Proteoglycan-Degrading Enzymes

A RADIOCHEMICAL ASSAY METHOD AND THE DETECTION OF A NEW ENZYME, CATHEPSIN F

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1. Polyacrylamide beads containing entrapped ^{35}S -labelled proteoglycan molecules have been prepared. 2. The measurement of release of radioactivity provides an extremely sensitive assay for proteoglycan-degrading enzymes, including proteinases and hyaluronidase. 3. The amount of label released is a logarithmic function of enzyme concentration or time of incubation. Experiments were made in an attempt to explain this. 4. Assays were made by the new method at several pH values, and with the inclusion of inhibitors to identify the proteoglycan-degrading enzymes of rabbit ear cartilage. 5. A previously undescribed proteinase, active against proteoglycan at pH 4.5 but unaffected by pepstatin, was discovered. The enzyme was named cathepsin F, and was partially purified and characterized; it was detected in human articular cartilage.

The destruction of the proteoglycan of articular cartilage in arthritis is recognized to be an important factor in the irreversible impairment of joint function. The idea that lysosomal enzymes play a part in this process (Dingle, 1962) has gained wide acceptance (Barrett, 1975). Subsequent work in our laboratory has suggested that non-lysosomal enzymes may also be involved, but that secretion of these may nevertheless be linked to changes in the lysosomal system (Werb & Dingle, 1976). Much has yet to be learned about the identity of the degradative enzymes, their cellular origin in the joint, and the control of their production. A major obstacle to research has been the lack of a convenient, sensitive and specific assay for proteoglycan-degrading activity applicable to enzymes at either acid or neutral pH values.

Proteoglycan, as it exists in cartilage, is a molecule in which sulphated polysaccharide chains are covalently linked to a protein backbone representing 5–10% of the total weight of the complex. The integrity of the protein backbone has a profound effect on the physical properties of the material, and the primary route of degradation of the proteoglycan under physiological conditions is undoubtedly proteolytic. Other possible forms of enzymic breakdown of the molecule include hydrolysis of glycosyl bonds (as by hyaluronidase or chondroitinases) or ester bonds (sulphatase). A new dimension has been added to the problem of elucidating the mechanism of enzymic degradation of proteoglycan *in vivo* by the discovery that the proteoglycan mol-

ecules are non-covalently associated as subunits of huge multimolecular aggregates that also contain hyaluronic acid and one or more 'link' proteins (Hardingham & Muir, 1974; Hascall & Heinegård, 1974), so that the retention of proteoglycan in cartilage matrix does not depend solely on the integrity of the molecule itself.

Because the protein component of proteoglycan is small in quantity, proteolytic degradation gives fragments that are chemically closely similar to the parent molecules, and such physical properties as intrinsic viscosity, sedimentation rate and diffusion coefficient must form the basis of assays. Assays based on viscometry and ultracentrifugation have been described (Woessner, 1973; Sapolsky *et al.*, 1973); other methods have used micro diffusion chambers (Weissman & Spilberg, 1968; Dingle *et al.*, 1971), a form of electrophoresis (Dziewiatkowski *et al.*, 1968) and a precipitation method (Sapolsky *et al.*, 1975). Ignarro *et al.* (1973) have described the use of discs of ^{35}S -labelled rabbit ear cartilage in an assay for proteoglycan-degrading enzymes. Although each of the above methods has allowed the detection of activity, it has been our experience that none combines convenience with high sensitivity and good quantification.

The present paper describes a new assay procedure based on the finding that intact proteoglycan molecules do not escape from cross-linked polyacrylamide gels, whereas degraded molecules do (Barrett, 1966); in the present method, microscopic beads of poly-

acrylamide gel containing ^{35}S -labelled proteoglycan have been used. The method is better in several respects than those available previously, and has been used in the detection and characterization of a new proteoglycan-degrading enzyme of cartilage, cathepsin F.

A preliminary account of part of this work has been presented (Blow, 1975).

Materials and Methods

The sources of materials obtained commercially were as follows: benzylpenicillin sodium and streptomycin calcium chloride, Glaxo Laboratories, Greenford, Middx., U.K.; Mycostatin, E. R. Squibb and Sons, Liverpool L4X 1QW, U.K.; $\text{Na}_2^{35}\text{SO}_4$ (code SJS-1) dispensed at 10mCi/ml, The Radiochemical Centre, Amersham, Bucks., U.K.; acrylamide, *NN'*-methylenebisacrylamide, ammonium persulphate, di-isopropyl phosphorofluoridate and *NNN'*-tetramethylethylenediamine from BDH Chemicals, Poole, Dorset, U.K.; sorbitan sesquileate, Phase Separations Ltd., Deeside Industrial Estate, Queensferry, Clwyd CH5 2LR, Wales, U.K.; papain (twice-crystallized; sodium acetate suspension), trypsin inhibitors of soya bean and lima bean, Sigma (London) Chemical Co., Kingston-upon-Thames, Surrey KT2 7BH, U.K.; phenylmethanesulphonyl fluoride, Calbiochem, London W1H 1AS, U.K.; and Trasylol was bovine pancreatic trypsin/kallikrein inhibitor from Bayer A.G., Wuppertal-Elberfeld, Germany. Purified bovine testicular hyaluronidase was the gift of Dr. B. Högborg, A.B. Leo, Helsingborg, Sweden. Alcian Blue 8GX was from G. T. Gurr, High Wycombe, Bucks., U.K., and chondroitin sulphate (from shark) was from Koch-Light Laboratories, Colnbrook, Bucks., U.K. Sepharose and Sephadex were from Pharmacia Fine Chemicals, London W5 5SS, U.K. Pepstatin was the gift of Professor H. Umezawa, Institute of Microbial Chemistry, Kamiosaki, Shinagawa-ku, Tokyo, Japan.

