APPENDIX

Proteinase activity in chondroitin lyase (chondroitinase) and endo- β -D-galactosidase (keratanase) preparations and a method to abolish their proteolytic effect on proteoglycan

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Significant amounts of proteinase activity have been found in chondroitin ABC lyase (EC 4.2.2.4), chondroitin AC II lyase and endo- β -D-galactosidase (keratanase) from commercial sources. It would appear, therefore, that certain earlier biochemical and histochemical studies, which employed these commercial enzyme preparations for their presumed ability to degrade only glycosaminoglycans, may require re-evaluation. A mixture of EDTA, *N*-ethylmaleimide, phenylmethanesulphonyl fluoride and pepstatin abolishes the effect of the contaminating proteinases on proteoglycan with less significant effect on the chondroitin lyase or keratanase activity.

Chondroitin ABC lyase (EC 4.2.2.4) and chondroitin AC lyase (EC 4.2.2.5) were purified from *Proteus vulgaris* ECTC 4636 and *Flavobacterium heparinum* ATCC 13125 respectively by Yamagata *et al.* (1968). It has been established that these enzymes act as an endo-eliminase (attacking hexosaminide bonds in a random fashion) to yield unsaturated disaccharides as final products. Hiyama & Okada (1975) have since described a chondroitin lyase (termed 'chondroitin AC II lyase') from *Arthrobacter aureescens* which exhibits a substrate specificity similar to chondroitin AC lyase but appears to be more active as an exo-eliminase (removing dissaccharide units stepwise from the non-reducing terminus) rather than an endo-eliminase (Hiyama & Okada, 1976). Nakazawa & Suzuki (1975), on the other hand, have purified endo- β -D-galactosidase (trivial name: keratanase) from *Pseudomonas* sp. IFO 13309 which attacks the endo-galactoside bonds of keratan sulphate to give rise to small oligosaccharides. All these enzymes have been used increasingly, as specific reagents for the degradation of chondroitin sulphate, dermatan sulphate or keratan sulphate.

In the course of studies on the structure of cartilage proteoglycan (Oike *et al.*, 1980), the need arose for the development of a method with the glycosaminoglycan-degrading enzymes to remove the bulk of the glycosaminoglycan moiety, without altering the protein moiety of the macromolecule. We have tested, therefore, commercial preparations of chondroitin ABC lyase, chondroitin AC II lyase and keratanase for proteinase activity that would be expected to cause the most confusion in isolation and characterization of the protein molecule.

Here, we report the presence of significant amounts of proteinase activity in the chondroitin lyase and keratanase samples and a method to abolish the effect of the contaminating proteinases on proteoglycan.

Experimental

Unless otherwise indicated, the materials and general methods used are the same as those employed in the accompanying paper (Oike *et al.*, 1980).

Chondroitin ABC lyase (EC 4.2.2.4) from *Proteus vulgaris* NCTC 4636 (Yamagata *et al.*, 1968), chondroitin AC II lyase from *Arthrobacter aureescens* (Hiyama & Okada, 1975) and keratan sulphate endo- β -D-galactosidase (keratanase) from *Pseudomonas* sp. IFO 13309 (Nakazawa & Suzuki, 1975) were products of Seikagaku Kogyo Co., Nihonbashi-honcho 2-9, Tokyo, Japan, and were kindly donated by Dr. M. Nomoto and Dr. S. Shibata of the company. These commercial enzyme preparations are available from the above company or from Miles Laboratories Inc., Elkhart, IN, U.S.A. In preliminary experiments, *F. heparinum* chondroitin AC lyase (EC 4.2.2.5) isolated in this laboratory (Yamagata *et al.*, 1968) was used instead of the chondroitin AC II lyase sample to prepare chondroitin sulphate-free cores and essentially identical results were obtained. Pepstatin, the inhibitor of acid proteinases (Aoyagi *et al.*, 1971), was a gift from Dr. H. Umezawa, Institute of Microbial Chemistry, Tokyo, Japan. The reagent is available commercially from Protein Research Foundation, 476, Ina, Minoh, Japan, or from Sigma Chemical

Co., St. Louis, MO, U.S.A. A standard preparation of chondroitin 6-sulphate was a gift from Dr. M. B. Mathews, University of Chicago, Chicago, IL, U.S.A. Bio-Gel A-1.5m was purchased from Bio-Rad Laboratories (Richmond, CA, U.S.A) and DEAE-cellulose (DE 52) from Whatman (Springfield Mill, Maidstone, Kent, U.K.).

[^{14}C]Serine-labelled non-proteoglycan protein was obtained from the PG-L/non-proteoglycan-protein fraction (see the Experimental section in the main paper, Oike *et al.*, 1980), as follows: a 6×10^5 c.p.m. portion of the [^{14}C]PG-L/non-proteoglycan-protein fraction from zonal sedimentation [the top one-

fourth; see Kimata *et al.* (1978) for the pattern of sedimentation] was dialysed against 0.02M-Tris/HCl, pH 7.2, containing 5mM-EDTA. The non-diffusible material was applied to a column (5cm $\times$ 0.9cm diam.) of DEAE-cellulose equilibrated with the same buffer. The column was washed with the same buffer. Washings (30ml) containing about 5×10^5 c.p.m. of ^{14}C or 1mg of Lowry protein were combined, dialysed against 5mM-Tris/HCl, pH 7.2, containing 1mM-disodium EDTA and freeze-dried.

