Cathepsin D from pig myometrium

Characterization of the proteinase

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The purification of cathepsin D from pig uterus by two-step affinity chromatography on concanavalin A- and pepstatin-Sepharose was described previously [Afting & Becker (1981) *Biochem. J.* **197**, 519–522]. In this paper, chemical and physical properties of the proteinase are presented. The purified enzyme showed three bands on SDS (sodium dodecyl sulphate)/polyacrylamide-gel electrophoresis, one main band corresponding to an M_r of 31 000 and two minor bands with M_r values of 43 000 and 15 000 respectively. Gel filtration on Bio-gel P-150 and sedimentation-diffusion equilibrium studies give an M_r for the main band of about 35 000. The pI of the enzyme was determined to be 7.2. Haemoglobin was the best substrate, with a K_m value of 6.4×10^{-6} M. It was hydrolysed with a pH optimum between 3.0 and 3.3 for a substrate concentration of 100 μ M. The proteinase was stable over the pH range of 3.5–6.5. At pH 6 the enzyme showed stability up to a temperature of 50°C; at pH 3 the activity was already decreased below 40°C. Carbohydrate studies resulted in the staining of all three bands on an SDS/polyacrylamide gel by thymol/ H_2SO_4 . After treatment with endo- β -N-acetylglucosaminidase H, all three bands were shifted to a region of lower M_r . Of various inhibitors tested, only pepstatin was strongly inhibiting, with a K_i of 2.1×10^{-9} M.

Cathepsin D is an acid proteinase which, as one of the main lysosomal endopeptidases, is thought to play an important role in the breakdown of intracellular and extracellular proteins. It is widely distributed in animals and has been purified from various tissues (Press *et al.*, 1960; Kress *et al.*, 1966; Barrett, 1970; Sapolsky & Woessner, 1972; Ferguson *et al.*, 1973; Reichelt *et al.*, 1974; Smith & Turk, 1974; Cunningham & Tang, 1976; Wiederanders *et al.*, 1976; Kregar *et al.*, 1977; Okitani *et al.*, 1981).

There have been many reports about isoenzymes and partly active/partly inactive forms of cathepsin D in various tissues with different M_r values. For example, Sapolsky & Woessner (1972) postulated twelve isoenzymes of cathepsin D in bovine uterus. Since our earlier studies on rat uterus had shown great increase and decrease of cathepsin D activity during pregnancy and involution, especially a sharp increase in activity on days 1 and 2

after parturition (Afting *et al.*, 1979), it can be assumed that this enzyme plays a key role in the rapid postpartal protein degradation in the myometrium. The potentially present isoenzymes could have a regulatory role, or some of them could contain inhibitor peptides that would be cleaved to activate the proteinase as has been described for other proteinases by Puizdar & Turk (1981).

In a previous paper (Afting & Becker, 1981) we described the purification of cathepsin D from pig uterine myometrium by the use of affinity chromatography on concanavalin A- and pepstatin-Sepharose. In the present paper physical and chemical properties of the proteinase are given.

Experimental

Materials

The following compounds were purchased from the indicated sources: Bio-gel P-150 from Bio-Rad, München, Germany; carrier ampholytes from Serva, Heidelberg, Germany; concanavalin A-Sepharose and Sepharose 4B-CL from the Deutsche Pharmacia, Freiburg, Germany; endo- β -N-

Abbreviation used: SDS, sodium dodecyl sulphate.

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acetylglucosaminidase H (EC 3.2.1.96) from Seikagaku Kogyo Co., Tokyo, Japan; Freund'sches Adjuvans from Behringwerke, Marburg, Germany; pepstatin 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.

Enzyme purification

The enzyme was purified from pig uterus as described by Afting & Becker (1981).

Activity assay

The assay of proteolytic activity was performed by the method of Anson (1938) with the modifications previously published (Afting & Becker, 1981).

Protein determination

Protein was determined by the method of Lowry *et al.* (1951), with bovine serum albumin as standard.

Polyacrylamide-gel electrophoresis

Polyacrylamide-gel electrophoresis was performed in 12.5% (w/v) gels as described by Laemmli & Favre (1973). Gels were stained for protein with Coomassie Brilliant Blue R250 and destained in methanol/acetic acid/water (8:3:29, by vol.), and for carbohydrate with thymol/H₂SO₄ by the method of Racusen (1979).

Isoelectric focusing

Gel electrofocusing was carried out as described by Wrigley (1971). Polyacrylamide gels (7.5%, w/v), containing 1% carrier ampholytes (pH 3–10), were used. After electrofocusing at 80 V for 15 h and 300 V for 1 h, one of the duplicate gels was stained with Coomassie Brilliant Blue R250 [trichloroacetic acid (10%, w/v) and sulphosalicylic acid (3%, w/v) were added to the staining solution to avoid staining by ampholines] for 30 min at 60°C and destained in ethanol/acetic acid/water (3:1:8, by vol.). The other duplicate gel was cut into 3 mm pieces and the pieces were minced and extracted with 0.4 ml of water; pH and proteolytic activity of the extracts were measured.