α_2 -Macroglobulin from human plasma was prepared as described by Barrett & Starkey (1973), chicken ovinhibitor as described by Barrett (1974), egg-white papain inhibitor essentially as described by Fossum & Whitaker (1968), and turkey ovomucoid as described by Lineweaver & Murray (1947). Human cathepsins B (specific activity 5.0 EC units/mg) and D [specific activity 820 units/mg (1 unit $\equiv \Delta A_{280}$ of 1.0 in 1 h; Barrett, 1970)] were prepared as described by Barrett (1973).

^{35}S -labelled and unlabelled cartilage proteoglycan

Bovine nasal cartilage was obtained from a local slaughterhouse, and used within a few hours of the death of the animal. The septal cartilage was sliced thinly (about 1 mm), and 10 g of tissue was placed in

25 ml of a modification of the synthetic culture medium BGJ₅ (Dingle *et al.*, 1967). The medium contained antibiotics at tenfold the normal concentration (i.e. 16 mg of streptomycin calcium chloride, 6.75 mg of benzylpenicillin and 0.833 mg of Mycostatin in 25 ml) together with 5 mCi of carrier-free $\text{Na}_2^{35}\text{SO}_4$ but no unlabelled sulphate (since the MgSO_4 of the normal formulation had been replaced by equimolar MgCl_2). The tissue was incubated under a gas phase of $\text{CO}_2/\text{O}_2/\text{N}_2$ (1:4:15) in a roller bottle for 6 h at 37°C. The radioactive culture medium was discarded, and the tissue rinsed with 25 ml of medium BGJ_w (Poole *et al.*, 1974). The tissue was extracted for 24 h with 100 ml of 4M-guanidinium chloride in 0.05M-Tris/HCl buffer, pH 7.6, with stirring at 25°C. The extract was separated by passage through a sintered-glass (no. 1) filter, and the tissue was rinsed on the filter with a further 50 ml portion of the 4M-guanidinium chloride, which was combined with the first filtrate. Ethanol (3 vol.) was added to the extract, and after 30 min the precipitated proteoglycan was sedimented by centrifugation for 5 min at 1250g. The supernatant solution was discarded, and the pellet redissolved in 150 ml of 1M-potassium acetate solution overnight at 4°C. The solution was passed through a sintered-glass (no. 1) filter, and reprecipitated with 3 vol. of ethanol. The proteoglycan, now in the form of its potassium salt, was collected by centrifugation as above, dissolved in 0.10M-NaCl and stored at -20°C in 15 ml portions. The total dry weight and radioactivity of the products were determined (see the Results section).

Unlabelled proteoglycan was prepared by extraction of nasal cartilage with 4M-guanidinium chloride and precipitation, exactly as described for the radioactive material.

Preparation of cross-linked polyacrylamide beads containing entrapped ^{35}S -labelled proteoglycan

The method was based on those described for polyacrylamide and agarose beads by Hjertén (1968).

The reaction vessel was a 3-litre round-bottom flask set up in a water bath at 15°C, fitted with a powerful mechanical stirrer operating at 350 rev./min and provision for flushing with O_2 -free N_2 . A mixture of toluene (1440 ml), carbon tetrachloride (360 ml) and sorbitan sesquileate (3 g) was placed in the flask and allowed to reach 15°C while being bubbled with N_2 , during 15 min. A solution of 15.0 g of acrylamide, 0.75 g of methylenebisacrylamide, 1.5 g of NaCl and 0.3 g of proteoglycan containing 34.5×10^6 c.p.m. of ^{35}S , in 10mM-Tris/acetic acid buffer, pH 7.8 (total volume 150 ml), was de-gassed under reduced pressure [~ 20 kPa (150 mmHg)], and 7.5 ml of 1.0% (w/v) ammonium persulphate was mixed in. Immediately thereafter, 15 ml of tetramethylethylenediamine was poured into the reaction vessel, followed by the solution of acrylamide and proteoglycan. An emul-

sion formed, and stirring under N_2 was continued for 1 h, during which time vinyl polymerization converted the aqueous droplets into polyacrylamide beads. Ethanol (1.05 litres) was poured slowly into the reaction flask, and stirring was then stopped to allow the beads to settle. The supernatant fluid was decanted, and the beads were covered with 2.0 litres of 1% (v/v) acetic acid. After 20 min with occasional stirring, the beads were sedimented by centrifugation at 700g during 15 min. Three further washes with 1% acetic acid were made in just the same manner, followed by a wash with water, after which the beads were stirred for 1 h in 6 litres of aq. 0.5% sodium dodecyl sulphate containing 0.5% (v/v) of conc. HCl. Once more, the beads were recovered by centrifugation, and washed with 1-litre quantities of water until the supernatant had no tendency to foam (at least four times). Finally, the beads were suspended in enough water to form a free-flowing slurry, freeze-dried, passed through a no. 36 sieve (425 μ m aperture, A. Gallenkamp type SE-105) and stored over P_2O_5 at 20°C.

Typically, 11.0g of beads was obtained by this procedure, with a specific radioactivity of 2800 c.p.m./mg, a recovery of 89% of the radioactivity.

Sepharose beads with covalently linked ^{35}S -labelled proteoglycan

Sepharose was 'activated' by a method based on those of Cuatrecasas & Anfinsen (1971) and Axen & Ernback (1971); it was then allowed to react with ^{35}S -labelled proteoglycan. The procedure was as follows.

