Cathepsin D

Purification from rat liver and immunohistochemical demonstration in rat incisor

A. LINDE, B. PERSLIDEN & L. RÖNNBÄCK

Laboratory of Oral Biology, Department of Histology, University of Gothenburg, Sweden

Linde, A., Persliden, B. & Rönnbäck, L. Cathepsin D. Purification from rat liver and immunohistochemical demonstration in rat incisor. *Acta Odontol. Scand.* 36, 117–126

Rat liver cathepsin D (EC 3.4.23.5) was purified using precipitation technique, ion exchange chromatography, molecular sieve chromatography and isoelectric focusing. Rabbit anti-rat cathepsin D IgG was prepared and rat incisor teeth were cross-sectioned in a cryostat. These sections were incubated with FITC-conjugated anti-rat cathepsin D IgG. Marked fluorescence, indicating the localization of cathepsin D, could be seen over the odontoblast and predentin area. No specific fluorescence could be demonstrated in the pulp connective tissue proper nor in the dentin

Key-words: Dentinogenesis; mineralization; proteoglycans

Anders Linde, Laboratory of Oral Biology, Department of Histology, Fack, S-400 33 Gothenburg 33, Sweden

INTRODUCTION

Proteoglycans (PGs), the protein complexes of glycosaminoglycans (GAGs), are synthesized at a relatively high amount at loci of biological mineralization and constitute a substantial portion of the organic matrix, e.g. in bone and dentin. However, there is a considerable loss of these macromolecular polyanions simultaneous with the actual mineralization of the organic matrix (2, 5, 9, 13, 14, 15, 25), the functional importance of which is not fully understood.

A possible enzymatic sequence for the degradation of PGs in the odontoblast-predentin region of the dentinogenically active rat incisor has been discussed (10, 11, 19). Cathepsin D (EC 3.4.23.5) is believed to be the enzyme mainly responsible for the degradation of PGs in cartilage (7, 8). We have earlier, by means of micro-dissection technique and biochemical analysis, demonstrated a strong activity of this enzyme in dentinogenically active odontoblasts from rat incisor teeth (19).

The aim of the present investigation was to purify cathepsin D from rat liver in order to obtain species-specific antibodies against this enzyme. By use of immunohistochemical technique the localization of cathepsin D in the odontoblast-predentin area of rat incisor teeth has been studied.

Received for publication, January 16, 1978

MATERIALS AND METHODS

The technique for the purification of cathepsin D was based on the procedure originally described by Barrett (1). Modifications and changes have been made and therefore the technique will be described.

All steps in the purification procedure were carried out in the cold (2 °C), unless otherwise stated. Chemicals were of analytical grade. Solutions used in the chromatographic procedures contained 1 % (v/v) n-butanol to prevent bacterial growth.

Homogenization: For each purification about 2.5 kg rat liver (Sprague-Dawley strain) were pooled. Five litres of 1 % NaCl containing 2 % (v/v) n-butanol were added, and the liver homogenized in a Sorvall Omni Mixer in portions of 200 g liver. $CaCl_2$, 110 g, was dissolved in the total tissue homogenate, and 750 ml 1 M $Na_2HPO_4 . 12\ H_2O$ were added slowly under thorough stirring. The pH was adjusted to pH 7.0 with 2 M Tris-HCl buffer pH 9.0, and after 1 h portions of the suspension were cleared by centrifugation at 15 °C for 30 min at 1500 *g*. The supernatants were pooled and adjusted to pH 3.6 with 5 M formic acid buffer, pH 3.0. The mixture was incubated overnight in a water bath at 37 °C.

Acetone fractionation: The autolysed suspension was cooled to 2 °C and 0.9 volumes of −12 °C acetone were added slowly under vigorous stirring over a period of 15 min. The resulting precipitate was removed by centrifugation at 1500 *g* for 15 min at 0 °C. Another 0.9 volumes of −12 °C acetone were added to the supernatant in the same way. Celite, 2 g/l, was added, and the mixture was stirred for 60 min on a magnetic stirrer.

The precipitate was collected by filtration and dialyzed against 50 mM Na_2-EDTA in 50 mM Tris-HCl buffer, pH 8.0, for 24 h. Further dialysis was performed against 2 mM Tris-HCl buffer, pH 9.0, until the pH of the enzyme preparation was above 8.2 and the conductivity was below 1.0 mmho/cm^2. The Celite was then removed by filtration.

