Carbohydrate Research, 255 (1994) 241–254
Elsevier Science B.V., Amsterdam

The sulphation pattern in chondroitin sulphate chains investigated by chondroitinase ABC and ACII digestion and reactivity with monoclonal antibodies

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(Received May 12th, 1993; accepted August 27th, 1993)

ABSTRACT

We have used progressive chondroitinase digestion of pig aggrecan in conjunction with ELISA assays and disaccharide analysis to derive information about the pattern of 4- and 6-sulphation in chondroitin sulphate chains. Digestion with chondroitinase ABC resulted in the release of mainly disaccharides from the nonreducing terminal of chondroitin sulphate chains but there was also the release of some tetra- and hexa-saccharides which were degraded to disaccharides with more extensive digestion. Chondroitinase ACII, in contrast, released only disaccharides. Analysis of the disaccharide composition of the intact and digested products at different stages of digestion showed that there was a slight increase in 6-sulphate content of the chains as they were shortened. Reaction of the partially digested proteoglycans with monoclonal antibodies 3-B-3 and 3-D-5 which recognise chains terminating in 6- or 4-sulphated disaccharides, respectively, showed major differences between chondroitinase ABC and ACII products. The results suggested that chondroitinase ABC preferentially cleaved next to 4-sulphated, rather than 6-sulphated disaccharides and this resulted in some oligosaccharides as well as disaccharide being released. Chondroitinase ACII also cleaved an additional disaccharide next to the linkage to protein of chondroitin sulphate, which was not removed by chondroitinase ABC and this disaccharide was mainly nonsulphated.

INTRODUCTION

Chondroitin sulphate is a long chain unbranched glycosaminoglycan which in vertebrate tissues contains predominantly 4- or 6-sulphated disaccharide units. In

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SSDI 0008-6215(93)E0397-J

cartilage aggrecan the chains are typically M_r 12–25 × 10^3 with a unimodal but polydisperse size distribution. The proportions of 4- or 6-sulphated disaccharides varies greatly with cartilage anatomical site, with species, and it changes during development and ageing[1]. Some sources, such as pig laryngeal cartilage, are predominantly 4-sulphated whereas others, such as human costal cartilage, are predominantly 6-sulphated. The functional significance of 4- and 6-sulphation is unclear. In the ordered states of stretched semi-crystalline fibres, 6-sulphation has a large effect on chain conformation as the large sulphate group extends away from the main sugar backbone, whereas the 4-sulphate is closer to the main axis[2]. Chain associations have also been suggested to be possible with 6-sulphated chains, but not with 4-sulphated chains[3]. In addition to 6- and 4-sulphated disaccharides chondroitin sulphate chains contain a smaller proportion (2–10%) of nonsulphated disaccharides and a few disulphated disaccharides.

There have been no methods developed that enable sequences of sulphation within chains to be determined, but the production of monoclonal antibodies that recognise specific epitopes within chondroitin sulphate suggests that distinctive sequences are present[4]. Furthermore, the expression of these epitopes has been shown to change during development and in pathology they have been found to be increased in cartilage associated with experimental and natural degenerative joint disease[4]. This suggests that the pattern of sulphation produced during chondroitin sulphate biosynthesis is much less random than has previously been assumed and results in specific sequences within chains[5]. We have previously shown that digestion of intact aggrecan with chondroitinase permits chain composition to be investigated from the nonreducing free end of the chain progressively towards the attachment to the protein core[6]. We have now extended these studies and compared chondroitinase ABC and ACII digests and used two monoclonal antibodies that recognise chain terminal structures in chondroitin sulphate to further characterise the products.

EXPERIMENTAL

Aggrecan was prepared from pig laryngeal cartilage by extraction in 4 M guanidine HCl and density gradient centrifugation as previously described[7], and was used as a monomeric A1D1 fraction. Chondroitinase ABC (*Proteus vulgaris*) and chondroitinase ACII (*Arthrobacter aurescens*) were obtained from Seikagaku Kogyo supplied by ICN Flow, (High Wycombe, Bucks, UK). Chromatographic media (Sepharose CL 2B, CL 6B) were obtained from Pharmacia LKB, (Uppsala, Sweden) and Biogel P4 from (Biorad, Hemel Hempstead, Herts, UK). Total sulphated glycosaminoglycan was determined by an automated modification[8] of the dye-binding assay with 1,9-dimethyl Methylene Blue[9]. Analysis of disaccharides of chondroitin sulphate was carried out by capillary zone electrophoresis[10] in collaboration with Dr. Stephen Carney (Lilly Research Centre, Windlesham, UK).

