Two-step affinity-chromatographic purification of cathepsin D from pig myometrium with high yield*

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Cathepsin D was purified by two-step affinity chromatography on concanavalin A- and pepstatin-Sepharose. The main purification was achieved by washing the enzyme bound to the pepstatin-Sepharose column with buffered 6M-urea. This step separated cathepsin D from all low- and high-molecular-weight impurities. Although the 1700-fold purified acid proteinase was homogeneous on sodium dodecyl sulphate/polyacrylamide-gel electrophoresis, it still showed microheterogeneity.

During our studies on the involution of the rat uterus *post partum*, we have described different proteinases and a proteinase-inhibitor protein which could be involved in the rapid intracellular proteolysis that occurs in the myometrium after delivery (Afting, 1979). Among the proteinases, a proteolytic activity with an acid pH optimum increased rapidly during the last third of pregnancy, increased abruptly in days 1 and 2 after parturition and disappeared gradually over the next 10 days *post partum* (Afting *et al.*, 1979). We have purified and characterized this enzyme, which turned out to be cathepsin D, known from a variety of other tissues (for a review see Barrett, 1977).

The purification procedures for cathepsin D published in recent years afforded a variety of different purification steps and resulted always in low recovery. This paper describes a rapid two-step affinity-chromatographic purification of cathepsin D from pig myometrium with high yield. This method is also applicable to the purification of the enzyme from other tissues and may help to isolate cathepsin D on a large scale to allow amino acid sequence studies as well as the elucidation of the glycosidic side chain of this glycoprotein.

Materials and methods

Materials

Concanavalin A-Sepharose and Sepharose 4B-

Abbreviation used: SDS, sodium dodecyl sulphate.

* Dedicated to Professor Dr. Helmut Holzer on the occasion of his 60th birthday.

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CL were obtained from Deutsche Pharmacia, Freiburg, Germany, and pepstatin was from the Protein Research Institute, Osaka, Japan. All other reagents used were of analytical grade and purchased from Merck, Darmstadt, Germany, or Roth, Karlsruhe, Germany.

Assay of cathepsin D

The acid proteinase activity was measured at 37°C with a solution of haemoglobin (12 mg/ml) in 0.2M-sodium citrate, pH 3.3, as substrate, by analogy with the method of Anson (1938). The incubation (final volume of mixture 0.7 ml, in 1.5 ml Eppendorf micro test tubes) was stopped after 30 min incubation by the addition of 0.5 ml of 10% (w/v) trichloroacetic acid. After 15 min on ice the tubes were centrifuged in an Eppendorf table-top centrifuge at maximal speed for 3 min, and 0.5 ml of the supernatant was neutralized with 0.25 ml of 1M-NaOH and assayed for protein by the method of Lowry *et al.* (1951). One unit of cathepsin D is defined as that amount of enzyme that causes a rate change of 0.1 A_{750} unit/h in the incubation mixture under the test condition described.

Determination of protein

Protein was measured as described by Lowry *et al.* (1951), with crystalline bovine serum albumin as standard.

Electrophoresis

SDS/polyacrylamide-gel electrophoresis was performed as described by Laemmli & Favre (1973), and disc electrophoresis as described by Maurer (1971); 7.5% polyacrylamide gels were used throughout.

Preparation and extraction of the tissue

Pig uteri were collected immediately after slaughter, kept on ice and used within 90 min after death. All operations were performed at 4°C. Fat and connective tissue were removed, the uterine horns slit open and the endometrium was scraped off. The external serosa was stripped off as far as possible. The strips of myometrium so obtained were put twice through a hand-powered meat mincer (1 mm holes), and the mince was washed with 3 vol. of 0.9% NaCl to get rid of most of the serum contaminants. The mince was then homogenized in 30 mM-veronal buffer, pH 7.4, for 3 × 30 s in an Omni-Mix blender at maximum speed. The soluble phase was separated by centrifugation for 10 min at 25 000g. The supernatant so obtained was used as crude extract.