The initial velocity of degradation of unlabelled glycosaminoglycans by chondroitin ABC lyase,

Fig. 1. Elution profiles from Sepharose CL-4B of products obtained after digestion of [^{14}C]PG-H with chondroitin ABC lyase, chondroitin AC II lyase or keratanase in the absence or presence of proteinase inhibitors

Portions (1 ml, 1×10^4 c.p.m.) of the digests were applied to the column and separated under conditions described in the Experimental section of the accompanying paper (Oike *et al.*, 1980). The profiles of the digests prepared with chondroitin ABC lyase, chondroitin AC II lyase and keratanase in the absence of proteinase inhibitors are shown in (a), (b) and (c) respectively, and those of the digests prepared in the presence of proteinase inhibitors in (d), (e) and (f) respectively. Intact [^{14}C]PG-H was eluted in the void volume (V_0) as shown in a (.....). Peaks PG(-CS)_{ABC}, PG(-CS)_{AC} and PG(-KS) were pooled as indicated by the horizontal bars and analysed as described in the accompanying paper (Oike *et al.*, 1980).

chondroitin AC II lyase or keratanase was determined by measurement of oligosaccharide products with the ferricyanide reagent (Park & Johnson, 1949), essentially as described by Nakazawa *et al.* (1975).

Results and Discussion

The results described below indicate that the commercial preparations of chondroitin ABC lyase, chondroitin AC II lyase and keratanase are contaminated with proteinases.

[^{14}C]Serine-labelled proteoglycan (PG-H) from chick-embryo cartilages was incubated for 40 min

with each commercial enzyme preparation under conditions which permit almost complete degradation of the chondroitin sulphate (in the case of chondroitin lyases) or the keratan sulphate (in the case of keratanase) moiety. Subsequent chromatography of the digestion products on Sepharose CL-4B indicated the appearance of radioactive peaks corresponding to small peptides (Fig. 1*a,b,c*).

More extensive proteolysis was demonstrated when a non-proteoglycan-protein fraction, prepared from [^{14}C]serine-labelled cartilages, was incubated for 4 h with each enzyme preparation (Fig. 2*a,b,c*).

We, therefore, investigated various combinations of enzyme concentration, incubation time and

Fig. 2. Elution profiles from Bio-Gel A-1.5 m of products obtained after digestion of [^{14}C]non-proteoglycan proteins with chondroitin ABC lyase, chondroitin AC II lyase or keratanase in the absence or presence of proteinase inhibitors

The conditions for enzymic digestion were those described for the digestion of PG-H (see the Experimental section) except that the substrate was replaced by 500 μg (as Lowry protein) of [^{14}C]non-proteoglycan proteins and the reaction was carried out for 4 h. Portions (0.4 ml, 5×10^4 c.p.m.) of the digests were applied to the column and separated under conditions described in the Experimental section of the accompanying paper (Oike *et al.*, 1980). The profiles of the digests prepared with chondroitin ABC lyase, chondroitin AC II lyase and keratanase in the absence of proteinase inhibitors are shown in (a), (b) and (c) respectively, and those of the digests prepared in the presence of proteinase inhibitors in (d), (e) and (f) respectively. Intact [^{14}C]non-proteoglycan proteins were eluted in the void volume (V_0) as shown in (a) (.....).

proteinase inhibitors to find satisfactory conditions for the degradation of the glycosaminoglycan chains without altering the protein portion of PG-H. The following procedures proved satisfactory for this purpose. All incubations were carried out in stainless-steel test tubes, because in detergent-free solutions, proteoglycan core molecules tend to adhere to glassware (Oike *et al.*, 1980). Note that the enzyme units indicated below are not nominal ones given by the manufacturer. Enzyme activities were assayed immediately before use; the assay procedures were those described by Yamagata *et al.* (1968) (for chondroitin ABC lyase), Hiyama & Okada (1975) (for chondroitin AC II lyase) and Nakazawa & Suzuki (1975) (for keratanase), except that incubations were carried out in the following buffers: 0.025 M-Tris/HCl, pH 8.0, containing 0.03 M-sodium acetate (chondroitin ABC lyase assay), 0.025 M-Tris/HCl, pH 7.4, containing 0.03 M-sodium acetate (chondroitin AC II lyase assay) and 0.025 M-Tris/HCl, pH 8.0 (keratanase assay). One unit of enzyme is defined as the amount that catalysed the release of 1 μ mol of unsaturated disaccharides from chondroitin 4-sulphate per min (chondroitin ABC lyase and chondroitin AC II lyase) or the release of 1 μ mol of reducing galactose end-groups from bovine corneal keratan sulphate per min (keratanase).

