The Degradation of Cartilage Proteoglycans by Tissue Proteinases

PROTEOGLYCAN HETEROGENEITY AND THE PATHWAY OF PROTEOLYTIC DEGRADATION

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1. CaCl_2 -extracted proteoglycan from bovine nasal cartilage was degraded by four tissue proteinases till no further decrease in hydrodynamic size was obtained. The proteoglycan and its final degradation products were then fractionated by Sepharose 2B chromatography. 2. The average size of the degradation products was least for cathepsin B and lysosomal elastase, and greatest for cathepsin D and cathepsin G. The latter two proteinases also produced degradation products that showed the widest range of sizes. 3. The structure of the degradation products ranged from peptides containing a single glycosaminoglycan chain to those containing twelve or more chains. Of the four proteinases, only cathepsin B produced peptides that contained a single chondroitin sulphate chain. 4. The proteoglycan was very heterogeneous with respect to size and chemical composition. Its behaviour on electrophoresis suggested that at least two genetically distinct core proteins might exist. 5. Irrespective of their structural variations, all proteoglycan molecules were able to interact with hyaluronic acid. In contrast, none of the degradation products were capable of this type of interaction. 6. A pathway for the proteolytic degradation of proteoglycans is postulated in which the sites of initial cleavage may be common to the majority of proteinases, whereas the production of the final clusters is dependent on the specificity of the proteinase. Only those proteinases of broadest specificity can produce single-chain chondroitin sulphate-peptides.

Cartilage proteoglycan is very heterogeneous with respect to both size and chemical composition (Hascall & Sajdera, 1969; Hoffman *et al.*, 1975; Thyberg *et al.*, 1975), even as extracted by a dissociative procedure at 4°C in the presence of proteinase inhibitors (Pearson & Mason, 1977). Heterogeneity, as it exists *in vivo*, may arise from a variety of sources, such as variations in the structure of the core protein or the arrangement of glycosaminoglycan chains along its length (Hopwood & Robinson, 1975; Hardingham *et al.*, 1976). In the preceding paper (Roughley & Barrett, 1977) we showed that the final products of the proteolytic degradation of proteoglycan, i.e. glycosaminoglycan 'clusters', differed with different proteinases. Comparison of the electrophoretic mobility of intermediate degradation products and the amino acid composition of the clusters led us to propose that the glycosaminoglycan chains are arranged in groups along the core protein. Proteolytic cleavage in the peptide regions separating the groups is common to all proteinases, whereas cleavage within the groups is dependent on the specificity of the proteinase. The results described in the present paper concern the variations in structure of the proteoglycan molecules that exist *in vivo* and the glycosaminoglycan 'clusters' produced by complete

proteolytic degradation of these molecules. The results are discussed in terms of proteoglycan heterogeneity and the pathway of proteolytic degradation of the proteoglycan molecules.

Methods

The procedures for the CaCl_2 extraction of proteoglycan and its degradation by exhaustive proteolysis are described in the preceding paper (Roughley & Barrett, 1977), together with the methods for uronic acid determination and hexosamine analysis. Chemicals used were either analytical grade or the best grade commercially available.

Sepharose 2B chromatography

A Pharmacia column (SR25/45) was packed with a bed of Sepharose 2B, then equilibrated with 0.2M-sodium acetate buffer, pH 5.5. The final bed volume was 190 ml. CaCl_2 -extracted proteoglycan subunit (PGS_{Ca}) or its final proteolytic degradation product (10 mg) was dissolved in the above buffer at a concentration of 2 mg/ml and chromatographed by downward elution at 20°C with a flow rate of 20 ml/h; 2 ml fractions were collected, and every fifth fraction was

assayed for uronic acid content. Material was isolated from fractions representative of different positions on the uronic acid elution profile. To each fraction 4 ml of ethanol was added, and the mixture kept at 4°C for 20 h. The precipitate was separated by centrifugation at $2500g_{av.}$ ($r_{av.} = 14\text{cm}$) for 5 min at 20°C, then washed with $2 \times 2\text{ml}$ of ethanol, and then with $2 \times 2\text{ml}$ of diethyl ether. After removal of the ether, the solid was dissolved in water to a concentration of approx. 1 mg/ml, then stored at -20°C.

