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The Degradation of Cartilage Proteoglycans by Tissue Proteinases

PROTEOGLYCAN STRUCTURE AND ITS SUSCEPTIBILITY TO PROTEOLYSIS

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1. Proteoglycan was obtained from bovine nasal cartilage by a procedure involving sequential extraction with a low-ionic-strength KCl solution, then a high-ionic-strength $CaCl_2$ solution. Purification was by CsCl-density-gradient centrifugation. 2. The $CaCl_2$-extracted proteoglycan was subjected to proteolytic degradation by papain, trypsin, cathepsin D, cathepsin B, lysosomal elastase or cathepsin G. Degradation was allowed to proceed until no further decrease in viscosity was detectable. 3. The size and chemical composition of the final degradation products varied with the different proteinases. Cathepsin D and cathepsin G produced glycosaminoglycan-peptides of largest average size, and papain produced the smallest product. 4. The KCl-extracted proteoglycan was intermediate in molecular size and composition between the $CaCl_2$-extracted proteoglycan and the largest final degradation products, and may have been formed by limited proteolysis during the extraction procedure. 5. It is postulated that the glycosaminoglycan chains are arranged in groups along the proteoglycan core protein. Proteolytic cleavage between the groups may be common to the majority of proteinases, whereas cleavage within the groups is dependent on the specificity of each individual proteinase.

Cartilage proteoglycan may be extracted by either a disruptive procedure, with high-speed homogenization of the tissue, or a dissociative procedure, involving extraction with a high-ionic-strength salt solution (Hascall & Sajdera, 1969). The latter technique is preferable because it minimizes mechanical and enzymic fragmentation of the macromolecules (Dunstone & Cleland, 1975). The availability of proteoglycan in its native state is a prerequisite for investigations of structure and function, and it is now known that proteoglycan molecules exist *in vivo* as macromolecular aggregates in association with hyaluronic acid (Gregory, 1973; Hardingham & Muir, 1974; Hascall & Heinegård, 1974). Further, the proteoglycan molecules do not have a unique structure; it is known that they differ in content of chondroitin sulphate, keratan sulphate and protein (Hoffman *et al.*, 1975), but much remains to be learnt about the structure of the core protein (Hopwood & Robinson, 1975) and the precise arrangement of glycosaminoglycan chains (Heinegård & Axelsson, 1977).

Information on the structure of proteoglycans has been gained by studying the products of proteolytic degradation. By using such an approach, Mathews (1971) postulated that the chondroitin sulphate chains are arranged in 'doublets' along the polypeptide core. However, Heinegård & Hascall (1974*b*) demonstrated that one-third of the length of the core protein is devoid of glycosaminoglycan chains, and it is this region that interacts with hyaluronic acid (Hardingham & Muir, 1974; Hascall & Heinegård, 1974). It was postulated that the remainder of the core protein contained the glycosaminoglycan chains arranged in groups of various sizes, characterized by the resistance of their internal peptide bonds to proteolytic attack.

The results described here concern the degradation of cartilage proteoglycan by six proteinases, of which four were purified from human tissues. These were cathepsin B, cathepsin D, lysosomal elastase and cathepsin G. For each enzyme a possible role has been postulated in pathological tissue damage (Dingle & Burleigh, 1974; Barrett, 1975; Malemud & Janoff, 1975; Barrett & Starkey, 1977), and each is capable of degrading cartilage proteoglycan to small fragments (Morrison *et al.*, 1973; Keiser *et al.*, 1976). Degradation was measured by viscometry, and the electrophoretic properties and chemical composition of the products were determined. The results are discussed in terms of proteoglycan structure and its susceptibility to proteolytic attack.