Sepharose 2B or 4B (25g of hydrated gel) was washed with water on a sintered-glass filter, and suspended in 50ml of water, in a 250ml beaker. The beaker was placed in a fume cupboard and equipped with a magnetic stirrer and a pH electrode. CNBr (1g) was dissolved in 40ml of water in a closed bottle; the solution was poured into the Sepharose slurry and the mixture was quickly adjusted to pH 11 and maintained at this value during 6 min by addition of 2M-NaOH. The 'activated' Sepharose obtained in this way was washed on the sintered-glass filter with cold 0.1M- $NaHCO_3$, re-suspended in the same solution and stirred overnight with ^{35}S -labelled proteoglycan (60mg, equivalent to 240×10^6 c.p.m.) in 0.1M- $NaHCO_3$.

The coupled Sepharose was rinsed briefly with 0.1M- $NaHCO_3$ and then stirred for 1 h in 1% sodium dodecyl sulphate with 1% glycine, in 0.1M- $NaHCO_3$. The material was washed thoroughly with water until the washings had little tendency to foam. The solid was further stirred for 1 h at 20°C in 1% bovine serum albumin in 1% NaCl, and then washed with 0.20M-NaCl on a sintered-glass funnel until the washings did not foam appreciably, and contained less than 1000 c.p.m. of ^{35}S /ml. The ^{35}S content

(c.p.m.) of a weighed sample of the damp gel was determined, and the gel was stored as a suspension containing about 40% (v/v) gel in 0.20M-NaCl/0.01% NaN_3 at 4°C.

Assay of proteoglycan-degrading enzymes with substrate in polyacrylamide beads

Dry polyacrylamide beads (250mg) were suspended in 20ml of water with magnetic stirring, and allowed at least 5 min for hydration. (The Sepharose beads were already hydrated.) The suspension was stirred while portions (0.5ml) were transferred to the 9ml polystyrene assay tubes (type PT 13/76; Luckham, Burgess Hill, Sussex, U.K.). Buffer (0.1 ml, 1M in buffer ion; see the Results section for buffers used) of the appropriate pH value was added to each tube, together with enzyme sample, activators or inhibitors and 0.145M-NaCl to make a total volume of 1 ml.

The tubes were incubated at 37°C in a roller-tube apparatus rotating at 3 rev./min. The apparatus had a capacity of 130 tubes.

The reaction (usually of 1–4 h duration) was stopped by the addition of 0.25ml of 5% (w/v) sodium dodecyl sulphate in 5% (v/v) conc. HCl. The tubes were centrifuged for 5 min at 1250g to sediment the beads, and 0.20ml samples removed for liquid-scintillation counting of radioactivity. Alternatively, samples of up to 0.20ml were used in chemical assays of the released glycosaminoglycan, by the Alcian Blue method (see below).

The proteoglycan-degrading activity of crude tissue extracts was assayed in the same way. It was for crude samples that the sodium dodecyl sulphate/HCl stopping reagent was particularly necessary. Without it, the radioactive products were lost from the supernatants by adsorption to the precipitates commonly formed during incubation at low pH values.

Liquid-scintillation counting

Aqueous samples of up to 0.20ml were mixed with 10ml of scintillant solution, in which the solvent was ethoxyethanol/toluene (3:7, v/v) (Hall & Cocking, 1965). The liquid-scintillation counter was a Packard Tri-Carb model 3375. Quench corrections by the automatic external-standard method were applied when necessary (as described by the manufacturers).

Determination of concentration of proteoglycan fragments by precipitation with Alcian Blue

The method was a minor modification of that of Whiteman (1973), in which the Alcian Blue-glycosaminoglycan complexes were redissolved in 2% (w/v) sodium dodecyl sulphate, in 0.05M-sodium acetate buffer, pH 5.8, rather than the 40% (w/v)

Mannoxol of the original method. Chondroitin sulphate served as standard.

Viscometry

Miniature Ostwald viscometers (type UM.4; A. Gallenkamp) were used, in a water bath (Grant Instruments, Cambridge CB2 5QZ, U.K.) at $37 \pm 0.01^\circ\text{C}$. The water flow-time was 20–25 s. The substrate was cartilage proteoglycan (5 mg/ml) in the appropriate buffer (see the Results section) (50 mM) containing 200 mM-NaCl.

Results

The assay

In principle, the assay depends on the fact that the radiochemically labelled proteoglycan molecules are trapped within beads of cross-linked polyacrylamide, and that the products of enzymic degradation are small enough to escape into solution, where they are detected radiochemically or chemically. The pore size of the beads is very important, so that enzyme can freely enter the beads, and degraded proteoglycan can leave, but also so that undegraded material is trapped within the beads. The pore size was altered experimentally by changing the ratio of acrylamide to methylenebisacrylamide, and the final composition of 15.0% (w/v) acrylamide with 0.75% of methylenebisacrylamide was found to give the best retention of undegraded labelled material, with release of the degraded material.

³⁵S-labelled proteoglycan. In four preparations, extraction of the ³⁵S-labelled cartilage slices (10 g) with 4 M-guanidinium chloride yielded 1.5 g of proteoglycan containing 1.0 mCi of ³⁵S (representing a 20% incorporation of the radioactive sulphate from the culture medium). On the basis of the work of Sajdera & Hascall (1970), it is assumed that the material is a complex mixture of the covalently linked sulphated glycosaminoglycan-protein complexes and associated proteins and glycoproteins, but since the ³⁵S label allows one to follow degradation of the proteoglycans specifically, no further characterization or fractionation of the mixture was considered necessary.

Polyacrylamide beads containing labelled proteoglycan. The beads were seen microscopically to be approximately spherical, and mostly in the range 30–80 μm in diameter, when hydrated (Fig. 1). Dry, they formed a fine white powder.