Epitopes of chondroitin sulphate recognised by monoclonal antibodies 3-B-3, 3-D-5, and 7-D-4 were determined by competitive ELISA[11]. The antibodies were provided by Dr. Bruce Caterson, Orthopaedic Research Dept., University of North Carolina, Chapel Hill, North Carolina, USA. For 3-B-3 and 3-D-5 ELISAs, chondroitinase ABC digested pig aggrecan was used as the standard antigen and for the 7-D-4 ELISA the standard antigen was shark cartilage proteoglycan, which was a gift also provided by Dr. Bruce Caterson. Epitope contents were calculated from standard curves produced with these antigens.

Digestion of aggrecan with chondroitinase ABC and ACII.—Pig aggrecan monomer was dissolved (0.2–1.0 mg/mL) in 0.1 M Tris · HCl, 0.1 M sodium acetate, pH 8.0, and was digested with chondroitinase ABC 0.005–0.011 units/mg aggrecan at 37°C for up to 18 h[12]. Digestion with chondroitinase ACII[13,14] was carried out with aggrecan (0.2–5.0 mg/mL) in 0.04 M sodium acetate, pH 6.0, containing proteinase inhibitors[14,15], 0.1 M 6-aminohexanoic acid, 5 mM benzamidine HCl, 10 mM sodium EDTA, 1 mM phenylmethanesulphonyl fluoride, 2 mM *N*-ethylmaleimide, and chondroitinase ACII (0.005–0.011 units/mg aggrecan). The progress of digestion was monitored by determining residual intact glycosaminoglycan by assay with 1,9-dimethyl Methylene Blue[8]. Samples of the digest were removed at timed intervals for further analysis after boiling for 5 min to inactivate the enzyme.

Proteinase inhibitors were included in chondroitinase ACII digests as some batches of enzyme contained low levels of proteinase activity[15], but no proteinase was detected in the chondroitinase ABC preparations used. The size of released oligosaccharides was determined by gel chromatography on Biogel P6 as described below.

To separate partially digested aggrecan from released disaccharides/oligosaccharides, 3 vol of cold (4°C) EtOH was added to each sample and after at least 3 h at 4°C the insoluble aggrecan precipitate was separated from the soluble oligosaccharides by centrifugation at 2000*g* for 10 min at 4°C. The precipitate was washed twice with EtOH and dried, and the supernatant was dried by evaporation and kept for oligosaccharide analysis.

The aggrecan fractions and released oligosaccharide fractions from the time course experiment with chondroitinase ABC were redigested with excess chondroitinase ABC (1 unit/mg aggrecan or oligosaccharide) for 5 h at 37°C to convert all chondroitin sulphate to disaccharides.

Digestion of aggrecan samples with papain.—Samples taken from the time course of digestion of aggrecan by chondroitinase ABC or ACII were mixed with an equal vol of 0.1 M sodium acetate pH6.0 containing 10 mM sodium EDTA and 10 mM cysteine. Papain (6 μg/mg aggrecan) was added and the digest mixture was incubated overnight at 60°C. The chain size of released chondroitin sulphate peptide was determined by gel chromatography on Sepharose CL-6B (see below).

Gel chromatography.—Samples of aggrecan (1.0–1.5 mg) were applied to a column of Sepharose CL-2B (9 × 130 mm) eluted with 0.5 M sodium acetate pH

6.8 at 4 mL/h at 4°C. Fractions of 1.0 mL were collected and assayed for sulphated glycosaminoglycan with 1,9-dimethyl Methylene Blue[8].

Samples of chondroitin sulphate chains (~ 2.5 mg) released from aggrecan by papain digestion were applied to a column of Sepharose CL-6B (6.5 × 1350 mm) eluted with 0.5 M sodium acetate pH 6.8 at 3 mL/h at 20°C. Fractions of 0.65 mL were collected and assayed for hexuronate by an automated carbazole reaction[16].

Samples of partial and complete digests of aggrecan with chondroitinase ABC and ACII containing 1 mg hexuronate were applied to a column (6.5 × 1350 mm) of Biogel P-4 eluted with 0.5 M sodium acetate pH 6.8 at 3 mL/h at 20°C. Fractions of 0.65 mL were collected and assayed for hexuronate by an automated carbazole reaction[16]. The excluded (V_o) and included (V_t) volumes of the Sepharose CL6B and Biogel P4 columns were determined with intact aggrecan and tritiated water respectively.