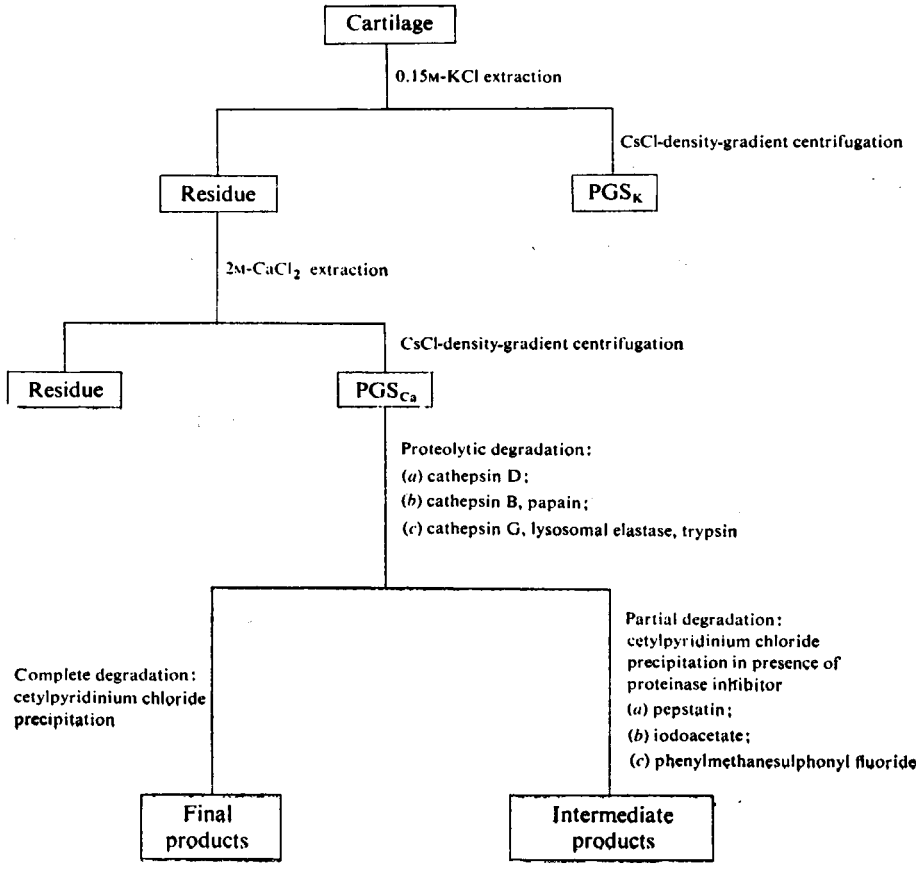
Methods

Chemicals used were either analytical grade or the best grade commercially available.

Extraction and purification of proteoglycan

Bovine nasal septa were obtained from 2-year-old animals within 30min of death. The tissue was kept at 0°C for up to 4h, during which time the cartilage was removed from the surrounding soft tissue and sliced; it was then stored at −20°C until required.

Cartilage slices (40g wet wt.) were extracted sequentially with (*a*) 0.15M-KCl, unbuffered (800ml), then (*b*) 2M-$CaCl_2$, buffered at pH5.8 with 0.05M-sodium acetate (800ml). Both extractions were for 20h at 4°C, and the extract was removed from the cartilage residue by filtration through a glass sinter (porosity 1). Each extract was dialysed against 10 litres of 0.05M-sodium acetate, pH5.8, at 4°C for 20h, then concentrated to 200ml by rotary evaporation at 25°C. Dialysis and concentration were repeated and the extracts were then filtered through a glass sinter (porosity 2). The density of each extract was adjusted to 1.69g/ml by the addition of solid CsCl, and the pH was re-adjusted to 5.8 by the addition of acetic acid. Centrifugation (Hascall & Sajdera, 1969) was performed in a MSE 65 centrifuge in an 8×50ml angle rotor at 90000$g_{av.}$ ($r_{av.}$ = 7.4cm) for 48h at 20°C. Fractions of the gradients were assayed for density, uronic acid and A_{280}. Fractions



Scheme 1. *Extraction and degradation of proteoglycan from bovine nasal cartilage*

Cartilage slices were extracted with 0.15M-KCl, then with 2M-$CaCl_2$. Proteoglycan subunit (PGS) was purified by CsCl-density-gradient centrifugation under first associative, then dissociative, conditions. Proteoglycan subunit was then subjected to degradation by (*a*) carboxyl, (*b*) thiol or (*c*) serine proteinases, and both intermediate and final degradation products were isolated by precipitation with cetylpyridinium chloride. Inhibitors specific for the three proteinase types, (*a*) pepstatin, (*b*) iodoacetate or (*c*) phenylmethanesulphonyl fluoride, were added during the isolation of intermediate products. For details of the experimental procedure see the Methods section.

having a density greater than 1.69g/ml were combined, dialysed exhaustively against water at 4°C, and freeze-dried for storage at −20°C. The KCl extraction and the $CaCl_2$ extraction yielded 0.35g and 2.42g respectively of proteoglycan.

Proteoglycan from each preparation was dissolved in a solution that contained 0.05M-sodium acetate, 4M-guanidinium chloride and CsCl to a density of 1.50g/ml at pH5.8. The centrifugation and fractionation procedures were repeated, and fractions having a density greater than 1.52g/ml were combined, dialysed exhaustively against water at 4°C, and freeze-dried for storage at −20°C; 250mg of the KCl-extracted material yielded 180mg of proteoglycan subunit (fraction PGS_K), and 1.50g of the $CaCl_2$-extracted material yielded 1.06g of proteoglycan subunit (fraction PGS_{Ca}) by this procedure (Scheme 1).