In the text the elution position of a fraction is described in terms of its partition coefficient, K , where $K = (V - V_0)/(V_t - V_0)$; V = elution volume of fraction, V_0 = void volume of column, V_t = total volume of column. V_0 was determined from the elution position of proteoglycan aggregate, and V_t was determined by the elution position of glucuronolactone. $K_{av.}$ refers to the elution position of the fraction having maximum uronic acid concentration.

Agarose/polyacrylamide-gel electrophoresis

Slab gels containing 0.6% agarose and 1.2% (w/v) polyacrylamide were prepared by a method based on that of McDevitt & Muir (1971). The slabs (70 mm $\times$ 80 mm $\times$ 2.5 mm) were formed between glass plates that were separated along two opposing edges by plastic strips and held in place by adhesive tape. The gel was held in position by Visking dialysis tubing taped along the lower edge. Samples (15 μl) were loaded into eight wells (10 mm $\times$ 4 mm) cut into the upper surface of the gel, and electrophoresis was carried out at 20 mA/gel. Sample preparation, conditions of electrophoresis, staining and destaining of gels, and calculation of results were as described by Roughley & Barrett (1977). The use of slab gels eliminated much of the variation found between individual cylindrical gels and so enabled small changes in mobility to be observed more accurately.

Interaction with hyaluronic acid

Hyaluronic acid from human umbilical cord (Miles Laboratories, Stoke Poges, Slough SL2 4LY, Berks., U.K.) was purified by fractionation on a cellulose column saturated with 1% (w/v) cetylpyridinium chloride (Antonopoulos *et al.*, 1961). Hyaluronic acid was eluted from the column with 0.3 M-NaCl containing 0.05% (w/v) cetylpyridinium chloride. On agarose/polyacrylamide-gel electrophoresis the purified material migrated as a broad zone of $R_{CS}^* 0.4$, which did not retain Toluidine Blue when destaining was performed with 3% (v/v) acetic acid.

Interaction between hyaluronic acid and the proteoglycan or its proteolytic degradation products

* Mobility relative to single-chain chondroitin sulphate obtained by alkaline degradation of proteoglycan.

was studied by agarose/polyacrylamide-gel electrophoresis. Samples were made up as follows: 15 μl of proteoglycan or degradation product (2 mg/ml), 15 μl of hyaluronic acid (2 mg/ml), 20 μl of aq. 50% (w/v) sucrose and 10 μl of aq. 0.05% (w/v) Bromophenol Blue. Interaction was indicated by a decrease in the electrophoretic mobility of the sample relative to that obtained in the absence of hyaluronic acid.

Results

CaCl₂-extracted proteoglycan

Most of the CaCl₂-extracted proteoglycan was of large molecular size, eluted in a single peak with K values in the range 0–0.5, but there was also a minor peak eluted near the total column volume (Fig. 1). The width of the major peak is indicative of the wide range of molecular sizes to be found in cartilage proteoglycan. Four fractions from the major peak were isolated for the determination of electrophoretic mobility and hexosamine content (Fig. 1). On

Fig. 1. Chemical and physical properties of bovine nasal proteoglycan

The CaCl₂-extracted proteoglycan was subjected to Sepharose 2B chromatography. The symbols V_0 and V_t refer to the void volume and total volume of the column. Fractions of various hydrodynamic sizes, (a), (b) etc., were investigated by hexosamine analysis and agarose/polyacrylamide-gel electrophoresis. The arrow alongside the gels depicts the position of the Bromophenol Blue band, and relative mobilities (R_{CS}) are quoted for the most intense regions of staining. Toluidine Blue staining is depicted as intense purple (■), intense blue (■) or diffuse (▨). For comparison, data are given for the unfractionated proteoglycan (●).

electrophoresis the unfractionated proteoglycan appeared as a broad area of purple staining, with two regions of maximal intensity. Each fraction from the Sepharose 2B column also showed diffuse staining, but contained only a single intense region. The average electrophoretic mobility of each fraction increased as the hydrodynamic size of the material within the fraction decreased. Interaction of the unfractionated proteoglycan with hyaluronic acid resulted in a decrease in its observed electrophoretic mobility. If the interaction was stable under the conditions of electrophoresis, staining would be expected as a discrete zone, whereas if the interaction was labile, staining should appear from the position of the aggregate to that of the proteoglycan. The latter situation was found. Each fraction from the Sepharose 2B column also showed this labile interaction, indicating that proteoglycan molecules of all sizes are capable of interacting with hyaluronic acid. The galactosamine/glucosamine molar ratio for the fractions ranged from 14.6 to 9.6, with the unfractionated proteoglycan having an intermediate value. As the size of the proteoglycan molecules decreased, the ratio of chondroitin sulphate to keratan sulphate (w/w) also decreased. Only galactosamine was detected in the minor peak. Thus the native proteoglycan is extremely heterogeneous with respect to both molecular size and glycosaminoglycan content. Further, the electrophoretic patterns suggest that there may be at least two polydisperse and heterogeneous populations of molecules, both of which are capable of interacting with hyaluronic acid.