Incubation of the hydrated beads under the conditions of the enzyme assay (see the Materials and Methods section), but without enzyme, resulted in release of a small percentage of the radioactivity (3–7% during 4 h at 37°C , for five typical batches of beads). These 'blank' values showed some dependence on time and pH of incubation (being greatest at neutral pH), suggesting the presence of traces of

Fig. 1. Microscopic appearance of hydrated polyacrylamide-proteoglycan beads

The beads were examined with dark-ground illumination. The horizontal bar represents 50 μm .

Fig. 2. Standard curve for cathepsin D

Assays by the radiochemical method were made with incubation at pH 4.5, 37°C for 1 h.

endogenous proteoglycan-degrading activity in the substrate preparations. Decreasing the total concentration of proteoglycan in the beads below that specified in the Materials and Methods section resulted in larger blank values, indicating that the proteoglycan itself contributed to the trapping effect.

Effect of enzyme concentration. Experiments with cathepsin D as degrading enzyme showed that the percentage release of radioactivity from the beads was not related linearly to the concentration of enzyme in the incubation mixture, but that good linear plots could be obtained by use of the logarithm of enzyme concentration. Inevitably, the curves became asymptotic as they approached the 0% and 100% values, but the range of linearity commonly extended at least from 20 to 70% release. A standard curve obtained with cathepsin D at pH 4.5 is shown in Fig. 2. With different batches of beads used over a period of some months, the slope [percentage release/log(enzyme concentration)] of such plots varied in the range 17.4–24.2. Standard curves were prepared

for each assay of unknown samples. The degradation of proteoglycan could also be detected as the release of polysaccharide precipitable with Alcian Blue (see the Materials and Methods section), and a similar dose-response curve was obtained (Fig. 3). The chemical modification of the assay procedure was less sensitive and rapid than the radiochemical method, but proved valuable in certain experiments in which the enzyme sample contained radioactivity.

Effect of duration of incubation. Reaction mixtures, pH4.5, containing 0.01 unit of cathepsin D were incubated for periods up to 24h. The results are shown in Fig. 4. The percentage release of radioactive material was clearly not linear with time, but was approximately logarithmic.

Effect of pH on activity. The activity of several proteoglycan-degrading enzymes in the standard assay was tested over the pH range 3.0-8.0. The buffers (0.1M) were sodium formate (pH3.0, 3.5), sodium acetate (pH4.0, 4.5, 5.0, 5.5), potassium phosphate (pH6.0, 6.5, 7.0) and Tris/HCl (pH8.0).

Maximal activity of cathepsin D was at pH4.5. Since values of percentage release of radioactivity are proportional to the logarithm of enzymic activity, it was thought that they should be corrected by use of a standard curve for cathepsin D at pH4.5. The resulting curve showed a remarkably sharp maximum, with activity falling to 20% or less of the maximal value at 0.5pH unit on either side (see Fig. 5). Greatest activity was obtained with other enzymes

Fig. 4. Effect of duration of incubation on release of radioactive material

The values for percentage release (means of triplicate determinations) have blank values (rising from 2.3 to 8.3%) subtracted, and are plotted against linear (○) or logarithmic (●) time scales. The line for the logarithmic plot is the linear regression obtained by the least-squares method ($r=0.983$), which gives $y = 22.31 \log x + 11.52$. The curve for the linear time scale also is calculated from this relationship.

Fig. 3. Activity of cathepsin D measured with unlabelled proteoglycan in polyacrylamide beads

The assays were at pH4.5, for 1h. Proteoglycan fragments released were assayed by the Alcian Blue method (see the Materials and Methods section), as chondroitin sulphate. The amounts of cathepsin D and chondroitin sulphate given are the totals for the 1.0ml incubation mixture.

Fig. 5. Effect of pH on the activity of cathepsin D Assays with the radiochemical assay were made by the standard method (see the Materials and Methods section), at the pH values indicated. The incubation time was 1h. The results (percentage release of ³⁵S) were converted into equivalent amounts of cathepsin D at pH4.5, by use of a dose-response curve.

as follows: papain, pH5.0-5.5; human cathepsin B, pH4.0-4.5; trypsin, pH9.0; bovine testicular hyaluronidase, pH5.0-6.0.

Specificity, sensitivity and precision of the assay. Standard curves were prepared for trypsin, papain, cathepsin B and hyaluronidase, at their respective

pH optima. For each, the percentage release of radioactivity was proportional to the logarithm of the enzyme concentration, as had been found for cathepsin D. Insufficient separate experiments were made to allow a definite decision as to whether there were consistent differences between enzymes in regard to the range of slopes of their standard curves, but in each of several experiments hyaluronidase gave rather steep slopes.

The reproducibility of assays was good. As an example, ten assay tubes were set up, each containing 0.01 unit of cathepsin D, at pH 4.5, with an equal number of blank incubation mixtures. After 4 h incubation, the percentage release of radioactivity was found to be 29.42 ± 0.99 (S.D., $n = 10$) as compared with 4.09 ± 0.17 (S.D., $n = 10$) in the blank tubes.

Assays can conveniently be compared as to sensitivity in terms of the amount of activity (here, that of cathepsin D in units of the standard haemoglobin assay) required to give a reading double that of the blank value for that assay. The present assay needs approx. 0.02 unit, compared with 0.2 for the haemoglobin assay, to give double the blank reading over 4 h at 37°C. In our hands, the corresponding value for the cartilage-disc method of Ignarro *et al.* (1973) is approx. 0.2 unit/ml.