Column chromatography: The crude enzyme preparation was applied to a DEAE-cellulose (Whatman DE 52) column equilibrated with a 14.4 mM Tris-HCl buffer, pH 7.8. Elution was performed by a linear gradient of NaCl in the same buffer. Protein amount (A_{280}) and enzyme activity (see below) in each fraction was determined. Conductivity was measured in the eluate to control the NaCl gradient. Fractions containing high enzyme activity were pooled. Buffer exchange to 60 mM acetate buffer, pH 4.75, and concentration were made in a Millipore ultrafiltration equipment using a PTGC filter (nominal mol.wt. limit 10.000). The enzyme was further chromatographed on a column of CM-cellulose (Whatman CM52), equilibrated with the 60 mM acetate buffer, pH 4.75. The column was washed with the same buffer, and the enzyme was eluted with a 250 mM acetate buffer, pH 5.5. Fractions were assayed for cathepsin D activity and protein as above.

In the first series of purifications, the final purification step was molecular sieve chromatography on Sephadex G-100. This was later replaced by isoelectric focusing.

Sephadex G-100 Chromatography: The enzyme preparation was desalted and concentrated by ultrafiltration as above and applied to a Sephadex G-100 column. The column was eluted with 0.1 M phosphate buffer, pH 7.2. Fractions were collected and tested for protein and enzyme activity as above.

Isoelectric focusing: In the second series of purifications, concentration and buffer change to 1 % glycine solution were made after CM-cellulose chromatography by means of ultrafiltration. Separation was performed on an LKB Ampholine 8100 column. The enzyme preparation was added to the light component of the sucrose gradient (5–50 %; v/v). Ampholines® (pH range 5.0–7.0) were used with a final concentration of 1 %. The column was run for

48 h at 600 V, except for the first 2 h which were run at 500 V. Fractions were tested for pH, protein (A_{280}) and enzyme activity. The presence of Ampholines® did not influence the enzyme assay. Fractions with high enzyme activity were combined, sucrose was removed and the preparation concentrated by ultrafiltration. The Ampholines® were removed by running the preparation on a Sephadex G-50 column (1.6 x 25 cm). Finally, the purified cathepsin D was stored in portions of 500 μg at −20 °C.

Assay of cathepsin D: The enzyme activity assay was modified (19). A 2 % acid denaturated haemoglobin solution was used as substrate. The sample was diluted to 200 μl with 0.1 M Na-citrate buffer, pH 3.0, and 300 μl haemoglobin substrate solution was added. After 1 h incubation in a water bath at 37 °C, the enzyme reaction was terminated by the addition of 1 ml 5.2 % trichloroacetic acid. The resulting precipitate was removed by centrifugation at 4000 *g* for 20 min, and the protein content in the supernatant was measured (20) with albumin as standard. When cathepsin D activity was measured in fractions from the column chromatography runs, the supernatant was directly monitored at 280 nm instead of measuring according to Lowry et al. (20).

Disc-electrophoresis (6, 21) was run to test the purity of the enzyme preparation. About 25 μg of protein were applied to 7 % polyacrylamide gels with an upper 3 % gel. The gels were run at 3 mA/gel for approximately 2 h in a Bio-Rad Gel Electrophoresis Cell 150 A. After electrophoresis, the gels were stained in a solution containing Coomassie Brilliant blue, methanol and glacial acetic acid (26). Destaining was performed in acetic acid-methanol-water (75:250:675, by vol.).

SDS-electrophoresis was run according to Weber and Osborn (26). Polyacrylamide gels (10 %) containing 0.1 % Na-dodecyl sulphate (SDS) were used. The protein sample (100 μg) was denaturated by boiling for 5 min in a 0.1 M Na-phosphate buffer, pH 7.0, containing 1 % SDS and 1 % (v/v) mercaptoethanol. The mixture was dialysed overnigth against a 10 mM Na-phosphate buffer, pH 7.0, containing 0.1 % SDS and 0.1 % mercaptoethanol. Albumin, ovalbumine, chymotrypsinogen, myoglobin and cytochrome c were used as marker proteins.

Production of antiserum to cathepsin D. One mg of the purified enzyme in 1.0 ml PBS buffer was mixed with 1.0 ml of Freund's complete adjuvant. Of this suspension 500 μl was injected into each footpad (3) of a rabbit once weekly for four weeks. Six weeks after the last injection, an intravenous injection of the pure antigen was administered. Ten days after this booster dose, the animal was bled through the marginal ear vein, and the blood was collected for IgG purification.

Purification of rabbit IgG from serum was done by using ion-exchange chromatography on QAE-Sephadex A-50 equilibrated with 0.1 M Tris-HCl buffer, pH 6.5. Portions of 12 ml serum previously diluted 1:1 with the equilibration buffer were applied to the column (2.6 x 30 cm). The IgG fraction was eluted with the same buffer containing 0.1 M NaCl. Flow rate was 12 ml/h and fractions of 3 ml were collected. The first peak represented the serum IgG fraction. After desalting and buffer change in the Millipore apparatus (PTGC filter), portions of 500 μl of anti-cathepsin D IgG were stored at −70 °C for further use.