Incubation with endo- β -N-acetylglucosaminidase H

The purified enzyme (100 μ g) was dissolved in 0.05 M-sodium citrate buffer, pH 5.5, and incubated with 0.02 unit of endo- β -N-acetylglucosaminidase H at 37°C for 17 h. The reaction was stopped by adding solubilizer and heating the samples for 10 min at 95°C.

Results

M_r

The enzyme, purified from fresh pig uterus by two-step affinity chromatography, showed three bands on SDS/polyacrylamide-gel electrophoresis (Fig. 1, lane 3), one main band (M_r 31 000) and two minor bands (M_r 43 000 and 15 000 respectively).

Gel-filtration on a calibrated Bio-Gel P-150 column as well as sedimentation-diffusion equilibrium studies gave an M_r of $35\,000 \pm 2\,500$ (results not shown).

Isoelectric point

Determination of the proteolytic activity and the pH values in extracted gel slices after isoelectric focusing yielded a pI of 7.2. When stained with Coomassie Brilliant Blue R250, the proteinase exhibited one band which appeared at a position corresponding to pH 7.2 (Fig. 2).

Fig. 1. SDS/polyacrylamide-gel electrophoresis of cathepsin D

Lane 1, M_r standards (from top to bottom: ovalbumin, 43 000; carboanhydrase, 30 000; soyabean trypsin inhibitor, 20 100; α -lactalbumin, 14 400); lane 2, cathepsin D after incubation with 0.01 unit of endo- β -N-acetylglucosaminidase H at 37°C for 17 h; lane 3, purified cathepsin D.

Fig. 2. Isoelectric focusing of purified cathepsin D. Gel electrofocusing was performed in 7.5% (w/v)-polyacrylamide gels containing 1% ampholytes (pH 3–10). Gel slices (3 mm) from an unstained gel were assayed for pH (○) and proteinase activity (●) (b). The activity corresponded to the stained band on a control gel (a).

Fig. 3. pH-dependence of activity and stability of cathepsin D

Buffers used: pH 2–6, citrate; pH 6–8, phosphate; pH 8–10, borate. The purified enzyme was assayed by the standard method at different pH values (○). For the determination of stability, samples of purified proteinase (2.5 μ g/150 μ l) were incubated at 37°C for 1 h at different pH values and assayed by the standard method (●).

Fig. 4. Thermal stability of proteinase activity. Cathepsin D (2.5 μ g/100 μ l) was exposed to different temperatures for 10 min in 50 mM-citrate buffer, pH 3.0 (○) and 50 mM-citrate buffer, pH 6.0 (●) respectively. The samples were cooled and assayed by the standard method.

pH-dependence of activity

The purified enzyme showed a pH optimum between 3.0 and 3.3 when assayed with haemoglobin as substrate for a concentration of 100 μ M. The proteinase was stable over the pH range 3.5–6.5. Above pH 7 there was a rapid decrease in activity over a prolonged incubation time (Fig. 3).

Temperature stability

Fig. 4 shows the heat inactivation of the purified enzyme at different pH values (3.0 and 6.0 respectively). At pH 6 the proteinase remains almost stable up to an incubation temperature of 50°C; at pH 3 the activity already starts to decrease at temperatures below 40°C.

Carbohydrate content

Incubation of an SDS/polyacrylamide gel, run with a sample of purified cathepsin D (Fig. 1, lane 3), in thymol/H₂SO₄ resulted in the staining of all of the three bands mentioned above (results not shown).

Incubation of a cathepsin D sample with endo- β -N-acetylglucosaminidase H followed by a SDS/polyacrylamide-gel electrophoresis of the mixture showed that all three bands were completely or partially shifted to a lower- M_r position, which confirmed the above results (Fig. 1, lane 2).

Substrate specificity

The substrate protein most effectively cleaved was haemoglobin, followed by bovine serum albumin, casein and actomyosin. Azocoll was not cleaved at all. Low- M_r substrates, such as *p*-nitro-anilides of different amino acids, *N*-acetyl-L-tyrosine ethyl ester or *N*-benzoyl-L-arginine ethyl ester were not cleaved by the proteinase.