Elution of volumes of samples (V_e) were used to calculate K_{av} from the relationship: $K_{av} = (V_e - V_o)/(V_t - V_o)$.

RESULTS

Pig laryngeal cartilage aggrecan is of high molecular weight (M_w ~ 2.0×10^6, M_n ~ 1.6×10^6) and contains a protein core (M_r 200–25000) to which is attached ~ 100 chondroitin sulphate chains and many fewer keratan sulphate chains[17]. The chondroitin sulphate accounts for 90% of the total glycosaminoglycans. The preparation of aggrecan from pig laryngeal cartilage was shown to be monomeric by gel chromatography on Sepharose CL-2B[17] (results not shown). A sample of the aggrecan was digested with chondroitinase ABC. The progress of the digestion was monitored with a dye-binding assay with 1,9-dimethyl Methylene Blue, which only detects intact chondroitin sulphate and not the digest products (Fig. 1). Samples of the digest were taken at timed intervals for analyses.

The effect of chondroitinase digestion on chondroitin sulphate chain length was assessed following papain digestion of the proteoglycan (Fig. 2). The average size of all chains was progressively reduced during digestion and there was, from the beginning, digest products of very low molecular weight. This showed that the enzyme had primarily an exo-action digesting chondroitin sulphate from the nonreducing end of the chain[18]. Further examination of the digest products on Biogel P4 showed, however, that although disaccharides were the major product, there were also significant amounts of tetra- and hexa-saccharides, particularly at the early stage of digestion (Fig. 3). The enzyme therefore released the terminal disaccharide, but also sometimes cleaved to remove 2 or 3 disaccharides from the end of the chain. After prolonged digestion the proportion of tetra- and hexa-saccharides decreased suggesting they were subsequently cleaved -into disaccharides by further enzyme action.

The partial digests thus contained oligosaccharide products as well as chondroitin sulphate chains of reduced length that remained attached to the aggrecan

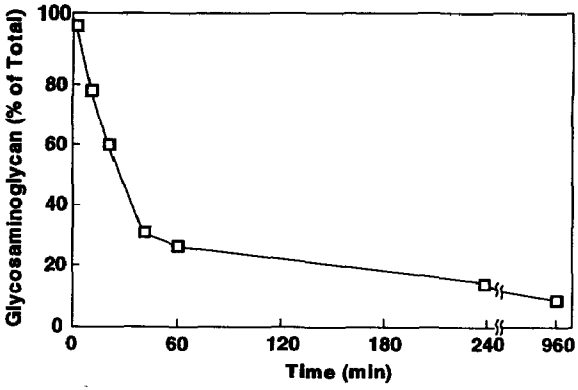


Fig. 1. Digestion of aggrecan with chondroitinase ABC. Porcine aggrecan was incubated with chondroitinase ABC (0.005 units/mg aggrecan) at 37°C for up to 18 h as described in the Experimental section. At timed intervals samples were removed, boiled, and assayed for total sulphated glycosaminoglycan by dye-binding assay[8].

protein core. Ethanol precipitation was used to separate the digested oligosaccharides (soluble) from the remaining aggrecan (insoluble). This enabled the digested regions of the chondroitin sulphate chains, which corresponded to regions away from their linkage to the protein core, to be analysed separately from the regions closer to the protein attachment. In order to determine the disaccharide composition it was necessary to redigest both fractions with chondroitinase ABC. This converted the mixture of released oligosaccharides to disaccharides and it completed the digestion of the residual chondroitin sulphate chains on the aggrecan

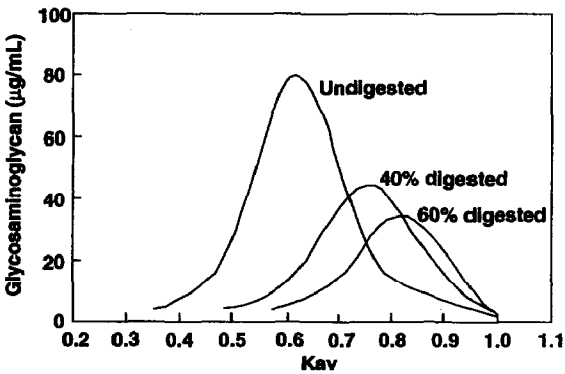


Fig. 2. Gel chromatography on Sepharose CL-6B of chondroitin sulphate chains from aggrecan digested with chondroitinase ABC. Samples of aggrecan from a time-course of digestion experiment with chondroitinase ABC (as in Fig. 1) were digested with papain (as described in the Experimental section) and the size of the released chains of chondroitin sulphate-peptide[17] was determined by gel chromatography on Sepharose CL-6B as described in the Experimental section. Fractions were assayed for total sulphated glycosaminoglycans by dye-binding assay[8].