Tests of possible explanations of the logarithmic dose-response of the assay

The logarithmic response of the assay gave it the advantage of an extremely wide range of sensitivity, such that a single incubation period, and a single dilution of samples, was satisfactory for the great majority of samples. It had the disadvantage that for precise quantification an appropriate standard curve was needed for each set of assays, and the choice of enzyme and conditions for the standard curve was not always easy. Experiments were therefore made in an attempt to explain and perhaps alter the form of the dose response.

Effect of size of beads. The polyacrylamide beads containing the radioactive substrate ranged in size from approx. 15 to 100 μm , and it seemed possible that the logarithmic response curve was a consequence of this. Beads were suspended in water and the most rapidly and most slowly sedimenting fractions were collected; they represented beads of 45 and 62 μm mean diameter respectively.

Dose-response curves were prepared for the two fractions, with cathepsin D at pH 4.5. The two curves were logarithmic and of similar slope. It was concluded that the heterogeneity of size of beads was not an important factor.

Effect of unlabelled external cartilage proteoglycan. The beads contain labelled proteoglycan, which is released from the beads by quite limited degradation.

Once outside the beads, further attack on the proteoglycan will not result in any further radioactivity being released, and it seemed possible that the logarithmic response curve was a result of the fact that the external proteoglycan acted as a competing unproductive substrate. In the standard assay, there is 0.25 mg of labelled proteoglycan in the beads in each tube. Assays were set up with 1.25 mg of unlabelled proteoglycan present outside the beads. No effect was observed on the slope of the dose-response curve, or on the curve of time versus release, so it was concluded that the logarithmic response was not a consequence of this effect.

Properties of Sepharose 4B-proteoglycan as substrate. ^{35}S -labelled proteoglycan was coupled to CNBr-activated Sepharose 2B or 4B (see the Materials and Methods section) to produce an alternative form of substrate. For practical purposes, the usefulness of this approach was limited by the fact that consistently only 5% of the labelled proteoglycan would react covalently with the Sepharose beads, regardless of the quantity of beads used. It was not found possible to increase the efficiency of conjugation by carrying out the reaction in 1 M-NaCl, or by subjecting the proteoglycan to a preliminary partial degradation with pepsin. It was concluded that only a small proportion of the proteoglycan molecules possessed amino groups available for reaction with the activated Sepharose. An additional 5% of the radioactive proteoglycan was bound non-covalently (perhaps by association with the covalently bound material) and eluted in the wash with sodium dodecyl sulphate.

The dose-response curves for the release of radioactive material from the Sepharose beads by a variety of enzymes were generally similar in shape to those obtained with the polyacrylamide beads (Fig. 6).

It was concluded that the logarithmic dose-response curve obtained in the standard assay is not a result of the immobilization of the substrate molecules by physical entrapment rather than covalent linkage.

Properties of ^3H -labelled immunoglobulin G entrapped in polyacrylamide beads as a substrate for proteolytic enzymes. Normal sheep immunoglobulin G was isolated by precipitation from 1.33 M- $(\text{NH}_4)_2\text{SO}_4$ and chromatography on DEAE-cellulose (Whatman DE-52). It was then labelled by treatment with $[\text{H}]$ acetic anhydride exactly as was described for α -crystallin (Blow *et al.*, 1975). The labelled protein was trapped in polyacrylamide beads exactly as described above for proteoglycan.

The dose-response curve for the effect of cathepsin D on the immunoglobulin G-polyacrylamide beads was very similar in shape to that obtained with the proteoglycan beads.

Fig. 6. Release of ^{35}S -labelled proteoglycan covalently linked to Sepharose beads by several enzymes

Assays were made by the standard method (see the Materials and Methods section), at the pH values indicated. The incubation time was 1 h. The enzymes were bovine testicular hyaluronidase, pH 4.5 (○); papain, pH 6.0 (△); bovine trypsin, pH 8.0 (□); chicken cathepsin D, pH 4.5 (●); pig pepsin, pH 4.5 (▲); and human cathepsin B, pH 5.0 (■). Incubation mixtures were supplemented with EDTA (2.0 mM) and cysteine (5.0 mM) for papain and cathepsin B, and with CaCl_2 (10 mM) for trypsin. The buffers (final concentration of buffer ion, 0.1 M) were sodium acetate (pH 4.5, 5.0), potassium phosphate (pH 6.0) and Tris/HCl (pH 8.0).

No definite explanation of the logarithmic response was indicated by the results of the above experiments. It seemed that this relationship of response to enzymic activity is not simply a peculiarity of the system in which cartilage proteoglycan is immobilized in polyacrylamide gel, but may apply equally to a variety of macromolecular substrates immobilized in a variety of ways.

Proteoglycan-degrading enzymes of rabbit ear cartilage: discovery of cathepsin F

Extraction of the proteoglycan-degrading enzymes of rabbit ear cartilage. Rabbit ear cartilage (6.2 g) was chopped finely and immersed in 60 ml of 200 mM-NaCl for 1 h. The cartilage was then dispersed by use of an Ultra-Turrax homogenizer (Scientific Instrument Centre, London WC1X 9JA, U.K.) during four 30 s bursts, each followed by 2 min cooling in ice/water. The homogenate was centrifuged at 15000 g for 30 min at 10°C and the supernatant further clarified by centrifugation at 100000 g for 30 min at 10°C to render it suitable for gel chromatography: 40 ml of extract was obtained, containing about 1.0 mg of protein/ml. Protein was determined by the method of Lowry *et al.* (1951), with bovine serum albumin as standard.