Degradation by cathepsin D

The final product from degradation by cathepsin D was eluted as a broad symmetrical peak with K values in the range 0.3–0.9 (Fig. 2). No material was recovered at either the void volume or the total column volume. The peak width was indicative of a wide range of molecular sizes, and fractions were selected to represent material of large, average and small size. The unfractionated degradation product showed two well-defined regions of purple staining on electrophoresis (Fig. 2). Each fraction also showed two distinct regions, but their relative amounts varied. The material of largest hydrodynamic size contained similar amounts of the two components, whereas that of smallest size contained predominantly the more-mobile component. The more-mobile component possessed greater heterogeneity in size than the less-mobile component. None of the fractions showed any interaction with hyaluronic acid on electrophoresis. When mixtures were electrophoresed, the mobilities were unaltered and the bands retained their sharpness. The galactosamine/glucosamine molar ratio for the fractions varied from 10.3 to 19.6, with the unfractionated

Fig. 2. Chemical and physical properties of the final products of proteoglycan degradation by cathepsin D. The degradation products of limiting size produced by the action of cathepsin D on the CaCl_2 -extracted proteoglycan were subjected to Sepharose 2B chromatography, and fractions of various hydrodynamic sizes were investigated by hexosamine analysis and agarose/polyacrylamide-gel electrophoresis. See legend to Fig. 1 for further explanation.

degradation product having an intermediate value (Fig. 2). The fraction of largest hydrodynamic size had the lowest ratio of chondroitin sulphate to keratan sulphate (w/w), whereas the fraction of average size had the highest ratio. Thus cathepsin D produced clusters which showed a wide range of heterogeneity in size, but all were larger than single-chain chondroitin sulphate. Bound keratan sulphate was most abundant in clusters of slowest mobility and largest size, which were electrophoretically distinct from the other clusters.

Degradation by cathepsin B

The final product from degradation by cathepsin B was eluted as a narrow asymmetric peak with K values in the range 0.6–1.1 (Fig. 3). No material was eluted near the void volume, but the peak overlapped the total column volume. Fractions were selected to represent material of varying size, the smallest being eluted at the total column volume. On electrophoresis the unfractionated degradation product showed a single broad band, whose lower area stained blue and whose upper area stained purple (Fig. 3). The material of largest hydrodynamic size appeared as a well-defined purple band, that of smallest size appeared as a well-defined blue band,

Fig. 3. Chemical and physical properties of the final products of proteoglycan degradation by cathepsin B
The degradation products of limiting size produced by the action of cathepsin B on the CaCl_2 -extracted proteoglycan were subjected to Sepharose 2B chromatography, and fractions of various hydrodynamic sizes were investigated by hexosamine analysis and agarose/polyacrylamide-gel electrophoresis. See legend to Fig. 1 for further explanation.

whereas that of intermediate size was of a composite nature. Thus the smallest material, eluted near the total column volume, had the characteristic blue staining of single-chain chondroitin sulphate, in contrast with the purple staining of multi-chain clusters. None of the fractions showed any interaction with hyaluronic acid, the staining pattern of mixtures being unaltered on electrophoresis. The galactosamine/glucosamine molar ratio of the fractions varied from 11.1 to 76.8, with the unfractionated material being of an intermediate value (Fig. 3). The proportion of keratan sulphate within each fraction decreased as the size of the product also decreased. In the smallest product, keratan sulphate was essentially absent. Thus cathepsin B produced clusters that showed little heterogeneity in size. Single-chain chondroitin sulphate was produced, and the bound keratan sulphate was associated with the clusters of larger size.