Proteolytic degradation of proteoglycan

Papain (EC 3.4.22.2), twice crystallized, was obtained from Sigma (London) Chemical Co., Kingston-upon-Thames, Surrey KT2 7BH, U.K. Digestion mixtures contained 25μg of enzyme/ml of proteoglycan solution (2mg/ml) in 50mM-Na_2HPO_4, pH6.0, containing EDTA and cysteine (each 5mM).

Trypsin (EC 3.4.21.4), grade IV, was obtained from Miles Laboratories, Stoke Poges, Slough SL2 4LY, Berks., U.K., and dissolved in 1mM-HCl at 1mg/ml. Digestion mixtures contained 20μg of enzyme/ml of proteoglycan solution (2mg/ml) in 50mM-Tris/HCl, pH7.5, containing 1mM-$CaCl_2$.

Cathepsin D (EC 3.4.23.5) was obtained from human liver as described by Barrett (1970, 1973) and used as an aqueous solution of concentration 0.54 mg/ml (specific activity 800 units/mg). Digestion mixtures contained 3.2μg of enzyme/ml of proteoglycan solution (2mg/ml) in 0.2M-sodium acetate, pH5.0.

Cathepsin B (EC 3.4.22.1) was obtained from human liver as described by Barrett (1973) and used as an aqueous solution of concentration 10.4mg/ml (specific activity 5 units/mg). Digestion mixtures contained 104μg of enzyme/ml of proteoglycan solution (2mg/ml) in 0.2M-sodium acetate, pH5.0, containing EDTA and cysteine (each 5mM).

Lysosomal elastase (EC 3.4.21.11) was obtained from human spleen as described by Starkey & Barrett (1976) and used as an aqueous solution of concentration 0.18mg/ml (specific activity 924nkat/mg). Digestion mixtures contained 0.36μg of enzyme/ml of proteoglycan solution (2mg/ml) in 0.2M-Tris/HCl, pH7.5.

Cathepsin G (EC 3.4.21.20) was supplied by Dr. J. Travis (Department of Biochemistry, University of Georgia, Athens, GA 30602, U.S.A.) as an aqueous 0.36mg/ml solution (specific activity 120nkat/mg). Isolation and purification from human granulocytes were by a modification of the procedure described by Baugh & Travis (1976). Digestion mixtures contained 0.72μg of enzyme/ml of proteoglycan solution (2mg/ml) in 0.2M-Tris/HCl, pH7.5.

The general procedure for proteolytic digestion was as follows. Fraction PGS_{Ca} (10mg) was dissolved in 5ml of buffer, and the solution was equilibrated at 40°C before measurement of its viscosity. Enzyme solution (see above) was then added, and the degradation was measured by viscometry (U-tube viscometer M4; Scientific Supplies Co., London EC1R 5EB, U.K.; water flow-time about 20s). After 24h at 40°C the fall in viscosity had ceased. A second portion of enzyme was then added and the incubation continued for a further 24h to ensure that proteolysis was complete. The final degradation products were then precipitated by the addition of 2ml of a 5% (w/v) solution of cetylpyridinium chloride containing 0.25M-$MgCl_2$. The precipitate was separated from the supernatant by centrifugation at $2500g_{av.}$ ($r_{av.}$ = 14cm) for 5min at 20°C, then resuspended in 2ml of a 1% (w/v) solution of cetylpyridinium chloride containing 0.05M-$MgCl_2$. The wash procedure was repeated three times. The final precipitate was dissolved in 1ml of propan-1-ol/water (3:2, v/v), then re-precipitated by the addition of 2ml of ethanol saturated with potassium acetate. After centrifugation at $2500g_{av.}$ (r_{av} = 14cm) for 5min at 20°C, the precipitate was washed with 2×2ml of ethanol, and then with 2×2ml of diethyl ether. The precipitate was dried at 20°C, then stored at −20°C as a solid or a 1mg/ml aqueous solution till required.

In other experiments, intermediate degradation products were isolated by the addition of a proteinase inhibitor together with the cetylpyridinium chloride to a sample of the digestion mixture. Pepstatin (5μg/ml), supplied by Professor H. Umezawa (Institute of Microbial Chemistry, Tokyo, Japan) was used to inhibit cathepsin D, iodoacetate (5mM) to inhibit cathepsin B, and phenylmethanesulphonyl fluoride (2mM) to inhibit cathepsin G and lysosomal elastase (Scheme 1). Purification of the intermediate degradation products was as described for the final degradation products.

In the above cetylpyridinium chloride-precipitation procedure, free peptides and keratan sulphate-peptides remain in solution (Heinegård & Hascall, 1974*a*). The product therefore contains all of the chondroitin sulphate with any covalently linked peptide and keratan sulphate.