A large part of the proteoglycan-degrading activity of the extract was found to be inhibitable by pepstatin, and therefore attributable to cathepsin D (Barrett & Dingle, 1972; Dingle *et al.*, 1972). Further assays were therefore made with 0.5 µg of pepstatin/ml (for 4 h at 37°C) in an attempt to identify the other degradative enzymes.

pH-stability of the cartilage enzymes. Extracts (5 ml) were dialysed against the following buffers at 4°C (all 40 mM sodium salts containing 200 mM-NaCl, 0.1% Brij 35 and 0.1% butan-1-ol): formate (pH 3.0), acetate (pH 4.5) and phosphate (pH 5.8 and 6.8). Samples were assayed at pH 4.5 after 3 days, and the percentage recovery of activity was calculated from the amounts of radioactivity released, by use of a dose-response curve for the enzyme. Approximate recoveries were as follows: pH 3.0, 30%; pH 4.5, 100%; pH 5.8, 60%; and pH 6.8, 40%.

We concluded that for the purposes of storage and purification, crude enzyme extracts are most stable at pH 4.5.

Effect of pH and pepstatin on the cartilage enzymes. A sample of the clarified cartilage extract (0.5 ml) was added to 1 ml of bead suspension and 0.5 ml of 0.4 M buffer as follows: pH 3.0–3.5 (sodium formate), 4.0–5.5 (sodium acetate), 6.0–7.0 (sodium phosphate) and pH 8.0 (Tris/HCl). At each pH value, triplicate determinations were made of activity in the absence of extract ('blanks'), in the presence of extract and in the presence of extract and pepstatin (final concn. 0.5 µg/ml). Incubation was for 4 h at 37°C, and 100 µl of each final supernatant was taken for liquid-scintillation counting of radioactivity. The results (Fig. 7) expressed as percentage release of radio-

Fig. 7. Effect of pH and pepstatin on the activity of cartilage extracts

Assays were done in the absence (●) and presence (○) of pepstatin (1 µg/ml), by the bead assay. See the text for further details.

activity (although this leads to bias in favour of small amounts of activity) showed a peak of pepstatin-resistant activity at pH 4.0–4.5. Like cathepsin D, the enzyme(s) responsible for this activity gave a logarithmic response in the assay.

Effect on the viscosity of proteoglycan in solution. The pepstatin-resistant enzyme(s) could be shown to degrade proteoglycan in solution, in a viscometric assay (Fig. 8). The incubation mixture contained 5 mg of proteoglycan/ml of 0.1 M-sodium acetate buffer, pH 4.5, and 0.2 M-NaCl (total vol. 2 ml).

In the absence of pepstatin, 0.8 ml of extract took 34 min to produce a 50% decrease in viscosity, whereas no decrease in viscosity occurred with a control sample of extract that had been heated to 100°C for 5 min. In the presence of pepstatin (1 µg/ml), the viscosity was halved in 160 min. It was concluded that 21% [(34/160) × 100] of the viscosity-decreasing activity of the extract was not attributable to cathepsin D. It seemed that the cartilage extracts contained a previously unrecognized enzyme capable of degrading proteoglycan at pH 4.5; this was tentatively designated 'cathepsin F'.

Additional pepstatin-resistant activity was detectable in assays with 2 mM-EDTA and 5 mM-cysteine; this activity was inhibited by leupeptin and egg-white papain inhibitor, which indicated that it was due to cathepsin B.

Separation of the enzymes by gel chromatography. A column (114 cm × 3.0 cm; 800 cm³) of Sephadex G-100 was equilibrated with 42.5 mM-KH₂PO₄/7.5 mM-K₂HPO₄ buffer, pH 5.85, containing 200 mM-NaCl and 0.1% Brij 35, and calibrated with Blue Dextran 2000 (void volume), bovine serum albumin (68 000 mol.wt.), chymotrypsinogen (25 000 mol.wt.), cytochrome *c* (12 400 mol.wt.) and ferricyanide (330 mol.wt.).

Fig. 8. *Viscometry of cartilage-extract proteoglycan-degrading activity*

Incubation (at 37°C) was in the absence (●) and presence (○) of pepstatin (1 µg/ml), or with a boiled extract (Δ). The relative viscosity was calculated, and expressed as a percentage of the zero-time value. See the text for further details.

Cartilage extract (35 ml) was run on the column at a flow rate of 4.2 ml/h per cm²; the effluent was monitored at 280 nm and 8 ml fractions were collected. The fractions were assayed for proteoglycan-degrading activity by the bead assay in the presence of various inhibitors and by viscometry, and for lysozyme by agar-gel radial diffusion (Schill & Schumacher, 1972), the latter assay being standardized with hen's-egg lysozyme. The results are shown in Fig. 9.

Most of the protein in the extract appeared near the void volume, whereas the proteoglycan-degrading activities were markedly retarded. In the absence of inhibitors, two major peaks of activity were detected, with mol.wts. of about 43 000 and 25 000, consistent with their representing cathepsins D and B. These identifications were confirmed by the inhibition of the first activity with pepstatin, and the second with leupeptin. In the presence of these inhibitors, a lower and broader peak of activity in the range 50 000–70 000 mol.wt. became apparent, and this was attributed to cathepsin F. The peak of lysozyme activity, eluted very late, was not associated with proteoglycan-degrading activity detectable at pH 4.5 or 7.0 in the bead assay or by viscometry.

The relative amounts of cathepsin D, B and F were estimated by a comparison of the activity (in cathepsin D units) of activated extracts in the presence of leupeptin and pepstatin (cathepsin F; 10 munits/ml), in the presence of pepstatin alone (cathepsin B + F = 50 munits/ml; therefore cathepsin B = 40 munits/ml) and in the absence of any inhibitors (cathepsins D + B + F = 180 munits/ml, therefore cathepsin D = 130 munits/ml). Expressed as units/g of ear cartilage this is equivalent to cathepsin D, 1.6 unit/g; cathepsin B, 0.5 unit/g and cathepsin F, 0.1 unit/g. This can be compared with 100 units of cathepsin D/g of human liver (Barrett, 1970).