Effect of inhibitors

Cathepsin D was not inhibited by chelating agents, metal ions or thiol reagents. It was only slightly inhibited by the serine-specific reagents phenylmethanesulphonyl fluoride or tosyl-phenylalanylchloromethane ('TPCK'), by phenylpyruvate and the two trypsin inhibitors kallikrein

Fig. 5. Effect of pepstatin on the activity of cathepsin D. Increasing amounts of inhibitor were added to a known amount of cathepsin D. The mixture was incubated for 15 min and the remaining enzyme activity was measured by the standard method. v_i , Enzyme activity after addition of pepstatin; v_0 , enzyme activity without pepstatin; $a = v_i/v_0$; I_0 , inhibitor concentration. The inset shows a replot from which K_i was calculated to be 2.1×10^{-9} M.

Fig. 6. Effect of substrate concentration on the enzyme activity (Lineweaver-Burk plot)

The purified enzyme was assayed in 0.2 M-sodium citrate buffer, pH 3.3, with haemoglobin concentrations ranging from $3.5 \mu\text{M}$ to $50 \mu\text{M}$. The K_m value was found to be 6.4×10^{-6} M.

and soyabean trypsin inhibitor. Of five inhibitor peptides from microbial sources, only pepstatin showed a strong inhibition at a concentration of 1.5 nM . The level of inhibition by pepstatin was about the same over the whole pH range where the proteinase was active. The K_i value was found to be 2.1×10^{-9} M (Fig. 5).

Effect of substrate concentration

The activity of cathepsin D was maximal from a substrate concentration of $30 \mu\text{M}$ upwards. There was no inhibition at higher concentrations of haemoglobin. The K_m value was determined as 6.4×10^{-6} M by means of a Lineweaver-Burk plot (Fig. 6).

Discussion

The cathepsin D that was purified from pig uterus for the first time in a homogeneous form has properties that are in general agreement with those of the enzyme purified from other tissues (Barrett, 1970; Ferguson *et al.*, 1973; Cunningham & Tang, 1976; Kregar *et al.*, 1977).

With regard to the M_r , most of the reports describe multiple forms of the enzyme with different M_r values (Sapolsky & Woessner, 1972; Huang *et al.*, 1979). There are, on the other hand, a few reports about a single polypeptide chain of cathepsin D (M_r 42000) with no multiple forms (Okitani *et al.*, 1981; Kazakova & Orekhovich, 1975). In our case only one major form (M_r 31000) and two minor forms (M_r 43000 and 15000 respectively) could be detected. Those variations raised the question of whether these multiple forms exist in nature or whether they arise during purification as a result of limited cleavage by proteinases present in the tissues or autolytic digestion to smaller active fragments, as in the case of pepsin. Although we used rapid purification steps, we never observed only the single chain form. Since, in addition, we did not observe a change in the electrophoretic pattern of cathepsin D even when we preincubated the crude extract for a prolonged time, we assume that the three forms are already present in the tissue, as has been described for cathepsin D from porcine spleen by Huang *et al.* (1980).

Studies of the biosynthesis of cathepsin D *in vivo* (Erickson *et al.*, 1981) have shown that it is synthesized in a precursor form (M_r 46000) that is converted into single-chain cathepsin D with an M_r of 44000. Those values, and the further processing to a heavy chain and a light chain with M_r values 30000 and 15000 respectively, correspond well with our results.

The fact that we could not detect any activity of the single chain form, in agreement with the results

of Hasilik & Neufeld (1980) for cathepsin D from human fibroblasts, and Puizdar & Turk (1981) for cathepsin D from bovine spleen, supports the idea that the single chain is an inactive intermediate form of the enzyme that is endoproteolytically processed to the active form with M_r 31 000. If both the single-chain form and the two-chain form of cathepsin D were active, as Huang *et al.* (1979) report, the physiological function of the conversion of the single chain into the two-chain form would not be clear.

Although the described isoenzyme forms and their M_r values sometimes varied, there did not seem to be a real difference between the cathepsins D from different tissues and different species. When we purified cathepsin D from pig spleen and human spleen we got the same electrophoretic pattern and the same M_r values for the different forms as we got for cathepsin D from pig uterus. Nevertheless, when we raised antibodies against cathepsin D from human spleen they cross-reacted neither with the enzyme purified from pig spleen nor with the enzyme from pig uterus. There does not seem to be an immunological identity between the cathepsin D from these two species.

Cathepsin D was assumed to be a glycoprotein on the following grounds. The enzyme was retained by a column of concanavalin A–Sephacrose 4B. Also, the protein bands seen on SDS/polyacrylamide gels gave positive reactions with the thymol/ H_2SO_4 reagent. When cathepsin D was incubated with endo- β -*N*-acetylglucosaminidase H, all three forms normally seen on SDS/polyacrylamide gels showed higher electrophoretic mobilities after treatment. Sensitivity to endo- β -*N*-acetylglucosaminidase H cleavage, together with the staining by the thymol/ H_2SO_4 reagent, suggested that all three forms were glycosylated and possessed a high proportion of mannose residues in their oligosaccharide moiety.