Specificity tests of the antigen-antibody reaction. The specificity of the antiserum was tested in the following ways:

1. A single precipitation line was obtained when the antiserum was tested by double diffusion in 1 % agar against purified cathepsin D (22).

2. Immunoelectrophoresis was run according to Campbell et al. (4) with the purified anti-cathepsin D IgG.

Conjugation with FITC: Conjugation of IgG with fluorescein isothiocyanate (FITC) was made according to Hijmans, Schuit & Klein (12). Separation of free FITC from IgG-bound FITC was made by molecular sieve chromatography. The column (1.6 x 20 cm) was loaded with Sephadex G-25 and eluted with PBS buffer, pH 7.2, at a speed of 12 ml/h.

Absorption of fluorescein-conjugated anti-cathepsin D. Antiserum absorbed with pure antigen was used as a control in the morphological experiments performed. Pure cathepsin D in small amounts was added to fluorescein conjugated anti-cathepsin D. The antiserum was incubated for 60 min at 37 °C, stored at 4 °C overnight and centrifuged at 10000 *g* for 30 min. This was performed 8–10 times until the antiserum was free from antibodies against cathepsin D. This was controlled by quantitative immunoelectrophoresis (17).

Immunohistochemistry. Experiments were carried out on 5–8 μm thick cryostate sections of frozen whole rat incisors or frozen rat incisor pulp. In all experiments, sections were treated for 30 min with anti-cathepsin D IgG conjugated FITC or with FITC conjugated anti-cathepsin D IgG absorbed with purified cathepsin D. After washing three times for 5 min in phosphate buffered saline, pH 7.2 (PBS) to remove excess fluorescent antibodies, the sections were mounted in buffered glycerol (9 parts of glycerol, 1 part of PBS buffer) under a coverslip and immediately examined in a dark field fluorescence microscope. Pictures were taken with Kodak Ectachrome high speed film in a Leitz incident fluorescence microscope and converted to black & white photographs.

Controls: Two types of controls were performed:

1. Tissue slices incubated with anti-cathepsin D absorbed with purified cathepsin D as described above. Antiserum or absorbed antiserum were diluted serially. Difference in fluorescence between test and control incubations was optimal at a dilution of 1:5 of the antiserum and the absorbed antiserum. This was thus used routinely.

2. Tissue slices, not treated with any antiserum, to determine whether any structures showed autofluorescence. This was negligible in our experimental set-up.

RESULTS

The results presented in the figures and the table are from representative runs. In the elution diagrams, the fractions indicated were transferred to the subsequent purification step.

With the exception of the elution profiles from the DEAE-cellulose separations, all results were highly reproducible. In the DEAE separations, the protein pattern differed considerably between different batches. However, cathepsin D was always eluted with the linear salt gradient at about 55 mM NaCl (Fig. 1).

The elution pattern from a CM-cellulose column is shown in Fig. 2. After this step enzymatically active fractions were pooled and subjected to further purification either on a Sephadex G-100 column (alt. I) or on an isoelectric focusing column (alt. II).

The result of molecular sieving on Sephadex G-100 is shown in Fig. 3a. The enzyme protein was eluted in one single peak. Marker proteins run on the same column revealed a linear relationship when plotted in a semilogarithmic diagram (Fig. 3b). An apparent molecular weight of 43 000 was obtained for rat liver cathepsin D. A similar value was obtained on the SDS-polyacrylamide gels.

Isoelectric focusing (alt. II) showed a spinous protein profile with several enzyme activity peaks (Fig. 4). The fractions indicated in Fig. 4 were pooled and are referred to below as purified rat liver cathepsin D.