Degradation of proteoglycan by cathepsin F. The 'bead assay' is not specific for the detection of proteolytic degradation of proteoglycans, and the finding that cathepsin F is unaffected by many proteinase inhibitors (see below) emphasized the need for proof of its endopeptidase action. The pepstatin-resistant lowering of viscosity of solutions of proteoglycan (see above) was, however, more consistent with the action of an endopeptidase, or possibly endoglycosidase, than a sulphatase or an exoglycosidase.

³⁵S-labelled proteoglycan was chromatographed on a column (1.4 cm × 30 cm) of Sepharose 2B before incubation with cartilage extract, and also after incubation with the extract for various periods at pH 4.5, in the presence of pepstatin. The column was equilibrated with 1.1 mM-KH₂PO₄/8.1 mM-Na₂HPO₄ containing 137 mM-NaCl.

Initially, the radioactive proteoglycan was largely excluded from the gel, and appeared as a peak at the

Fig. 9. Distribution of proteoglycan-degrading activities after chromatography on Sephadex G-100 gel of rabbit ear cartilage extract

Proteoglycan-degrading activity was assayed in the presence of no inhibitor (----), in the presence of pepstatin (····) and in the presence of pepstatin and leupeptin (-.-.-). Protein (—), lysozyme (---). See the text for further details. The elution volumes and molecular weights of the standards used to calibrate the column are indicated by the arrows.

void volume, with some trailing. Incubation with the cartilage extract caused progressively increasing retardation on the column, consistent with a decrease in molecular size, but always there was only a single, broad peak of radioactivity in the effluent fractions.

These results were thought to be consistent only with endopeptidase activity. Any form of attack on the polysaccharide chains would be expected to release radioactive material of relatively low molecular weight that would appear as a new, greatly retarded peak in gel chromatography.

It was concluded from the viscometric and gel-chromatography experiments that cathepsin F has endopeptidase activity against proteoglycan.

Stability. Over a period of 2 years, there was no substantial loss of cathepsin F activity in rabbit ear cartilage stored at -20°C . In high-speed homogenates of the tissue, the enzyme showed unusual heat-stability, and as shown above was stable to storage at 4°C when dialysed against sodium acetate buffer, pH4.5.

In contrast with the stability of unpurified cathepsin F, the enzyme purified by chromatography on Sephadex G-100 was very labile, showing a loss of activity of approx. 50% during 24h at 4°C .

Effect of potential inhibitors. The enzyme purified on Sephadex G-100 (specific activity fivefold that of a crude extract) was assayed in the presence of EDTA (5mM), cysteine (5mM), Trasylol (100 $\mu\text{g/ml}$), phenylmethanesulphonyl fluoride (1.0mM), soya-bean trypsin inhibitor (100 $\mu\text{g/ml}$), di-isopropyl-

phosphorofluoridate (1.0mM), lima-bean trypsin inhibitor (100 $\mu\text{g/ml}$) or chloroquine (20mM). Only with chloroquine was significant inhibition noted, the release of radioactivity corresponding to that with 40%-less-uninhibited enzyme, though no correction for possible quenching of radioactivity was made in these experiments.

Purified ovomithin and egg-white papain inhibitor (100 $\mu\text{g/ml}$) had negligible effect on the cathepsin F activity, though both chicken-egg white (100 μl) and turkey-egg white (100 μl) gave complete inhibition. α_2 -Macroglobulin was not tested on the purified material, but since all of the pepstatin-resistant activity in crude preparations was sensitive to this inhibitor it was assumed that cathepsin F is sensitive to α_2 -macroglobulin.

Intracellular localization of cathepsin F. Rabbit ear cartilage (2.0g) was chopped finely and placed in 15ml of culture medium (BGJ_w) containing 2mM-cysteine, 5mM-EDTA and 2mg of papain/ml. The mixture was incubated at 37°C for 3h with disruption of the tissue by use of a Pasteur pipette at 10min intervals. The suspension of cartilage cells obtained in this way was separated from residues of tissue by filtration through gauze, and the cells were sedimented by centrifugation at low speed (1700g). The cells were washed three times in fresh culture medium (10ml) by centrifugation (1700g) and resuspension. The second and third washes contained 0.1mM-Tos-Lys-CH₂Cl (7-amino-1-chloro-3-L-tosylamidoheptan-2-one, 'TLCK'), a powerful irreversible inhibitor of

papain. The cells, in the form of a dense suspension (2.0 ml), were mixed with 2.0 ml of 10% (w/v) gelatin solution in 0.25 M-sucrose containing 10 mM-Tris/HCl buffer, pH 7.2, at 37°C. The mixture was allowed to solidify at 4°C, and was then chopped into small pieces, which were homogenized in 10 vol. of the Tris-buffered sucrose solution by eight passes through the automatic homogenizer described by Dingle & Barrett (1969). The homogenate was warmed to 37°C to allow the gelatin to dissolve, cooled in ice and centrifuged for 3000 g-min. The pellet was retained and a further 200000 g-min pellet prepared from the supernatant.

The two pellets were resuspended in the buffered sucrose to the original volume of the homogenate, and portions of these, together with the final supernatant, were assayed for cathepsin F activity. Assays with ³⁵S-labelled proteoglycan in polyacrylamide beads were made at pH 4.5 with 1 mM-Tos-Lys-CH₂Cl and 1 µg of pepstatin/ml to give specificity for cathepsin F.