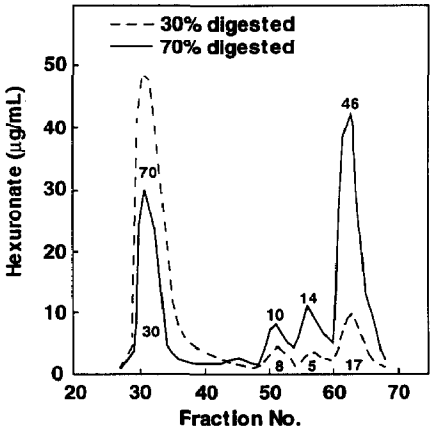


Fig. 3. Gel chromatography on Biogel P4 of chondroitinase ABC digest products released from aggrecan. Samples from two partial digests of aggrecan with chondroitinase ABC (as in Figs. 1 and 2) were chromatographed on Biogel P4 as described in the Experimental section. Fractions were assayed for hexuronate[16]. Results are shown for an early digest (30% digested) and for a later digest (70% digested). Numbers show the percentage of total hexuronate in each peak.

fraction. The proportion of 4- and 6- sulphated disaccharides in each fraction was then determined by capillary zone electrophoresis. The results showed that early released digest products were consistently more abundant in 4-sulphated disaccharides than the chains remaining attached to the proteoglycan (Fig. 4). This suggested that 4-sulphated disaccharides were preferentially more abundant towards the nonreducing ends of the chondroitin sulphate chains.

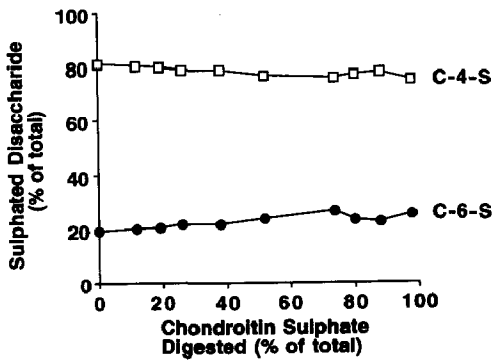


Fig. 4. Changes in the proportion of 4-sulphated and 6-sulphated disaccharides in aggrecan during digestion with chondroitinase ABC. Aggrecan was digested with chondroitinase ABC as described in the Experimental section. At timed intervals samples were taken and the extent of digestion was determined by assaying the total sulphated glycosaminoglycan remaining. The proportion of 4-sulphated and 6-sulphated disaccharide in undigested chondroitin sulphate chains was determined by capillary zone electrophoresis following ethanol precipitation and complete digestion with chondroitinase ABC as described in the Experimental section.

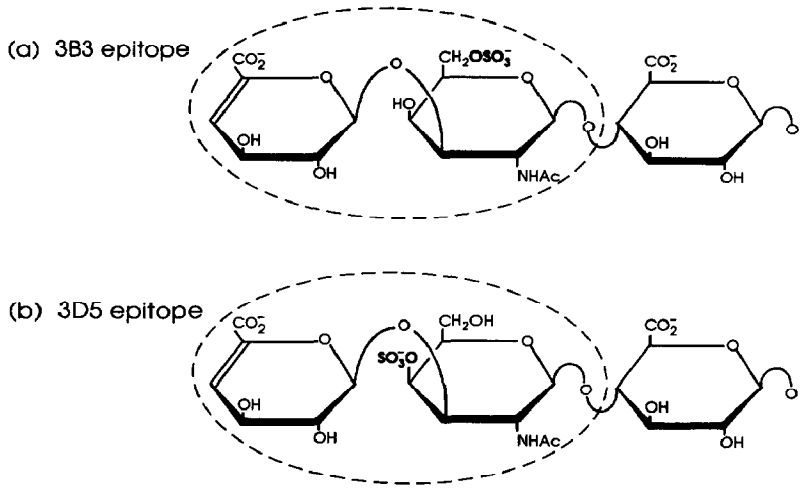


Fig. 5. Chondroitin sulphate epitopes of monoclonal antibodies 3-B-3 (a) and 3-D-5 (b).