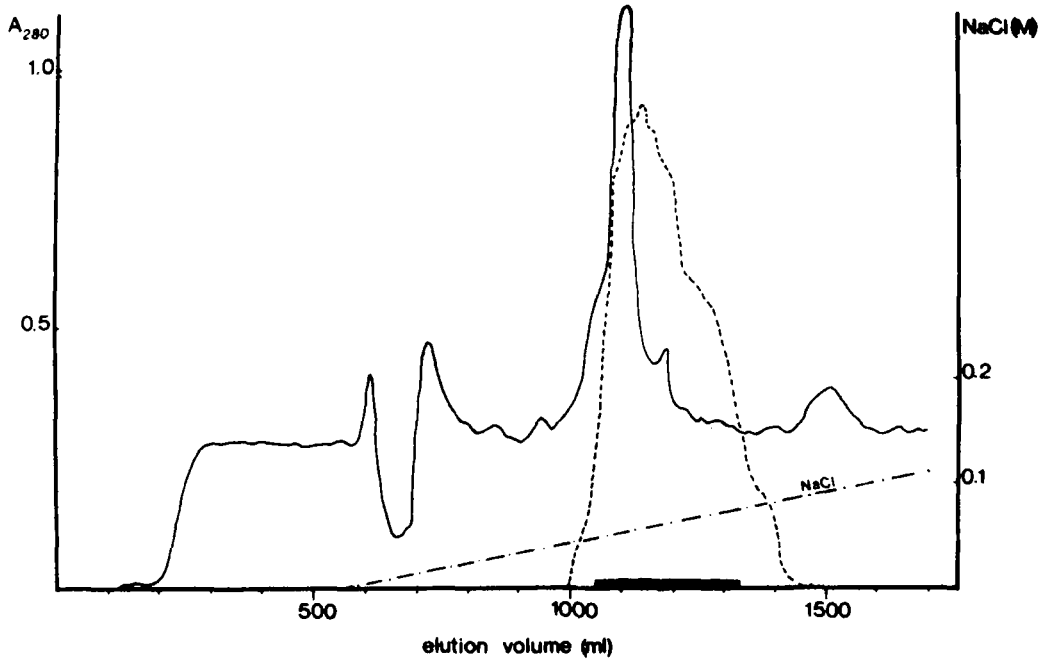


Fig. 1. DEAE-cellulose (Whatman DE52) ion exchange chromatography. Bed dimensions 2.6 x 40 cm. Flow rate 110 ml/h. Fraction volume 9.5 ml. Protein (———) is expressed as optical density at 280 nm. Enzyme activity (- - - -) was assayed as described in the text. The enzyme was eluted by a linear NaCl gradient (—·—·—). Pooled fractions are marked (▬▬) above the abscissa. It can be seen that the enzyme was eluted at 55 mM NaCl.

Table 1 shows the protein amount, enzymatic activity, specific enzyme activity and enzyme activity yield at the different steps in the purification procedure. It can be seen that the termination of the purification sequence by isoelectric focusing (alt. II) gave a higher specific catheptic activity and a better enzyme activity yield, compared to the Sephadex G-100 alternative. As will be seen below, this also gave a more pure product.

Fig. 5 shows polyacrylamide gel electrophoresis on material obtained after the purification procedure. Only one protein containing band could be seen when using isoelectric focusing as last purification step.

The antiserum towards cathepsin D, tested on double-diffusion agar, revealed only one precipitation line against purified enzyme. In the same way immunoelectrophoresis according to Campbell et al. (4) demonstrated only one single band (Fig. 6).

The degree of conjugation between FITC and IgG, known as the F/P-ratio (27), was calculated and expressed as micrograms of FITC/mg IgG. Using a normogram reading, the F/P-ratio varied between 0.5–2.5 μg/mg.

Fig. 7 shows the immunohistochemistry on the cryostat sectioned rat incisor. A marked fluorescence could be seen over the odontoblasts in the pulp sections (Fig. 7a) and in the odontoblast-predentin area in the sectioned whole teeth (Fig. 7b). Fig. 7c shows a control section, incubated with the absorbed antiserum. Only scanty distribution and autofluorescence could be seen over the pulp connective tissue proper.

From the pulp sections an intense fluorescence in the adhering odontoblasts was

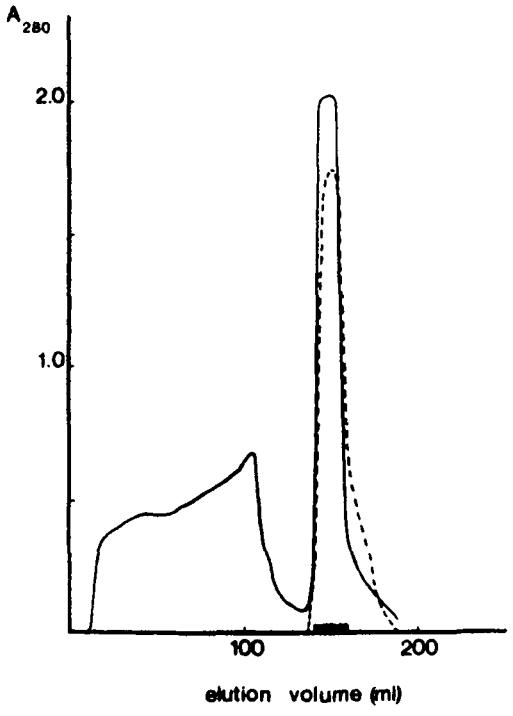


Fig. 2. CM-cellulose chromatography on Whatman CM52. Bed dimensions (1.6 x 10 cm). Flow rate 30 ml/h. Fraction volume 3 ml. Protein content (———) and enzyme activity (- - - -) were measured as described in Fig. 1. The enzyme was eluted by a 250 mM acetate buffer, pH 5.5. Fractions (3 ml) marked (▬▬) were collected and transferred to the next purification step.