Some 46% of the cathepsin F activity was found in the 3000 g-min pellet (including cells and nuclei), 44% in the 200000 g-min pellet (including mitochondria and lysosomes) and 10% in the final supernatant (including microsomal fraction and cytosol). With due allowance for probably incomplete breakage of the cells, this distribution would be consistent with an association of cathepsin F with one of the membrane systems of the cell. The activity of the 20000 g-min fraction was not increased or solubilized by treatment with 0.1% Triton X-100 or by freezing and thawing, and thus did not show that 'latency' typical of lysosomal enzymes. Certain of the lysosomal enzymes do behave in this way, however, and the possibility that cathepsin F occurs with the majority of other cellular acid hydrolases, in lysosomes, remains open.

Presence of cathepsin F in human and rabbit articular cartilage. Samples of human articular, rabbit articular and rabbit ear cartilage were homogenized as described previously, except in that the extraction medium was 0.1% Triton X-100 in 200 mM-NaCl, and that the volume used was 20 ml/g wet wt. of tissue. Incubation was at 37°C for 24 h in the presence of 1 µg of pepstatin/ml, in the bead assay at pH 4.5 or 7.0. Results are expressed as percentage release of radioactivity, above the blank value. Samples (0.2 ml) of homogenates from six different extracts of human articular cartilage gave an average of 35% release (range 21–53%), those from five different extracts of rabbit articular cartilage gave an average of 50% release (range 42–63%) and those from ten different extracts of rabbit ear cartilage gave an average of 65% release (range 43–82%), at pH 4.5. Assays at pH 7.0 gave only 3% release for human cartilage extracts and less than 1% for rabbit cartilage (ear or articular).

Discussion

The new assay for proteoglycan-degrading enzymes has very great sensitivity, and is applicable to large numbers of samples. The method also has the advantage of complete specificity for enzymes degrading proteoglycan, and yet is sensitive to virtually any type of degradation of the molecule; it even could be expected to show a positive reaction to sulphatase attack, which would be missed by most other methods. The logarithmic dose response of the assay makes careful standardization more critical than it would otherwise be, but carries with it the advantage of an extremely wide usable range of sensitivity. A possible limitation stems from the fact that the enzyme molecules must be of small enough molecular size to enter the beads. Activity of high-molecular-weight enzymes, or enzymes associated with membrane fragments, could therefore be missed.

We are not able to offer any complete explanation of the logarithmic relationship of percentage release of radioactivity to enzymic activity (i.e. concentration of enzyme, or incubation time). Professor J. E. Scott (personal communication) has pointed out to us that logarithmic isotherms for adsorption of enzyme to substrate particles could be involved, but an explanation is also required for the dependence of percentage release on the logarithm of incubation time. Since physical trapping of the large proteoglycan molecules (and their aggregates) probably plays a large part in their retention in cartilage matrix, as in the polyacrylamide beads, it is clearly possible that the logarithmic relationship applies to physiological systems, as well as the assay *in vitro*.

Because most of the extracellular matrix of cartilage is believed to be at near-neutral pH, there is currently much interest in the possible existence in the tissue of enzymes active at higher pH values than the well-characterized lysosomal proteinases, cathepsins B and D. In the present study of rabbit ear cartilage, as well as work along similar lines with human articular cartilage (B. Hazleman, D. Y. Bulgen, J. T. Dingle & A. J. Barrett, unpublished work), very little neutral activity has been detected. On the contrary, a new acidic activity has been identified, and this is attributable to a tissue proteinase that has tentatively been named cathepsin F. The desirability of naming an enzyme before it has been thoroughly characterized may be questioned, but we consider that it is more helpful than harmful, and does facilitate discussion of the evidence.

The molecular size of the products of action of cathepsin F is characteristic of the products of proteolytic degradation, and the crude enzyme is completely inhibited by α₂-macroglobulin, which seems specific for endopeptidases (Barrett & Starkey, 1973; Barrett *et al.*, 1974). On the other hand, cathepsin F was unaffected by a range of inhibitors,

some of which would have been expected to affect a proteinase of any of the four recognized catalytic classes, namely the serine, thiol, carboxyl and metallo-proteinases. Several proteinases have already been reported to show similarly anomalous behaviour (Barrett, 1977). Our preliminary results indicate that cathepsin F may be rather selective for the degradation of proteoglycan, having little or no action on haemoglobin. Cathepsins A and C are exopeptidases, not known to have any action on proteoglycan, whereas cathepsin E is inhibited by pepstatin, like cathepsin D (Barrett & Dingle, 1972).

The pH optimum of cathepsin F is in the region of 4.0–4.5, and the enzyme has a mol.wt. of approx. 50000. Activity was stable in the tissue and in extracts kept at pH 4.5, but rapidly disappeared after purification of the enzyme on Sephadex G-100.

Fractionation of subcellular particles of rabbit ear chondrocytes showed almost half of the activity in the fraction composed largely of mitochondria and lysosomes, and, being an acid hydrolase, it seems probable that cathepsin F is a lysosomal enzyme, although we did not detect typical latency.

The bulk-phase pH value of cartilage matrix is approximately neutral, but it has been argued that the chondrocytes may well acidify their immediate environment, creating pericellular regions favourable to the action of acid hydrolases (Dingle, 1975). Even so, the amount of cathepsin F activity was not impressive, being much less than that of cathepsin B or cathepsin D in the bead assay. Conceivably, only a fraction of the cathepsin F in the tissue was extracted in a form of sufficiently small molecular size to be retarded on Sephadex G-100, and to be detected in the bead assay. Cathepsin F was found in the articular cartilage of rabbit and man, but it would be premature at this stage to attempt any definite conclusions in regard to its possible physiological significance.

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