Alkaline degradation of proteoglycan

Fraction PGS_{Ca} (10mg) was dissolved in 5ml of 0.5M-NaOH. The solution was flushed with N_2 for 5min, then kept at 4°C for 72h in a sealed tube. The pH of the solution was adjusted to 7.0 with 1M-acetic acid, and water was then added to give a final volume of 10ml. The product was precipitated and stored as described for the enzymic products.

Electrophoresis in agarose–polyacrylamide gels

Cylindrical gels containing 0.6% agarose and 1.2% (w/v) polyacrylamide were prepared by the method of McDevitt & Muir (1971), except that the agarose and acrylamide solutions were mixed at 43°C and the gels cast at the same temperature. The composite gels were retained in Perspex tubes by 5% (w/v) polyacrylamide plugs, and electrophoresis was carried out at 4mA/gel as described by Roughley & Mason (1976). Gels were stained with Toluidine Blue overnight at 20°C, and destained with 3% (v/v) acetic acid at 35°C for 6h. Samples for loading on to the gel were in a volume of 30μl made up as follows: 15μl of proteoglycan or degradation product (1mg/ml solution), 10μl of aq. 50% (w/v) sucrose, and 5μl of aq. 0.05% Bromophenol Blue. Electrophoresis was carried out until the Bromophenol Blue band had migrated 2.5–3.0cm into the gel.

The mobilities of the bands were calculated as R_{BPB} (where BPB = Bromophenol Blue) values. For glycosaminoglycan and proteoglycans, R_{BPB} values of this type vary slightly in different gel preparations. To obtain a more reproducible measure of electrophoretic mobility, the R_{BPB} values were converted into R_{CS} values (where CS = single-chain chondroitin sulphate obtained by alkaline degradation).

Analytical methods

Uronic acid was determined by the method of Bitter & Muir (1962) with D-glucuronolactone as standard. Galactosamine/glucosamine molar ratios and amino acid analyses were performed on an amino acid analyser (Locarte Co., London W12 9RT, U.K.) by use of a single-column system. For galactosamine/glucosamine molar ratios, 50μg of proteoglycan or degradation product was hydrolysed in 1ml of 4M-HCl at 100°C for 8h. The sample was dried under vacuum, then dissolved in 1ml of sodium citrate buffer, pH2.2. A portion (0.5ml) of this solution was used for hexosamine analysis (single-buffer system, sodium citrate, pH5.28). For amino acid analysis, 2mg of material was hydrolysed in 1ml of 6M-HCl under vacuum at 105°C for 20h. The sample was dried under vacuum, then dissolved in 1ml of sodium citrate buffer, pH2.2. Solutions were filtered to remove charred material, then 0.4ml was used for amino acid analysis (three-buffer system, sodium citrate, pH3.25, 4.25 and 6.65). All analyses were run in duplicate. Total protein content was calculated from the results of amino acid analysis.

Results

Viscosity changes on proteolysis

Change in viscosity is a sensitive measure of proteolytic degradation of proteoglycan, since cleavage along the core protein produces a significant decrease in hydrodynamic size. All the proteinases produced a large decrease in the specific viscosity of the proteoglycan solution, which was rapid at first, but then slowed with time (Fig. 1). The final products produced by the various proteinases differed in specific viscosity. On the reasonable assumption that the specific viscosity of the digestion mixtures is directly related to the hydrodynamic size of the proteoglycan degradation fragments, the viscosity data imply that the final fragments produced by trypsin are larger than those produced by papain, the final fragments produced by cathepsin D are larger than those produced by cathepsin B, and the final fragments produced by cathepsin G are larger than those produced by lysosomal elastase. Clearly, regions of the core protein vary in their susceptibility to attack by different proteinases.

In control experiments, proteoglycan was incubated at 40°C in the absence of proteinases. In all cases the decrease in viscosity after 24h was insignificant compared with that produced by the proteinases (Fig. 1). Samples from the control experiments showed no change on agarose/polyacrylamide-gel electrophoresis.

Electrophoretic mobility of the products

The results of electrophoretic analysis of the final products of proteolytic degradation of fraction PGS_{Ca} are shown in Fig. 2. The starting material showed a broad zone of purple staining, with two

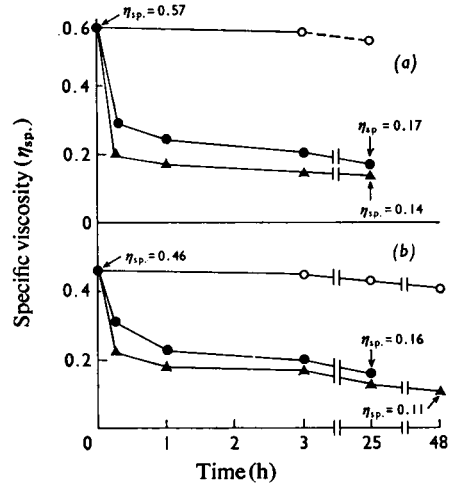


Fig. 1. *Effect of proteolytic degradation on the viscosity of proteoglycan solutions*

The $CaCl_2$-extracted proteoglycan (2mg/ml) was subjected to degradation by (*a*) cathepsin G (●) or lysosomal elastase (▲) in 0.2M-Tris/HCl, pH7.5, and (*b*) cathepsins D (●) or B (▲) in 0.2M-sodium acetate, pH5.0, as described in the Methods section. Control incubations were made in the same buffers (○).