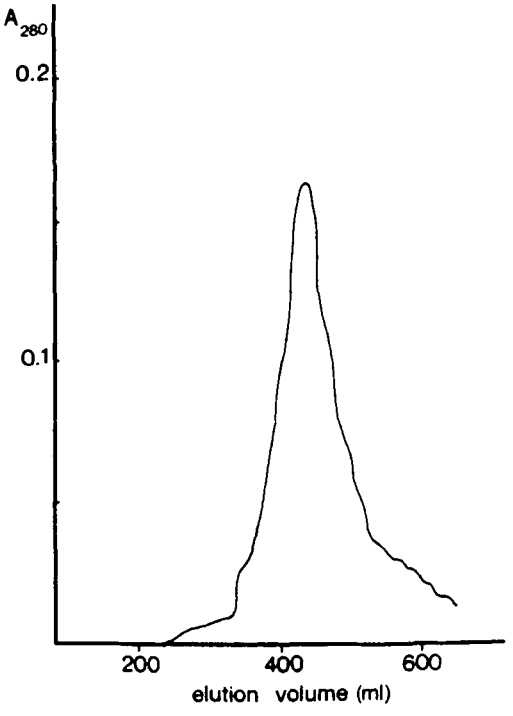


Fig. 3a. Sephadex G-100 molecular sieve chromatography on a 2.5 x 160 cm column. Protein profile (———) represents A_{280}. Elution of the 8.5 ml fractions was made with 0.1 M phosphate buffer, pH 7.2, at a flow rate of 18 ml/h.

thus obvious. In the whole rat incisor sections a similar intense fluorescence was seen over the odontoblast-predentin area, but it was not possible to determine whether this fluorescence was derived from the extracellular predentin as such also.

DISCUSSION

Only fractions with strong enzyme activity were collected and carried over to the next separation step (Fig. 1, 2). This precaution was taken to assure a highly pure preparation for immunization. The yield of cathepsin D (5–7 mg/2.5 kg liver) might therefore seem low compared with the values reported by Barrett (1), but he studied human and chicken liver and the data are therefore not completely comparable.

The apparent molecular weight of the present preparation, 43 000, is comparable with the previously published results (1, 24, 28). The additional separation step after ion exchange chromatography, molecular sieve chromatography on Sephadex G-100 (alt. I), improved the results. Comparison of polyacrylamide gels before and after this step revealed that several unindentified protein bands could be removed from the preparation. This, however, contrasts

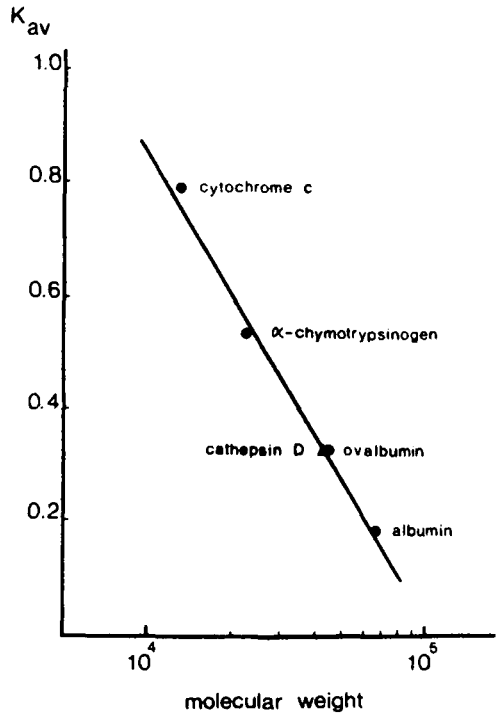


Fig. 3b. K_{av}-values plotted vs. molecular weight for four reference substances with known molecular weight. Cathepsin D (▲) was eluted in the same position as ovalbumin, indicating an apparent molecular weight of 43 000. Note that the abscissa is logarithmic.