Degradation by cathepsin G

The final product from degradation by cathepsin G was eluted as a broad peak extending from the void volume to the total column volume (Fig. 4), indicative

Fig. 4. Chemical and physical properties of the final products of proteoglycan degradation by cathepsin G
The degradation products of limiting size produced by the action of cathepsin G on the CaCl_2 -extracted proteoglycan were subjected to Sepharose 2B chromatography, and fractions of various hydrodynamic sizes were investigated by hexosamine analysis and agarose/polyacrylamide-gel electrophoresis. See legend to Fig. 1 for further explanation.

of a wide range of molecular sizes. Fractions were selected to represent material of large, average and small sizes. On electrophoresis the unfractionated degradation product showed a single major region of intense purple staining, with a region of diffuse staining immediately above (Fig. 4). The material from each fraction showed single intense regions of staining, and with that of largest size, diffuse staining was also seen above the intense band. The electrophoretic mobility of the product increased as its hydrodynamic size decreased, with the diffuse staining in the unfractionated material arising from the material of largest size. None of the fractions showed any interactions with hyaluronic acid, the staining pattern being unaltered on electrophoresis of mixtures of hyaluronic acid and the fractions. The galactosamine/glucosamine molar ratio of the fractions varied from 16.4 to 21.5, with the unfractionated material being at the bottom of the range (Fig. 4). The proportion of chondroitin sulphate relative to keratan sulphate was greatest for the material of largest size and least for that of average size, although the variations were not large. Thus cathepsin G produced clusters that showed great heterogeneity in size, some being comparable with the

size of small proteoglycan molecules, though not possessing the ability to interact with hyaluronic acid. No single-chain chondroitin sulphate was produced, and keratan sulphate was associated with all cluster sizes.

Degradation by lysosomal elastase

The final product from degradation by lysosomal elastase was eluted as a narrow symmetrical peak with K values in the range 0.6–1.0 (Fig. 5). No material was isolated near the void volume, indicating that the product was all of small size. Fractions were selected to represent material of large, average and small size eluted before the total column volume. The unfractionated degradation product showed a single well-defined region of purple staining on electrophoresis (Fig. 5). Each fraction also showed single well-defined bands whose electrophoretic mobility increased only slightly as the size of the material contained within them decreased. None of the products showed any interaction with hyaluronic acid, the sharpness and mobility of the staining being identical in the absence and presence of hyaluronic

acid. The galactosamine/glucosamine molar ratio of the fractions varied from 21.3 to 33.4, with the unfractionated material being at the bottom of the range (Fig. 5). The ratio of chondroitin sulphate to keratan sulphate was greatest for the material of largest size and least for that of average size, although the variations were not large. Thus elastase produced clusters showing little heterogeneity in size. The clusters were all larger than single-chain chondroitin sulphate, and keratan sulphate was associated with clusters of all sizes.

Discussion

Proteoglycan heterogeneity may arise from a variety of sources. Firstly, the glycosaminoglycan chains may be heterogeneous with respect to both chain length and degree of sulphation, secondly, the core protein may possess a multiplicity of structures, and finally, the arrangement of glycosaminoglycan chains along the core protein may vary. Seno *et al.* (1975) showed that 4- and 6-sulphation can occur within the same chondroitin sulphate chain, and Murata & Bjelle (1976) showed that the ratio of 4- to 6-sulphation varies in different proteoglycan molecules. Further, Wasteson (1971) demonstrated that chondroitin sulphate chains had lengths varying from about 6 to 80 disaccharide repeating units, with an average value of 40. In contrast, Hopwood & Robinson (1975) identified three pools of chondroitin sulphate, each having a narrow molecular-weight distribution, and each occurring on a genetically distinct core protein. However, Heinegård & Hascall (1974a) showed that all proteoglycan molecules, irrespective of their chemical composition, contained chondroitin sulphate of similar average chain lengths. Hardingham *et al.* (1976) suggested that all proteoglycan molecules were formed on a genetically unique core protein, and variations in proteoglycan structure arose from proteolytic cleavage within the glycosaminoglycan-attachment region of the core protein. Rosenberg *et al.* (1976) provided evidence that the core protein of bovine articular-cartilage proteoglycan contained a hyaluronic acid-binding region of constant size and composition and a glycosaminoglycan-attachment region of variable length and composition. As the size of the proteoglycan molecules decreased there was a decrease in the ratio of chondroitin sulphate relative to keratan sulphate. A similar variation was observed in bovine nasal-cartilage proteoglycan by Heinegård (1977). Hoffman *et al.* (1975) also provided evidence for a continuous variation in glycosaminoglycan composition ranging from proteoglycan molecules that contained essentially no keratan sulphate to those that contained keratan sulphate as the predominant glycosaminoglycan. As the keratan sulphate is concentrated in that part of the glycosaminoglycan-