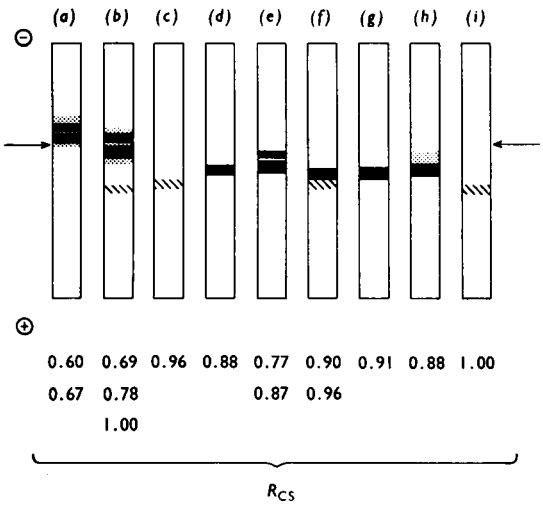


Fig. 2. *Agarose/polyacrylamide-gel electrophoresis of proteoglycan and its final degradation products*

Proteoglycan extracted with KCl (PGS_K) or $CaCl_2$ (PGS_{Ca}) and the final degradation products produced by the action of alkali or proteinases on the $CaCl_2$-extracted material were subjected to electrophoresis as described in the Methods section. The result of Toluidine Blue staining is shown in terms of intense purple staining (■), intense blue staining (▩) or diffuse staining (▒); the arrow indicates the position of the Bromophenol Blue marker. R_{CS} values for intense regions of staining are given beneath each gel. Key to gels: (*a*) PGS_{Ca}, (*b*) PGS_K; degradation of PGS_{Ca} by (*c*) papain, (*d*) trypsin, (*e*) cathepsin D, (*f*) cathepsin B, (*g*) lysosomal elastase, (*h*) cathepsin G and (*i*) NaOH.

regions of maximal intensity. The diffuse nature of the staining is indicative of the heterogeneity of the proteoglycan. A similar broad zone of diffuse staining was also seen for fraction PGS_K, but with higher mobility. This material also showed a well-defined band of blue staining similar to that obtained with the single-chain chondroitin sulphate produced by alkaline degradation of fraction PGS_{Ca}.

All the proteinases produced final degradation products whose electrophoretic mobilities were intermediate between those of fraction PGS_{Ca} and chondroitin sulphate. Papain, trypsin and elastase produced fragments that showed single well-defined areas of staining. With the fragments produced by trypsin or elastase the band stained purple, whereas with the fragments produced by papain, a blue colour was obtained. The mobility of the fragments produced by papain was greater than those of the fragments produced by elastase or trypsin. Cathepsin B produced fragments that showed a well-defined but broad area of staining. The upper part of this band was similar to that of the fragments produced by elastase in both colour and mobility, whereas the lower part was similar to that of the fragments produced by papain. Cathepsin D produced fragments showing two well-defined areas of purple staining. The more mobile band was broader and comparable in appearance with that of the product of trypsin degradation. Cathepsin G produced fragments that showed purple staining in the same mobility range as the fragments produced by cathepsin D. However, the staining of the less-mobile component was of a more diffuse nature.

On the basis of electrophoretic mobility and appearance, it is possible to divide the final degradation products into two classes: (*a*) those with greatest mobility, which stain blue, similar to free chondroitin sulphate, and (*b*) those with lesser mobility, which stain purple. Proteinases that produced fragments of greatest mobility also produced the greatest decrease in the viscosity of the digestion mixture (Fig. 1) and hence produced the greatest decrease in hydrodynamic size. Thus it is reasonable to assume that as the fragments increase in mobility, they also decrease in size.

The gel-electrophoretic patterns for intermediate degradation products were similar for proteolysis by cathepsins D, B or G, or lysosomal elastase. Staining with Toluidine Blue was diffuse in appearance, and as the specific viscosity of the digestion mixture decreased, the average mobility of the degradation products increased. No staining was observed at the position of the final degradation product until the viscosity of the digestion mixture had neared its limiting value, and little further change in hydrodynamic size was occurring. Only at this stage were there noticeable variations in the products produced by different proteinases.