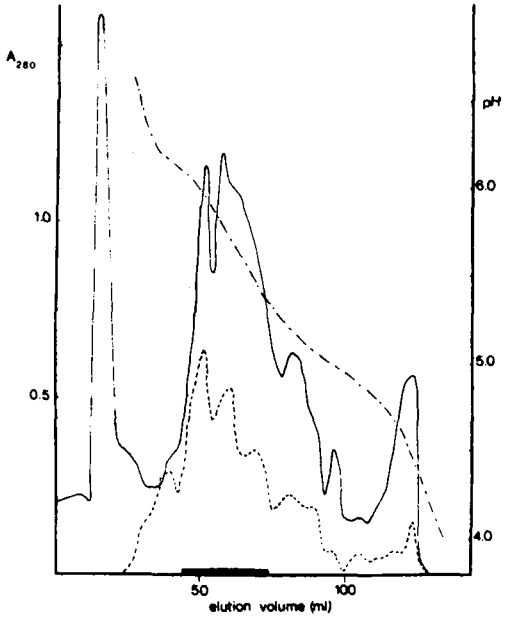


Fig. 4. Elution profile from isoelectric focusing on LKB Ampholine 8100 column. Protein (———) and enzyme activity (- - - -) are expressed as in Fig. 1. The pH gradient is indicated (—·—·—). Fractions marked (▬▬) were collected, concentrated to 1 mg/ml and referred to as purified cathepsin D.

to the results on human and chicken enzyme (1). To control the controversial data obtained by separation on Sephadex G-100, molecular sieve chromatography was replaced by isoelectric focusing. The results (Fig. 4) revealed a spinous distribution profile of enzyme activity possible due to the presence of isoenzymes, over a pH range of 5.1–6.0. After this step, only one protein band could be detected by polyacrylamide gel electrophoresis (Fig. 5).

The immunization method and the immunohistochemical technique were all based on preparations which included isoelectric focusing as the last separation step.

Cryostat sectioning of whole teeth was considered the method of choice in order to preserve the morphological relationship between the pulp connective tissue and the dental hard tissue. However, distortions in the morphology were frequent. Fixation of the sections with ice-cold acetone or 4 % paraformaldehyde in PBS buffer did not improve the histology of the sections and was therefore omitted. Dilution series of FITC-conjugated IgG were made, and the optimal dilution 1:5 was used routinely.

The finding of FITC labelled anti-cathepsin D antibodies in the predentin and odontoblast area further confirms our earlier findings of a high catheptic activity in this region (19). The localization of cathepsin D in the mineralization area of dentin is a new finding and should be compared with the results of Poole, Hembry and Dingle (23). These authors studied diaphysis in cultured

Table 1. *Cathepsin D yield after each purification step in a representative preparation from 2.5 kg rat liver with isoelectric focusing as the last step (alt. II). The values for alt. I with Sephadex G-100 as the last step is from a separate run. Total protein (g) was assayed by the method of Lowry et al. (1951) using albumin as a standard. The total cathepsin D activity after each step is expressed as amount liberated protein (g) during a 1 h incubation at 37 °C with hemoglobin as a substrate and measured by the Lowry method. The specific activity describes the total enzyme activity per total protein after each step. The enzyme activity yield (%) relates the remaining enzyme activity after each purification step to the total activity in the liver homogenate*

	Protein (g)	Activity (g/h)	Spec.act.	Yield (%)
Homogenate	213	57.5	0.27	100
Ca^{2+}-sup.	62.3	30.0	0.48	52.2
Autolysed extract	71.3	26.7	0.37	46.4
Acetone precipitate	0.780	22.5	29	39.1
DEAE-chromat.	0.176	15.0	85	26.1
CM-chromat.	0.040	6.05	151	10.5
Sephadex G-100 (alt. I)	0.007	1.55	221	2.7
Isoelectr. focusing (alt. II)	0.012	2.99	249	5.2

Fig. 5. Polyacrylamide gel electrophoresis of material obtained after the last step in the purification procedure. Sample of about 25 μg protein was applied to a 7 % polycrylamide gel with an upper gel of 3 %. The gel was run for 2 h at 3 mA and stained with Coomassie Brilliant blue.

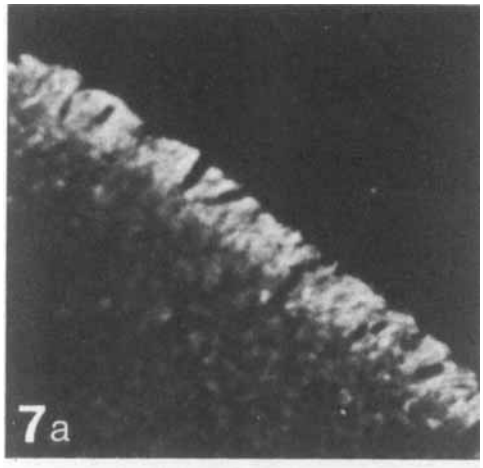
Fig. 6. Immunoelectrophoresis in 1 % agar system. Electrophoresis was run for 3 h at a voltage of 4 V/cm. The purified anti-cathepsin D IgG fraction was placed in the well and the system was allowed to precipitate for 24 h at 2 °C. The agar plate was dried and stained with Coomassie Brilliant blue. The picture was taken with Kodak Plus-X film in a Leitz Reprovit IIa equipment. A: cathepsin D, B: cathepsin D diluted 1:1 with PBS buffer, pH 7.2, C: rabbit anti-cathepsin D IgG.