Chondroitin ABC lyase digestion

A stainless-steel test tube containing freeze-dried proteoglycan sample (about 1 μ mol as hexuronate) was placed in an ice-bath, to which was added 1.0 ml of an enzyme reagent containing 100 μ mol of Tris/HCl, pH 8.0, 30 μ mol of sodium acetate, 100 μ g of bovine serum albumin, 10 μ mol of disodium EDTA, 10 μ mol of *N*-ethylmaleimide, 5 μ mol of phenylmethanesulphonyl fluoride, 0.36 μ mol of pepstatin and 1.5 units of chondroitin ABC lyase. Since it is difficult to dissolve phenylmethanesulphonyl fluoride and pepstatin directly in water, the reagents were previously dissolved in methanol to give a concentration of 0.1 M and 7.2 mM respectively. The final concentrations of the four proteinase inhibitors in the reaction mixture are: 10 mM-EDTA, 10 mM-*N*-ethylmaleimide, 5 mM-phenylmethanesulphonyl fluoride and 0.36 mM-pepstatin (hereinafter this mixture will be referred to as 'inhibition mixture'). The tube was then placed in a 37°C-water bath. After incubation for 40 min, the reaction was terminated by addition of 1 ml of 8 M-guanidinium chloride containing 0.4% (w/v) Triton X-100 and the inhibition mixture.

Chondroitin AC II lyase digestion

The procedures were as described above except that 1 ml of an enzyme reagent containing 100 μ mol of Tris/HCl, pH 7.4, 30 μ mol of sodium acetate, the

inhibition mixture and 1.0 unit of chondroitin AC II lyase was used.

Keratanase digestion

The procedures were as described for the chondroitin ABC lyase digestion except that 1 ml of an enzyme reagent containing 100 μ mol of Tris/HCl, pH 8.0, the inhibition mixture and 0.05 unit of keratanase was used.

Fig. 1(*d,e,f*) and Fig. 2(*d,e,f*) indicate that the mixture of proteinase inhibitors abolishes the effect of the contaminating proteinases on both [¹⁴C]PG-H and ¹⁴C-labelled non-proteoglycan protein, i.e. the release of ¹⁴C-labelled small peptides from these macromolecules was completely blocked by the inclusion of the inhibition mixture in the incubation media. Omission of EDTA, *N*-ethylmaleimide, phenylmethanesulphonyl fluoride or pepstatin from the incubation of chondroitin ABC lyase with ¹⁴C-labelled non-proteoglycan protein caused the appearance of 6, 8, 42 or 16% respectively, of the total radioactivity in retarded fractions (elution patterns not shown), indicating that inclusion of all the four inhibitors is essential for complete inhibition. Further analyses of the digestion products, obtained from [¹⁴C]PG-H and [³⁵S]PG-H in the presence of the proteinase inhibitors, indicate that chondroitin ABC lyase (or chondroitin AC II lyase) and keratanase released the bulk of the chondroitin sulphate and the keratan sulphate respectively, without altering the protein portion of the macromolecule (Oike *et al.*, 1980).

In a separate experiment, unlabelled chondroitin 6-sulphate (0.8 μ mol as hexuronate) was treated with chondroitin ABC lyase (0.04 unit) or chondroitin AC II lyase (0.04 unit) in the absence or presence of the proteinase inhibitors. Measurements of the initial velocity of enzyme reactions indicated that the proteinase inhibitors had a depressant effect (80% inhibition) on the activity of chondroitin ABC lyase whereas they had no effect on the chondroitin AC II lyase activity at all. The inhibitory effect on chondroitin ABC lyase is primarily brought about by phenylmethanesulphonyl fluoride. With corneal keratan sulphate (0.4 μ mol as galactose) and keratanase (0.02 unit), the initial velocity of keratan sulphate hydrolysis in the presence of the inhibitors was 70% of that in the absence of the inhibitors. The results suggest that enzyme concentration and incubation time must be rigorously controlled, particularly when the glycosaminoglycan moiety of a relatively large amount of proteoglycan is to be degraded by digestion with chondroitin ABC lyase or keratanase in the presence of the proteinase inhibitors.

The presence of proteinases in the commercially available preparations of chondroitin lyases and keratanase has important implications for the use of

these enzymes in histochemical and physiological studies. For example, the disappearance of some activities of a tissue or cell by treatment with chondroitin AC II lyase is considered to be convincing evidence that the material participating in the activities is chondroitin sulphate proteoglycan. In view of the findings reported here it is advisable that such effects of the enzyme should either be interpreted with more caution or the specificity of the enzyme action should be supported by further experiments. Our method with a mixture of proteinase inhibitors provides a tool to overcome these limitations. Quintarelli *et al.* (1978) also used proteinase inhibitors to validate their results with *Streptomyces* hyaluronate lyase working with cartilage proteoglycans.

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