The partially digested proteoglycans were also investigated for their reactivity with monoclonal antibody 3-B-3. This antibody[19] reacts with terminal disaccharides of chondroitin sulphate chains only if glucuronate is terminal and the adjacent *N*-acetylgalactosamine is 6-sulphated (Fig. 5a), but it reacts with either the unsaturated hexuronate product of chondroitinase digestion, or with a saturated (but terminal) glucuronate. Reactivity with this antibody is thus a measure of the number of chains with a terminal 6-sulphated disaccharide. In the competitive ELISA assay oligosaccharide and free chondroitin sulphate chains show very little competition and the assay is therefore only sensitive to epitopes on chondroitin sulphate chains attached to aggrecan. The native pig cartilage aggrecan that had not been treated with chondroitinase ABC showed no detectable interaction with this antibody in the ELISA assay. This showed that these chains do not terminate with GlcA-GalNAc6-SO_3^-. However, following chondroitinase digestion reactivity appeared (Fig. 6) and it increased markedly as chains became shorter, with a maximum reactivity when 80% of the chains were digested. This profile of reactivity (Fig. 7) suggested that terminal 6-sulphated disaccharides were of very low abundance early in digestion. This result suggested a more biased distribution of 6-sulphated disaccharides than was predicted from the disaccharide analysis (Fig. 4).

The distribution of 4- and 6-sulphated disaccharides along the chains was investigated further in partial digests prepared with chondroitinase ACII instead of ABC. As with chondroitinase ABC, the average length of all chondroitin sulphate chains remaining attached to the proteoglycan was progressively shortened during digestion and only low molecular weight digest products were released (Fig. 8). However analysis of the size of the released products showed that in contrast to chondroitinase ABC digests the ACII digests contained only disaccha-

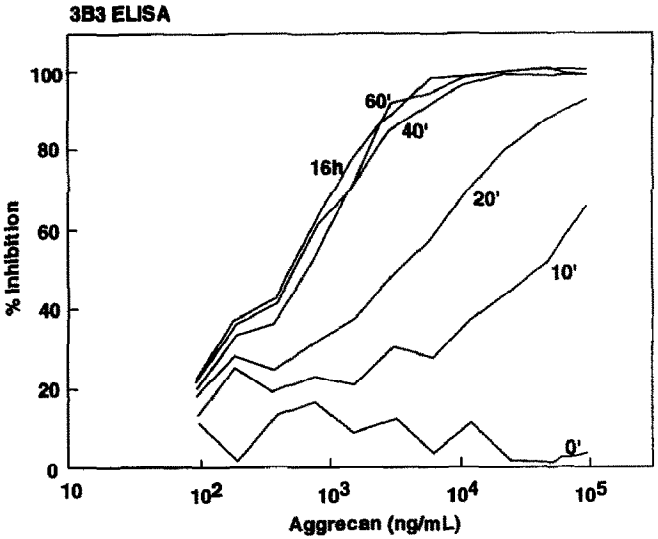


Fig. 6. Determination of 3-B-3 epitope by competitive ELISA in chondroitinase ABC digested aggrecan. Aggrecan was digested with chondroitinase ABC as described in the Experimental section. At timed intervals samples were taken (see Fig. 1) and assayed by ELISA for reactivity with monoclonal antibody 3-B-3. The extent of digestion was determined by assaying the total sulphated glycosaminoglycan[8]. Undigested aggrecan (time zero sample) has negligible reactivity with monoclonal antibody 3-B-3.

rides at all times of digestion (results not shown). The ACII enzyme thus acted more exclusively as an exo-enzyme as it released only disaccharides from the aggrecan chondroitin sulphate chains. Samples from partial digests were investigated to test their reactivity with monoclonal antibody 3-B-3 (Fig. 7). The result differed from that obtained with chondroitinase ABC digests, as more reactivity

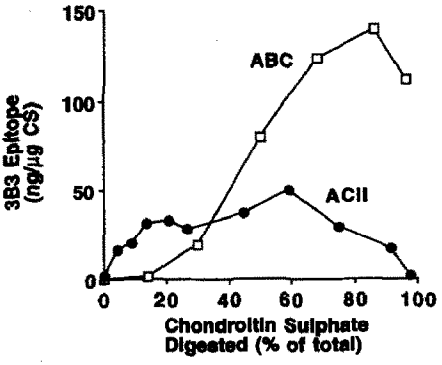


Fig. 7. Changes in the content of 3-B-3 epitope in aggrecan during digestion with chondroitinase ABC and ACII. Aggrecan was digested with chondroitinase ABC or chondroitinase ACII as described in the experimental section. At timed intervals samples were taken and assayed by ELISA with monoclonal antibody 3-B-3 and the extent of digestion was determined by assaying the total sulphated glycosaminoglycan[8].