Glycosaminoglycan and protein content of the products

The galactosamine/glucosamine molar ratio (Table 1) is a measure of the chondroitin sulphate/keratan sulphate weight ratio, since their repeating disaccharide units are of similar molecular weight, but those of chondroitin sulphate contain galactosamine, whereas those of keratan sulphate contain glucosamine. All the proteinases produced final degradation products enriched in chondroitin sulphate relative to fraction PGS_{Ca}. Within the limits of experimental error, the fragments produced by papain gave a ratio consistent with the cleavage of all keratan sulphate from chondroitin sulphate-peptides. For the fragments produced by cathepsin B, elastase and trypsin, the ratio indicated that 50–60% of the keratan sulphate remained bound to chondroitin sulphate-peptides, whereas for the fragments produced by cathepsins D and G, 80–85% of the keratan sulphate remained bound.

The protein content (Table 1) of the fragments was

Table 1. *Hexosamine and protein content of proteoglycan and its final degradation products*
Results are given for proteoglycan extracted with KCl (PGS_K) or $CaCl_2$ (PGS_{Ca}), and for the final degradation products produced by the action of alkali or proteinases on the $CaCl_2$-extracted material. See the Methods section for details of experimental conditions.

Proteoglycan	Treatment	Total protein (% of dry weight)	GalN/GlcN molar ratio
PGS_K	—	6.0	14.7
PGS_{Ca}	—	6.1	12.1
PGS_{Ca}	Papain	1.4	92.3
PGS_{Ca}	Trypsin	3.1	22.8
PGS_{Ca}	Cathepsin D	4.8	14.5
PGS_{Ca}	Cathepsin B	2.2	21.7
PGS_{Ca}	Elastase	3.8	22.5
PGS_{Ca}	Cathepsin G	4.5	14.3
PGS_{Ca}	NaOH	0.2	132.1

Table 2. *Amino acid composition of proteoglycan and its final degradation products*
Results are given for proteoglycan extracted with KCl (PGS_K) or $CaCl_2$ (PGS_{Ca}), and for the final degradation products produced by the action of proteinases on the $CaCl_2$-extracted material. Tr, trace. See the Methods section for details of experimental conditions.

	Content (residues/1000 residues)							
	Proteoglycan		Proteolytic degradation products of fraction PGS_{Ca} by:					
Amino acid	PGS_{Ca}	GS_K	Papain	Trypsin	Cathepsin D	Cathepsin B	Elastase	Cathepsin G
Asp	66	64	47	51	58	49	57	58
Thr	60	58	32	47	57	46	47	56
Ser	126	139	260	165	146	204	166	147
Glu	144	142	117	152	148	112	145	147
Pro	92	90	87	106	101	98	96	97
Gly	125	137	177	163	140	154	153	139
Ala	70	70	70	59	66	76	66	71
CyS	Tr	Tr	0	0	0	0	0	0
Val	68	65	55	65	58	51	61	59
Met	4	3	0	Tr	Tr	Tr	Tr	Tr
Ile	36	35	48	36	33	36	34	32
Leu	77	78	82	85	79	74	81	80
Tyr	19	15	3	5	13	9	10	14
Phe	37	34	17	29	36	35	32	36
His	25	27	Tr	11	24	27	24	23
Lys	14	14	Tr	10	12	11	8	11
Arg	37	30	8	14	27	18	20	28

also less than that of fraction PGS_{Ca}. Enzymes that produced the greatest loss in protein were those that caused the greatest cleavage of free keratan sulphate and the greatest decrease in hydrodynamic size. Fraction PGS_K had a protein content similar to that of fraction PGS_{Ca}, but contained a lower proportion of keratan sulphate. The galactosamine/glucosamine molar ratio was similar to that of the final fragments produced by cathepsins D and G, though these fragments were of smaller size.

Amino acid analysis of the products

The amino acid analyses for the proteoglycan and the final degradation products are shown in Table 2. If an amino acid occurs mainly in peptides that retain chondroitin sulphate chains after proteolysis, then its relative abundance will increase in the precipitated degradation product. All the final enzymic-degradation products showed an enrichment in serine, which links chondroitin sulphate to the core protein (Muir, 1958). The serine content was greatest for the chondroitin sulphate-peptides produced by papain, and decreased with increasing average size of the fragments produced by the other enzymes. Analysis of the fragments produced by papain indicated that serine, glycine, glutamic acid, proline and leucine were most abundant in the chondroitin sulphate-peptide linkage region. Comparison of the multiple

chain fragments with fraction PGS_{Ca} indicated that aspartate, threonine, valine, tyrosine, lysine and arginine were always most easily separated from chondroitin sulphate in the course of proteolysis. The amino acid composition of the fragments bore a direct relationship to their average size. Amino acids such as serine and glycine increased, whereas others such as threonine and aspartate decreased with decreasing size. The amino acid composition of fraction PGS_K was intermediate between those of fraction PGS_{Ca} and the largest of the final degradation products.