Concanavalin A–Sephacel chromatography

The crude extract containing cathepsin D was combined with concanavalin A–Sephacel (1 g wet wt. of gel/10 ml of crude extract) and stirred slowly for 30 min. The gel was collected by suction on a Büchner funnel, washed with 500 ml of 30 mM-veronal buffer, pH 7.4, containing 0.6 M-NaCl and 1 mM-mercaptoethanol, and poured into a column (2.5 cm × 50 cm). The column was washed with the same buffer at a constant flow rate of 20 ml/h until no protein could be measured in the eluate. The proteinase was eluted from the concanavalin A–Sephacel gel with 0.1 M-sodium phosphate buffer, pH 6.5, containing 0.2 M- α -methyl mannoside, 0.6 M-NaCl and 1 mM-mercaptoethanol. The eluate with cathepsin D activity can be fractionated and pooled, but generally this eluate was directly pumped on to the pepstatin affinity column.

Preparation of the pepstatin–Sephacel affinity column

Pepstatin was coupled to aminoethyl–Sephacel as the *N*-hydroxysuccinimide ester derivative (Cuatrecasas & Parikh, 1972), as described by Cuatrecasas (1970). The aminoethyl–Sephacel was prepared as reported by Shaltiel (1974).

Chromatography on pepstatin–Sephacel

The pooled fractions, or generally the eluate, from concanavalin A–Sephacel chromatography were directly applied to a pepstatin–Sephacel column (1 cm × 6 cm) at a flow rate of 20 ml/h. The column was washed with 100 ml of 50 mM-citrate buffer (pH 3.3)/0.6 M-NaCl, then with 25 ml of the same buffer containing 6 M-urea, followed by 100 ml of the citrate/NaCl buffer again. The activity was eluted with 0.05 M-NaHCO₃/0.6 M-NaCl (pH 8.2) at a flow rate of 30 ml/h and collected in 8 ml fractions. The active fractions were pooled, dialysed overnight against 2 mM-sodium phosphate buffer, pH 6.5, and

freeze-dried. The dry snow-white residue was dissolved in 2 ml of 10 mM-sodium phosphate buffer, pH 6.5. In the freeze-dried form, the cathepsin D could be stored at –20°C for several months without loss of activity.

Results and discussion

The lysosomal glycoprotein cathepsin D was isolated from most other enzymes in the crude extract by chromatography on concanavalin A–Sephacel. The enzyme was eluted from the affinity column with 0.2 M- α -methyl mannoside, resulting in a 13.4-fold purification (Table 1). A linear gradient of α -methyl mannoside, tried to separate the cathepsin D from other glycoproteins, resulted in no further purification or separation (results not shown).

The main purification step of cathepsin D was the highly specific affinity chromatography on pepstatin–Sephacel (Table 1). Pepstatin, an inhibitor of carboxy-proteinases (Umezawa, 1972), shows a very strong inhibition of and interaction with cathepsin D (Knight & Barrett, 1976). The activity completely stuck to the column, while most of the inactive proteins passed through (Fig. 1). If the cathepsin D was directly eluted after prolonged washing with 30 mM-citrate buffer (pH 3.3)/0.6 M-NaCl, the enzyme preparation was still contaminated by many other proteins, which were also bound to the affinity column by specific or unspecific hydrophobic interactions (Fig. 2b). On the other hand, if the column was further developed with 6 M-urea in 30 mM-citrate buffer (pH 3.3)/0.6 M-NaCl, a protein peak could be eluted which showed no acid-proteolytic activity (Fig. 1). In SDS/polyacrylamide-gel electrophoresis this peak contained only huge amounts of high- and low-molecular-weight-protein-bands (Fig. 2c), but the cathepsin D band was not seen (Fig. 2d).

Fig. 1. Chromatography of cathepsin D on a pepstatin–Sephacel column

○, Protein concn.; ●, cathepsin D activity. For full details see the Materials and methods section.

