregar, and V. Turk, *Croat. Chem. Acta* 52 (1979) 411.
8. M.L. Anson, *J. Gen. Physiol.* 22 (1939) 79.
9. O.H. Lowry, M.J. Rosebrough, A.L. Farr, and R.J. Randall, *J. Biol. Chem.* **193** (1951) 265.

10. B. J. Davis, *Ann. N. Y. Acad. Sci.* **23** (1964) 404.
11. P. D. Weston, *Immunology* **17** (1969) 421.
12. O. Ouchterlony and L. A. Nilson, *Handbook of Experimental Immunology* D. M. Weir (Ed.) Blackwell Sci. Publ., Oxford 1973, p. 19-20.
13. J. J. Scheidegger, *Int. Arch. Allergy* **7** (1955) 103.
14. G. Mancini, A. O. Carbonara and J. F. Heremans, *Immunochemical Techniques for the Identification and Estimation of Macromolecules.* 2nd edition. J. Clausen (Ed.) Elsevier North Holland, Amsterdam 1981, p. 229-230.
15. P. Flodin and J. Killander, *Biochim. Biophys. Acta* **63** (1962) 403.
16. M. Musi, A. O. Carbonara, O. V. Kazakova, and F. M. Baccino, *IRCS Med. Sci.* **10** (1982) 565.
17. C. G. Knight and A. J. Barrett, *Biochem. J.* **155** (1976) 117.

SAŽETAK

Imunoafinitetna kromatografija humanog katepsina D

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S pomoću imunoafinitetne kromatografije s vezanim protutijelom, dobivenim protiv humanog katepsina D, na CNBr-aktiviranoj Sepharose 4B izolirali smo katepsin D iz ljudske jetre i mišića. Eluirani enzim pokazivao je jednu liniju sa specifičnim protutijelom i na imunodifuzijskoj i na imunoelektroforetskoj ploči. Aktivnost izoliranog katepsina D potvrdili smo mjerenjem aktivnosti na hemoglobinski substrat i mjerenjem inhibicije pepstatinom. Opisana je i jednostavna radijalna imunodifuzija u agarskom gelu, kojom smo kvantitativno mjerili katepsin D u ljudskom tkivu dobivenom biopsijom u veoma malim količinama. Pokazali smo, da se katepsin D iz ljudskih mišića i iz ljudskoga gingivalnog fluida imunološki ne razlikuje od ljudskog katepsina D iz jetre