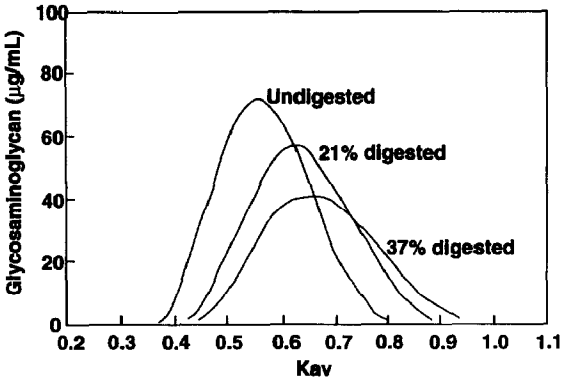
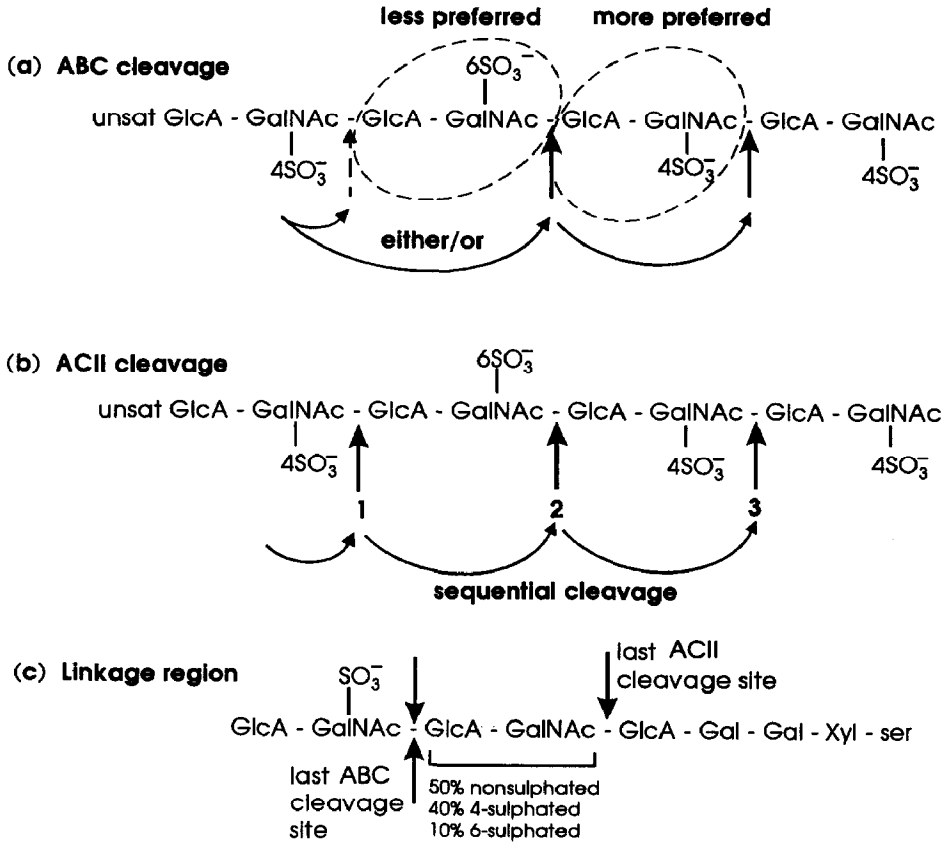


Fig. 8. Gel chromatography on Sepharose CL-6B of chondroitin sulphate chains from aggrecan digested with chondroitinase ACII. Samples of aggrecan from a time-course of digestion experiment with chondroitinase ACII were digested with papain and the size of the released chondroitin sulphate peptide[17] was determined by gel chromatography on Sepharose CL-6B as described in the experimental section. Fractions were assayed for total sulphated glycosaminoglycan by dye-binding assay[8].