Discussion

Proteoglycan extracted with high-ionic-strength salt solutions shows variations in composition and size when fractionated on CsCl density gradients (Hascall & Sajdera, 1970). Hoffman *et al.* (1975) have demonstrated that the composition may vary from 1% keratan sulphate, 3% protein in the largest proteoglycan molecules, to 30% keratan sulphate, 45% protein in the smallest proteoglycan molecules. Fraction PGS_{Ca} described in the present paper contained 87% chondroitin sulphate, 7% keratan sulphate and 6% protein, and its amino acid analysis was in good agreement with those reported previously for proteoglycan from bovine nasal cartilage (Mayes *et al.*, 1973; Hascall & Sajdera, 1970) and pig laryngeal cartilage (Hardingham & Muir, 1974).

The variations in proteoglycan structure are representative of the material found within the cartilage, and do not arise by proteolysis during the extraction procedure. Oegema *et al.* (1975) showed that the inclusion of certain proteinase inhibitors in the extraction mixture did not significantly alter the sedimentation coefficient of proteoglycan from bovine nasal cartilage. By using the same cartilage, Pearson & Mason (1977) showed that the inclusion of EDTA, phenylmethanesulphonyl fluoride, iodoacetate and benzamidine did not alter the gel-electrophoretic pattern of proteoglycan extracted with 2M-$CaCl_2$ at 4°C. Thus, in the dissociative extraction procedure, proteolytic activity is prevented, probably by the high salt concentration.

In contrast, proteolytic degradation can occur during extraction with low-ionic-strength salt solutions. Pearson & Mason (1977) showed that the inclusion of the above proteinase inhibitors in a 0.15M-KCl extract produced a proteoglycan whose behaviour on agarose/polyacrylamide-gel electrophoresis was similar to that of the 2M-$CaCl_2$-extracted material. The action of proteinases may explain the variability in composition of proteoglycan extracted at low ionic strength obtained by other workers (Mayes *et al.*, 1973; Hardingham & Muir, 1974). Fraction PGS_K described in the present paper was intermediate in composition and size between fraction PGS_{Ca} and the largest of the final degradation products. This is consistent with fraction PGS_K being a product of limited proteolysis of the proteoglycan that occurs *in vivo*, rather than a distinct species (Palmoski *et al.*, 1974).

It has now been established that the intact proteoglycan from bovine nasal cartilage has an average mol.wt. of 2300000 (Pasternak *et al.*, 1974), and is composed of a core protein of average mol.wt. 200000 (Hascall & Riolo, 1972), to which are attached chondroitin sulphate chains of average mol.wt. 20000 (Wasteson, 1971) and keratan sulphate chains of average mol.wt. 7000 (Hopwood & Robinson, 1974). Molecular weights of this order imply that fraction PGS_{Ca} extracted in the present work contains about 100 chondroitin sulphate chains and 25 keratan sulphate chains per molecule. Further, Heinegård & Hascall (1974*b*) have shown that one terminus of the core protein is free from glycosaminoglycan chains and is involved in the interaction of proteoglycan with hyaluronic acid. A peptide was isolated from this region which had a mol.wt. of 90000 and contained about 10% of the keratan sulphate. This implies that about 120 glycosaminoglycan chains are attached to the remainder of the core protein, with a mol.wt. of 130000. Although the precise arrangement of glycosaminoglycan chains along the core protein is not known, the chains are on average separated by only ten amino acid residues. Thus some of the interchain peptides may be so short and of such a structure as to be resistant to all but the most non-specific proteinases.

Mathews (1971) concluded that papain degradation of proteoglycan produced single-chain chondroitin sulphate-peptides, whereas degradation with trypsin followed by chymotrypsin produced a fragment that had a molecular weight about twice that of the papain product. It was proposed that chondroitin sulphate chains were arranged in 'doublets' along the core protein, and that the separation within doublets was less than ten amino acid residues, whereas the separation between doublets was about 35 amino acid residues. Support for this type of structure was presented by Morrison *et al.* (1973). Cathepsin B was shown to give a degradation product of similar size to that produced by papain, whereas cathepsin D produced fragments of similar size to those produced by trypsin. However, Heinegård & Hascall (1974*a*) showed that although papain produced only single-chain glycosaminoglycans, trypsin followed by chymotrypsin produced 'clusters' containing one to ten glycosaminoglycan chains. It was postulated that these clusters represented isolated groups of chondroitin sulphate chains arranged along the core protein, and that at least 50% of the keratan sulphate was associated with the groups. Support for the existence of groups of variable size was presented by Keiser *et al.* (1976), who showed that lysosomal

elastase produced fragments containing one to five chondroitin sulphate chains, whereas with cathepsin G the fragments were larger, containing 5–12 chains.