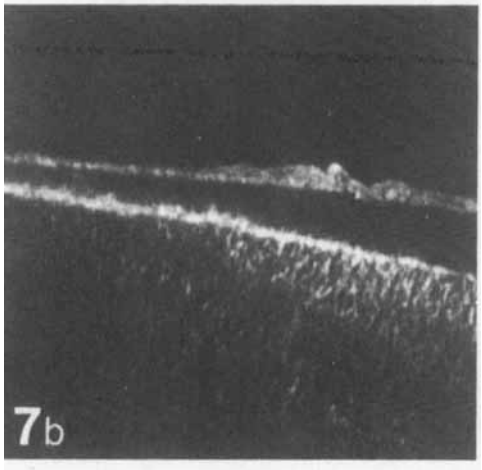
bone and were able to demonstrate cathepsin D in the osteoblast layer adjacent to the mineralization front by an immunohistochemical technique. Together, these results show a high cathepsin D activity adjacent to the mineralization front of mineralizing tissues and might be taken to indicate some important function for the enzyme in biological mineralization.

The fact that cathepsin D degrades PGs to smaller fragments makes it possible for other lysosomal enzymes present in odontoblasts (10, 11) to act in order to complete the degradation of PGs in predentin (18). Whether this digestion takes place in the extracellular space, in predentin or in digestion vacuoles is not known. Light microscopical immunohistochemistry was not able to resolve the subcellular localization of cathepsin D in this region.

Further studies with a similar technique on the ultrastructural level are in progress and might elucidate the exact localization for the suggested PG degradation. This information could be of value for the understanding of the turnover of PGs in this area and its connection with the major features of the calcification.

Acknowledgements. The authors gratefully acknowledge the technical assistance by Mrs. Agneta Höglund. Valuable help with cryostate sectioning has been given by the Dept. of Oral Pathology, University of Göteborg. The investigation was supported by the Swedish Medical Research Council, The Faculty of Odontology, University of Göteborg and Göteborgs Tandläkaresällskap.





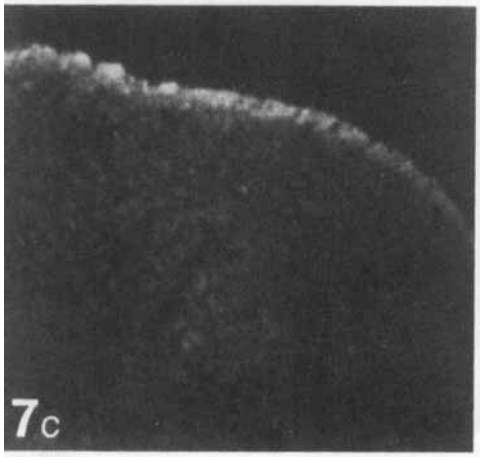


Fig. 7a–c. Cryostate sections (5–8 μm) of rat incisor pulp (Fig. 7a, c) and whole tooth (Fig. 7b). The sections in Fig. 7a and 7b were incubated for 30 min with 50 μl of FITC-conjugated anti-cathepsin D IgG. After extensive washing with PBS buffer the sections were mounted with glycerol. The fluorescence is located mainly in the odontoblast layer (7a) and in the odontoblasts-predentin region (7b). Only slight autofluorescence can be seen in the control section (7c) incubated in the same way with FITC-conjugated antibodies previoysly absorbed with purified antigen. The original pictures were taken with Kodak Ectachrome high speed film and converted into black & white photographs. Magnifications. 7a: x200, 7b: x80, 7c: x50.