was detected at early stages of digestion and as digestion approached completion there was a complete loss of reactivity. This result showed that at early stages of digestion ACII enzyme action produced partly digested aggrecan with a higher 3-B-3 epitope content than ABC digestion. The ABC digestion therefore contained fewer chondroitin sulphate chains that terminated in 6-sulphated disaccharides than those from the ACII digestion. As the ABC enzyme releases tetra- and hexa-saccharides as well as disaccharides it seems probable that the difference between the epitopes present on the enzyme products reflects the difference in the enzyme actions (Schemes 1a and 1b). The ABC enzyme does not initially cleave at every disaccharide linkage and the results suggest that it cleaves less frequently next to 6-sulphated disaccharides than the ACII enzyme as it creates fewer 3-B-3 epitopes. The distribution of 4- and 6-sulphated disaccharides along the chondroitin sulphate chains may thus influence the pattern of initial cleavage with chondroitinase ABC and create a difference between the ABC and ACII digests. This effect is most noticeable early in digestion. The increase in 3-B-3 epitope later in ABC digests and the conversion of tetra- and hexa-saccharides to disaccharides shows that all disaccharide bonds are eventually cleaved and this suggests that the effect is only a difference in the rate of cleavage. We had previously shown[20] that chondroitinase ABC had a higher V_{max} with chondroitin 4-sulphate than with chondroitin 6-sulphate, although both were fully digested by the enzyme.

Further support for this interpretation was obtained by investigating interaction with another monoclonal antibody 3-D-5 which has recently become available and reacts with an epitope comprising a terminal 4-sulphated disaccharide[21,22] (Fig. 5b). Comparison of the reactivity with this antibody showed that the ABC digests were more reactive at early stages of digestion than the ACII digests (Fig. 9). This result is entirely compatible with chondroitinase ABC preferentially cleaving adjacent to 4-sulphated disaccharides.



Scheme 1.

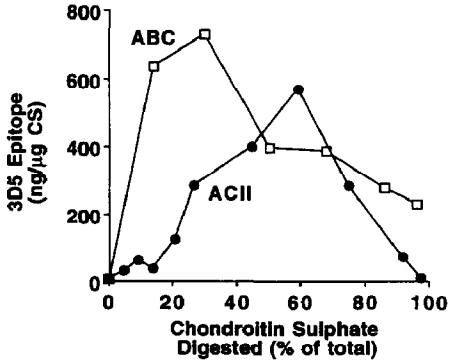


Fig. 9. Changes in the content of 3-D-5 epitope in aggrecan during digestion with chondroitinase ABC and ACII. Aggrecan was digested with chondroitinase ABC or chondroitinase ACII as described in the Experimental section. At timed intervals samples were taken and assayed by ELISA for reactivity with monoclonal antibody 3-D-5. The extent of digestion was determined by assaying the total sulphated glycosaminoglycan[8]. Undigested aggrecan (time zero sample), has negligible reactivity with monoclonal antibody 3-D-5.

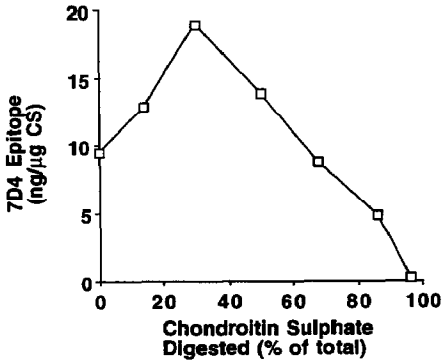


Fig. 10. Changes in the content of 7-D-4 epitope in aggrecan during digestion with chondroitinase ABC. Aggrecan was digested with chondroitinase ABC as described in the experimental section. At timed intervals samples were taken and assayed by ELISA with monoclonal antibody 7-D-4 and the extent of digestion was determined by assaying the total sulphated glycosaminoglycan[8].

Reactivity of the partial digests was also tested with monoclonal antibody 7-D-4 which recognises a nonterminal epitope in chondroitin sulphate[21,22]. The results (Fig. 10) showed that reactivity increased after brief digestion, but subsequently decreased to well below the initial value. Prolonged digestion resulted in complete loss of reactivity. This showed that the epitope recognised by monoclonal antibody 7-D-4 is within the chondroitin sulphate chain and is suggested to be part way along the chain.