Our results support the concept that the glycosaminoglycan chains are arranged in groups along the core protein of the proteoglycan, but distinguish these groups from the clusters of glycosaminoglycan chains obtained by complete proteolysis. We define a 'cluster' as a fragment obtained by exhaustive proteolysis of the proteoglycan, which contains two or more glycosaminoglycan chains linked to a peptide not susceptible to degradation by the proteinase. The resistance to proteolysis of peptides separating glycosaminoglycan chains within a cluster may be due to either steric hindrance by the presence of glycosaminoglycan chains, or the lack of a sensitive amino acid sequence. The resistant peptide sequences are not necessarily shorter than the susceptible sequences between clusters. Some clusters may correspond to the intact groups of glycosaminoglycan chains, whereas the majority are formed by cleavage within groups, the position of such cleavages depending on the specificity of the proteinase.

The smallest fragments produced by proteolytic degradation of fraction PGS_{Ca} were single-chain glycosaminoglycan-peptides, and, of the enzymes under investigation, only papain carried this process to completion. Single chondroitin sulphate chains produced by either papain or alkali gave rise to a well-defined blue band on agarose/polyacrylamide-gel electrophoresis, in contrast with the purple staining observed with the clusters produced by the other enzymes (Fig. 2). The electrophoretic behaviour of the fragments produced by cathepsin B indicated that both single chains and small clusters were produced. About one-half of the keratan sulphate contained in the proteoglycan was associated with the clusters. Elastase and trypsin each produced only small clusters, which had electrophoretic mobilities and keratan sulphate contents similar to those produced by cathepsin B, but contained more protein. Cathepsins D and G produced both small and large clusters that contained more keratan sulphate and protein than the clusters produced by the other enzymes.

As single-chain chondroitin sulphate-peptides were produced by only two of the enzymes, it can be concluded that the peptide sequences between adjacent chondroitin sulphate chains are mostly resistant to proteolysis, and chondroitin sulphate chains never occur in isolation on the core protein. Except for papain, each enzyme separated less than one-half of the keratan sulphate from the clusters (Table 1). Hence more than one-half the keratan sulphate chains must also be linked to chondroitin sulphate by peptide regions resistant to proteolysis, either within or at the end of the clusters. Since papain cleaves all sequences between adjacent chains, it seems unlikely that steric hindrance of access of proteinases to the core protein is a major factor restricting proteolysis. It seems more probable that variations in amino acid sequence distinguish sensitive from resistant peptides. The electrophoretic heterogeneity of the clusters liberated by several of the proteinases is therefore inconsistent with a repeating amino acid sequence between adjacent chains. If different enzymes attack along different peptide regions, the sequential action of two proteinases would produce clusters of smaller size than those produced by the action of either alone. This has been reported for cathepsin D and trypsin (Morrison, 1970).

The results of amino acid analysis (Table 2) bore a direct relationship to the average size of the clusters produced by a particular proteinase. For example, the relative abundance of serine and glycine increased, whereas that of threonine and aspartic acid decreased as the average size of the clusters decreased. Amino acids associated with chondroitin sulphate after papain digestion are clearly those close to the points of attachment of chondroitin sulphate chains, whereas the multi-chain fragments produced by the other enzymes also contain amino acids close to the points of attachment of keratan sulphate chains. The data would be consistent with the existence of unique sequences at the points of attachment of the two types of glycosaminoglycan chain, although the possibility of some variability cannot be excluded. Variations in the position of cleavage by different proteinases then depends on variable amino acid sequences between the constant peptide regions. Further, the electrophoretic patterns of the intermediate degradation products suggest that the initial points of cleavage may be common to all proteinases. These cleavages would occur in the peptide regions most susceptible to proteolysis, which by definition are the regions that separate the groups of glycosaminoglycan chains on the proteoglycan core protein.

In conclusion, a model of proteoglycan structure is postulated in which the core protein is composed of a globular hyaluronic acid-binding region and an extended glycosaminoglycan-attachment region containing groups of chains (Hardingham *et al.*, 1976). The groups may vary in both size and glycosaminoglycan composition, and although keratan sulphate chains may sometimes occur in relative isolation, the chondroitin sulphate is always associated with groups of two or more chains. The peptide regions separating the groups are probably longer than the regions within a group, and are readily susceptible to degradation by most proteinases. In contrast, proteolysis within the groups is more dependent on the amino acid sequence of the interchain peptides, and the structure of the limiting fragments varies with different proteinases.

A detailed study of the structural variations that occur within the clusters has provided further information on the proteolytic degradation of proteoglycan molecules. This work, together with a study of proteoglycan heterogeneity, is presented in the following paper (Roughley, 1977).

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