Fig. 5. Chemical and physical properties of the final products of proteoglycan degradation by lysosomal elastase

The degradation products of limiting size produced by the action of lysosomal elastase on the CaCl_2 -extracted proteoglycan were subjected to Sepharose 2B chromatography, and fractions of various hydrodynamic sizes were investigated by hexosamine analysis and agarose/polyacrylamide-gel electrophoresis. See legend to Fig. 1 for further explanation.

attachment region adjacent to the hyaluronic acid-binding terminus (Heinegård & Axelsson, 1977), these observations are in good agreement with the proteoglycan structure postulated by Hardingham *et al.* (1976).

The appearance of the CaCl_2 -extracted proteoglycan (PGS_{Ca}) on agarose/polyacrylamide-gel electrophoresis would suggest the existence of at least two proteoglycan populations, both of wide heterogeneity, corresponding to the regions of intense Toluidine Blue staining (Fig. 1). The molecules in the less-mobile population are of larger average size than those in the more-mobile population, though there is considerable overlap in size between the two populations. All the molecules, irrespective of size or electrophoretic mobility, are able to interact with hyaluronic acid, suggesting that even if more than one core protein exists, the hyaluronic acid-binding region is a common feature. A similar electrophoresis pattern has been shown to occur for the proteoglycan of human epiphyseal cartilage (Stanescu & Maroteaux, 1975). In contrast, the proteoglycan of foetal cartilage showed only a single region of intense Toluidine Blue staining, of similar electrophoretic mobility to the less-mobile component of the proteoglycan obtained from mature tissue. It is possible that foetal cartilage contains a single proteoglycan population, and that, during development, a second population is synthesized, which is of greater electrophoretic mobility and smaller size. During development in human costal (Mathews & Glagov, 1966) and tracheo-bronchial cartilage (Mason & Wusteman, 1970) there is an increase in keratan sulphate relative to chondroitin sulphate. Thus one might expect the proteoglycan population of greater electrophoretic mobility to possess a lower galactosamine/glucosamine molar ratio than the population of slower mobility. Such a variation in hexosamine molar ratio with size and electrophoretic mobility has been observed for the CaCl_2 -extracted proteoglycan (Fig. 1).

Irrespective of whether there is more than one genetically distinct core protein, further heterogeneity may arise by variations in the arrangement of glycosaminoglycan chains. At present little is known about such heterogeneity, but information has been gained by studying the products formed by proteolytic degradation of the proteoglycan (see the preceding paper, Roughley & Barrett, 1977). The proteinases used in the present investigation each produced final degradation products having a range of sizes and compositions. K_{av} values for the fragments produced by cathepsin D, cathepsin G, lysosomal elastase and cathepsin B were 0.57, 0.64, 0.82 and 0.88 respectively. Cathepsins G and D produced clusters exhibiting the widest range of molecular sizes, some of those produced by cathepsin G being of a size comparable with that of the smallest proteoglycans. Lysosomal elastase and cathepsin B

produced clusters that were of narrower size range and smaller average size. Only cathepsin B produced fragments small enough to be eluted at the total column volume. These fragments corresponded to peptides containing a single chondroitin sulphate chain, and the electrophoresis patterns of the Sepharose 2B fractions indicated that such peptides were not produced by the other proteinases.

The galactosamine/glucosamine molar ratios of the fractionated final degradation products indicate that the amount of keratan sulphate contained within the cetylpyridinium chloride-precipitable clusters may vary with both the size of the cluster and the proteinase used in its production. Keiser *et al.* (1976) have shown that clusters produced by elastase contain up to five chondroitin sulphate chains, whereas those produced by cathepsin G may contain as many as 12 chains. On this basis it is possible to estimate the size and composition of the precipitable clusters. In addition to single-chain chondroitin sulphate-peptides, cathepsin B produces clusters of smaller average hydrodynamic size than those produced by elastase. These clusters probably contain two to four chondroitin sulphate chains, whereas those produced by elastase contain two to five chains. As with the other proteinases, some clusters of all sizes contain keratan sulphate in addition to chondroitin sulphate. Cathepsin D produces clusters having size variations equivalent to the smaller clusters produced by cathepsin G and the larger clusters produced by elastase, and which probably contain two to ten glycosaminoglycan chains. Keratan sulphate is concentrated in the clusters of largest size which migrate as a distinct band on electrophoresis. These clusters may have arisen from that part of the core protein adjacent to the hyaluronic acid-binding region, which is rich in keratan sulphate chains (Heinegård & Hascall, 1974b; Heinegård & Axelsson, 1977). Most of the clusters produced by cathepsin G contain 2–12 glycosaminoglycan chains; however, much of the keratan sulphate cannot be accounted for within these clusters and must therefore be associated with the small proportion of material that is eluted near the void volume (Fig. 4). This material may be of similar origin to the largest clusters produced by cathepsin D, but of greater size and heterogeneity.