The glycosylation of the 43 000- M_r form excluded the possibility of it being 'procathepsin D' described by Erickson *et al.* (1981), since that form was supposed to be non-glycosylated. From our results we assumed that it was the single-chain form which was partly cleaved into the two-chain form.

The results obtained with different substrates agreed with other reports on cathepsin D where haemoglobin was also found to be the best substrate (Wiederanders *et al.*, 1976; Cunningham & Tang, 1976). The K_m value of 6.4×10^{-6} M indicated a relatively high substrate affinity of the proteinase. Similar K_m values for haemoglobin as substrate have been found by other groups (Sapolsky & Woessner, 1972; Wiederanders *et al.*, 1976). With respect to the substrate specificity, the enzyme is clearly different from a collagenolytic

cathepsin in rat uterus that has been described by Etherington (1976). We could not detect the inhibition at high concentrations of haemoglobin that has been described for cathepsin D (Yamamoto *et al.*, 1978). Isoelectric focusing resulted in one active band at a pH value of 7.2, which was confirmed by the staining of a band at the same pH value by Coomassie Blue. Only very faint additional bands could be seen on the stained gel, but they did not show any activity. There are many different pI values reported for cathepsin D, ranging from 5.5 (Sapolsky & Woessner, 1972) to 7.68 (Cunningham & Tang, 1976). One of the isoenzyme forms from bovine uterus showed the same isoelectric point as our enzyme (Sapolsky & Woessner, 1972). Since only the main band on SDS/polyacrylamide gels with an M_r of 31 000 showed activity, we assumed that it was this form we found after isoelectric focusing.

Of various inhibitors tested, among them phenylpyruvate, which has been reported as an inhibitor of cathepsin D from rabbit liver (Barrett, 1975), none had any significant effect on enzyme except pepstatin. It was effective at a concentration which was comparable with that of the enzyme and can therefore be classified as a tight-binding inhibitor (Ackermann & Potter, 1949; Morrison, 1969). The formation of the enzyme-inhibitor complex decreases the free inhibitor concentration to such an extent that the Michaelis-Menten model is no longer valid. Taking these inhibitor concentrations into account, we measured [by the methods of Easson & Stedman (1936) and Straus & Goldstein (1943)] a K_i of 2.1×10^{-9} M. A further division of the tight-binding inhibitors into fast and slow tight-binding inhibitors has been made according to the rate of establishment of the equilibrium between inhibitor and enzyme (Williams & Morrison, 1979; Morrison, 1982). Investigations on the inhibition of pepsin by pepstatin (Rich & Sun, 1980) showed that in this case pepstatin acts as a slow tight-binding inhibitor. In order to exclude inaccurate inhibitor constants due to an inappropriate binding time of a slow tight-binding inhibitor, the cathepsin D/pepstatin mixture was preincubated for 15 min and the remaining activity subsequently tested with haemoglobin as substrate with an additional incubation time of 30 min. Since, as compared with pepsin, cathepsin D lacks suitable low- M_r substrates, the binding kinetics of pepstatin as a slow or fast tight-binding inhibitor of cathepsin D could not be monitored. Finally, the many isoenzymes which had been reported by Sapolsky & Woessner (1972) in bovine uterus were not confirmed by our own work on pig uterus. We obtained results similar to those described for other tissues, namely a main, active form with M_r 31 000

(on SDS/polyacrylamide gels) and two minor forms, with M_r values 43000 and 15000 respectively.

Our finding that cathepsin D is more stable at a higher pH than at pH 3, where it is active, could indicate a mechanism similar to that described for pepsin by Herriot (1941), who reported a stabilization of pepsin by a peptide at pH values above 4. Similar results for cathepsin D were reported by Puizdar & Turk (1981). One could therefore assume that cathepsin D is normally stabilized by a protein/peptide (possibly corresponding to the 15000- M_r protein band) at higher pH values, and kept stable. This would be important during the post-partial involution of the myometrium, where sequestration by autophagic vacuoles could take place (Glaumann *et al.*, 1981). When primary lysosomes fuse with the autophagosomes, the lysosomal enzyme is protected against the pH shift to higher values that may possibly occur temporarily. After acidification of the secondary lysosome the inhibitor peptide is dissociated at the lower pH and the resulting form is the active proteinase with M_r 31000. In addition, the complex-formation between the protein/peptide (15000- M_r protein band) and the active proteinase (31000- M_r protein band) at higher pH values could be the main reason why the enzyme is much more resistant to increasing temperatures (Fig. 4) at pH 6 than at pH 3.

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