REFERENCES

1. Barret, A.J. Cathepsin D: Purification of isoenzymes from human and chicken liver. Biochem. J. 1970, 117, 601–607
2. Baylink, D., Wergedal, J. & Thompson, E. Loss of proteinpolysaccharides at sites where bone mineralization is initiated. J. Histochem. Cytochem. 1972, 20, 279–292
3. Bernier, G.M. & Cebra, J.J. Frequency distribution of α, γ, κ and λ polypeptide chains in human lymphoid tissues. J. Immunol. 1965, 95, 246–253
4. Campbell, D.H., Garvey, J.S., Cremer, N.E. & Sussdorf, D.H. Methods in Immunology, W.A. Benjamin Inc., New York, 1963, pp 149–155
5. Campo, R.D. & Dziewiatkowski, D.D. Turnover of the organic matrix of cartilage and bone as visualized by autoradiography. J. Cell. Biol. 1963, 18, 19–29
6. Davis, B.J. Disc electrophoresis II. Method and application to human serum proteins. Ann. N.Y. Acad. Sci. 1964, 121, 404–427
7. Dingle, J.T., Barrett, A.J. & Weston, P.D. Cathepsin D: Characteristics of immunoinhibition and the confirmation of a role in cartilage breakdown. Biochem. J. 1971, 123, 1–13
8. Dingle, J.T., Barrett, A.J. & Poole, A.R. Inhibition by pepstatin of human cartilage degradation. Biochem. J. 1972, 127, 443–444
9. Engfeldt, B. & Hjerpe, A. Glycosaminoglycans of dentine and predentine. Calc. Tiss. Res. 1972, 10, 152–159
10. Engström, C., Linde, A. & Persliden, B. A fluorimetric study of β-glucuronidase and β-galactosaminidase from the pulp of the rat incisor. Archs oral Biol. 1972, 17, 1421–1430
11. Engström, C., Linde, A. & Persliden, B. Acid hydrolases in the odontoblast-predentin region of dentinogenically active teeth. Scand. J. Dent. Res. *1976, 84, 76–81*

12. Hijmans, W., Schuit, H.R.E. & Klein, F. An immunofluorescence procedure for the detection of intracellular immunoglobulins. Clin. Exp. Immunol. 1969, 4, 457–472
13. Hirschman, A. & Dziewiatkowski, D.D. Protein-plysaccharide loss during endochondral ossification: Immunochemical evidence. Science 1966, 154, 393–395
14. Höhling, H.J., Hall, T.A., Boyde, A. & von Rosenstiel, A.P. Combined electron probe and electron diffraction analysis of prestages and early stages of dentine formation in rat incisors. Calc. Tiss. Res. 1968, 2, Suppl 5–5a
15. Höhling, H.J., Nicholson, W.A.P., Schreiber, J. & Zessack, U. The distribution of some elements in predentin and dentin of rat incisors. Naturwissenschaften 1972, 59, 423
16. Joustra, M. & Lundgren, H. Preparation of freeze-dried, monomeric and immunochemically pure IgG by a rapid and reproducible chromatographic technique. In: Protides of the biological fluids. Proceedings of the 17th Colloquium Bruges. (Ed: H. Peeters), 1969, 511–515 pp
17. Laurell, C-B. Quantitative estimation of proteins by electrophoresis in agarose gel containing antibodies. Analyt. Biochem. 1966, 15, 45–52
18. Linde, A. Glycosaminoglycans of the odontoblast-predentine layer in dentinogenically active porcine teeth. Calc. Tiss. Res. 1973, 12, 281–294
19. Linde, A. & Persliden, B. Cathepsin D activity in isolated odontoblasts. Calc. Tiss. Res. 1977, 23, 33–38
20. Lowry, O.H., Rosebrough, N.J., Farr, A.L. & Randall, R.J. Protein measurement with the Folin phenol reagens. J. Biol. Chem. 1951, 193, 265–275
21. Ornstein, L. Disc electrophoresis – I: Background and theory. Ann. N.Y. Acad. Sci. 1964, 121, 321–349
22. Ouchterlony, Ö. Diffusion-in-gel methods for immunological analysis. In: Progress in Allergy VI. Kallos, P. & Waksman, B. H., eds. S. Karger, Basel 1962, 49–51 pp
23. Poole, A.R., Hembry, R.M. & Dingle, J.T. Extracellular localization of cathepsin D in ossifying cartilage. Calc. Tiss. Res. 1973, 12, 313–321
24. Smith, R. & Turk, V. Cathepsin D: Rapid isolation by affinity chromatography on haemoglobin-agarose resin. Eur. J. Biochem. 1974, 48, 245–254
25. Sundström, B. New aspects on the utilization of inorganic sulphate during dentine formation. Histochemie 1971, 26, 61–66
26. Weber, K. & Osborn, M. The reliability of molecular weight determinations by dodecyl sulphate-polycrylamide gel electrophoresis. J. Biol. Chem. 1969, 244, 4406–4412
27. Wells, A.F., Miller, C.E. & Nadel, M.K. Rapid fluorescein and protein assay method for fluorescent-antibody conjugates. Appl. Microbiol. 1966, 14, 271–275
28. Woessner, F.J. Purification of cathepsin D from cartilage and uterus and its action on the protein-polysaccharide complex of cartilage. J. Biol. Chem. 1973, 248, 1634–1642