The final degradation products, irrespective of their size, showed no interaction with hyaluronic acid, indicating that glycosaminoglycan-peptides containing 1–12 chains are not capable of aggregate formation. As the interaction with hyaluronic acid is dependent on the presence of a functional hyaluronic acid-binding region (Hardingham *et al.*, 1976), all the proteinases studied must be capable of cleaving within this region. *In vivo* the hyaluronic acid-binding region may be protected from proteolysis by its interaction with hyaluronic acid and link proteins (Heinegård & Hascall, 1974b). However, this inter-

action is not sufficient to protect the glycosaminoglycan-attachment region from proteolytic attack. Thus all the proteinases may destroy the integrity of the cartilage by facilitating the diffusion of glycosaminoglycan-peptides from the extracellular matrix.

The results presented here provide further evidence that the glycosaminoglycan clusters obtained as the final products of proteolysis do not correspond to isolated groups of glycosaminoglycan chains arranged along the proteoglycan core protein. As not all proteinases are capable of releasing single-chain chondroitin sulphate-peptides, it would appear that none of the proteoglycan molecules contain isolated

chondroitin sulphate chains. It is possible that the biosynthesis of one chondroitin sulphate chain facilitates chain initiation on serine residues that are in close proximity. Glycosylation by membrane-bound multienzyme complexes, as postulated by Horwitz & Dorfman (1968), would enhance the possibility of the co-operative biosynthesis of adjacent chondroitin sulphate chains.

In conclusion, the results in this and the preceding paper (Roughley & Barrett, 1977) provide information on the pathway of the proteolytic degradation of cartilage proteoglycan as depicted in Fig. 6. All proteoglycan molecules contain a hyaluronic acid-binding region that is devoid of glycosaminoglycan chains, and a glycosaminoglycan-attachment region along which the chondroitin sulphate and keratan sulphate chains are arranged in isolated groups of variable composition. The initial cleavages may occur at sites that are common to the majority of proteinases, probably in the peptide regions that separate the groups of glycosaminoglycan chains. Further cleavages occur within the groups, in peptide regions whose susceptibility to proteolysis is more dependent on the specificity of the proteinase. Such cleavages may eventually lead to the production of clusters that cannot be degraded further by the proteinase, and whose structure varies for different proteinases. With many proteinases the final cleavages separate external keratan sulphate-peptides from fragments containing chondroitin sulphate chains (Roughley, 1977). Some proteinases also have the ability to separate terminal chondroitin sulphate-peptides, although complete degradation to single-chain glycosaminoglycan-peptides is a rare occurrence. With the six proteinases under investigation, the largest clusters were produced by cathepsins D and G, and the smallest by lysosomal elastase, trypsin and cathepsin B. The former proteinases retained about 80% of the keratan sulphate within the clusters, whereas the latter retained only 50%. Cathepsin B also released some single-chain chondroitin sulphate, but only papain resulted in complete degradation of the proteoglycan to single-chain glycosaminoglycans.

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Fig. 6. Schematic representation of bovine nasal cartilage proteoglycan degradation by proteinases

The proteoglycan core protein contains a glycosaminoglycan-attachment region along which chondroitin sulphate and keratan sulphate chains are arranged in groups. The sites of initial cleavage are in the peptide regions between the groups (1) and are common to the majority of proteinases. The sites of further cleavage, in the peptide regions within the groups (2), are dependent on the specificity of the proteinase. The final cleavages (3) remove external keratan sulphate (KS) and chondroitin sulphate-peptide (CS) from the multiple glycosaminoglycan-peptides. Steps (1), (2) and (3) are followed sequentially by all proteinases, though the number of cleavages in each category is different.

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