Table 1. *Purification of cathepsin D*

	Volume (ml)	Activity (units/ml)	Protein (mg/ml)	Total activity (units)	Sp. activity (units/mg)	Purification (-fold)	Recovery (%)
Crude extract	658	105.8	6.7	69 600	15.8	1	100
Concanavalin A eluate	600	105.6	0.5	63 400	211.2	13.4	91
Pepstatin eluate	64	380.6	0.014	24 400	27 190	1720	35
After freeze-drying	2	12 000	0.44	24 000	27 300	1730	34

Fig. 2. *SDS/polyacrylamide-gel electrophoresis of cathepsin D*

Lanes: (a) molecular-weight standards (RNA polymerase β -chain, 155 000; bovine serum albumin, 68 000; RNA polymerase α -chain, 39 000; soya-bean trypsin inhibitor, 21 500); (b) cathepsin D eluted from pepstatin-Sepharose without prior washing with 6M-urea; (c) proteins eluted from pepstatin-Sepharose with 6M-urea; (d) cathepsin D after elution from the urea-washed pepstatin-Sepharose column.

Fig. 3. *Microheterogeneity of myometrial cathepsin D* Polyacrylamide-gel electrophoresis of purified cathepsin D was performed at pH 8.9 (a). The upper (cathodic) part of the gel, indicated by a bar, was cut into 55 pieces each 0.5 mm thick and tested for cathepsin D activity (b). In slices 1–14 no activity could be measured.

The cathepsin D, which in solution is irreversibly inactivated by 6M-urea, retained its activity on the pepstatin affinity column when washed with the same concentration of buffered urea. If the cathepsin D was eluted from the pepstatin-Sepharose column without the 6M-urea washing, the same yield of total activity was obtained. The fall in recovery on the pepstatin affinity gel (Table 1) is therefore not due to a partial inactivation of the enzyme. Probably this could be the result of the separation of 'activator-proteins' at this step, as suggested by Huang & Tang (1977).

Since cathepsin D seems to be one of the main lysosomal endopeptidases responsible for intracellular protein degradation, many purification studies have been reported [for reviews see Smith & Turk (1974), Wiederanders *et al.* (1976), Barrett (1977) and Huang *et al.* (1979)]. However, the yields have always been low, and in those cases

Cathepsin D was eluted from the urea-washed column by a pH-shift to pH 8.2, normally by using 0.05M-NaHCO₃/0.6M-NaCl as eluent. The eluted enzyme was homogeneous, as judged by SDS/polyacrylamide-gel electrophoresis, with mol.wt. 30 000 (Fig. 2d). This was in good agreement with the molecular weight of 32 000 determined by gel chromatography on Bio-Gel P 40 (results not shown). Only when the SDS/polyacrylamide gel was heavily overloaded, an additional faint protein band of 43 000 mol.wt. was visible. This could be a cathepsin D precursor, which was described by Erickson & Blobel (1979).

where a pepstatin column was used in the course of purification, the cathepsin D preparation eluted without the prior urea washing of the enzyme bound to the affinity gel was impure (Fig. 2b) and required additional purification steps with low recovery. The two-step purification procedure reported in the present paper has a high yield and the purification is more than 1700-fold.

Despite the high purification and the homogeneity in SDS/polyacrylamide-gel electrophoresis (Fig. 2d), the enzyme was still micro-heterogeneous. As shown in Fig. 3(a), in polyacrylamide disc-gel electrophoresis at pH 8.9 one prominent and two minor bands could be seen. When the upper part of the gel, indicated by the bar in Fig. 3(a), was cut into 55 slices and the slices were tested for cathepsin D activity, all three bands were correlated with a proteolytic activity (Fig. 3b). This microheterogeneity could possibly result from isoenzymes differing in amino acid composition and/or core glycosylation, or could be caused by different lysosomal populations found in the various cell types of the myometrial layer of this organ.

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