DISCUSSION

The present results reveal several features of the chondroitin sulphate chains in pig aggrecan. The absence of reactivity of the intact proteoglycan with monoclonal antibody 3-B-3 and 3-D-5 show that chains do not terminate in glucuronate residues. This suggests that the chains terminate with a GalNAc residue, although it might be possible that a terminal GlcA substituted in some unknown way may also make the chain unreactive with these antibodies. The profiles of reactivity of the partially digested aggrecan preparations digested with chondroitinase ABC or ACII have complex shapes that are likely to be contributed to by several factors. Firstly, aggrecan, with 100 chondroitin sulphate chains attached, is potentially a very polyvalent antigen and in these experiments the valency for these antibodies, 3-B-3 and 3-D-5, dramatically changes as digestion proceeds. The proteoglycan thus begins with no 3-B-3 or 3-D-5 epitopes, but half way through digestion, from the disaccharide composition, it is likely to contain about 80 chains with 3-D-5 epitopes and about 18 chains with 3-B-3 epitopes. With chondroitinase ABC, at the end of digestion, the protein core retains 3-B-3 and 3-D-5 epitopes attached to the linkage region, but in the ACII digest the chondroitin sulphate linkage region finish with none of these epitopes remaining. The profile of reactivity with the

ACII digests is thus more "bell" shaped than those with ABC digests and the maximum reactivity achieved with ACII is also likely to be strongly limited by the fact that both the starting material and the limit digest are unreactive with the antibodies.

The influence of the changing valency of the antigen may have a nonlinear effect on its competitiveness in the assay[23]. Furthermore, the way in which the terminal epitopes compete together in the assay may also change as chains are reduced in length. The measurement of competitiveness in the assay is thus unlikely to be a simple measure of a quantitative difference in the number of epitopes on each proteoglycan. However, the comparison of profiles produced by analysing the ABC and ACII digests with the same antibody should give a reliable indication of the relative abundance of epitopes created by the different enzymes at each stage of digestion, as factors such as chain length will be similar and only numbers of epitopes will differ. It is on this basis that the profile of the ABC digests in the 3-B-3 assay is very different from the profile of the ACII digests and suggests a preferred action of the ABC enzyme that creates few 3-B-3 epitopes early in digestion.

Some of the complexity of interpretation of the assay profiles is also revealed by the results with mAb 7-D-4, which show an apparent increase in epitope content at early stages of digestion. It is unlikely that this increase is real, as only the chain lengths are changing, but it suggests that epitopes part way along chains may become more accessible to the antibodies and thus more competitive in the assay when the chains are reduced in length. This result also suggests that there can be significant removal of the distal part of the chondroitin sulphate chains before the 7-D-4 epitope itself becomes digested and is eventually destroyed. Thus the 7-D-4 epitopes are suggested to be not very close to either end of the chondroitin sulphate chains, but are distributed more towards the middle of the chains.

It had previously been shown that chondroitinase ABC digestion leaves intact one disaccharide adjacent to the neutral oligosaccharide that links chondroitin sulphate to protein, whereas chondroitinase AC (I or II) releases even this disaccharide[18]. This was also evident from the present results with pig aggrecan. After exhaustive ABC digestion the proteoglycan retained reactivity with both antibodies 3-B-3 and 3-D-5, but there was no reactivity with either antibody after ACII digestion. An analysis of the disaccharides released from aggrecan by ACII digestion after complete ABC digestion showed 50% nonsulphated disaccharide, 40% 4-sulphated disaccharide, and 10% 6-sulphated disaccharide. The first disaccharide adjacent to the linkage region in pig aggrecan chondroitin sulphate is thus preferentially non-sulphated (Scheme 1c). The ability of ACII enzyme to cleave closer to the linkage region than ABC is consistent with our observations on the selective action of ABC enzyme (Scheme 1a). The ability of ABC to cleave off di-, tetra-, or hexa-saccharides suggests that its substrate specificity depends at least in part on the structure of the disaccharides on the reducing terminal side of the cleavage site. This would explain its failure to cleave the disaccharide next to the

linkage region, as there is no GlcA-GalNAc disaccharide on the reducing terminal side of it. In contrast ACII enzyme functions as an exo-disaccharide lyase and is active down to, and including, the disaccharide adjacent to the linkage (Scheme 1c). The ACII enzyme thus shows no substrate specificity that extends to sugar residues on the reducing terminal side of the GalNAc-GlcA bond that is being cleaved. This contrasts with the structural specificity of the ACII enzyme at the hexuronate residue in the cleavage site itself, where dermatan sulphate sequences containing iduronate are not a substrate for ACII, but are a substrate for the ABC enzyme.

The disaccharide composition of chondroitin sulphate chains was shown to change as digestion proceeded. The change was not large, but as it represents the average composition of all chains, it suggests a consistent variation in 4- and 6-sulphation with chain length. It would require other strategies to detect the presence of specific sequences of sulphation that are not common to all chains. It is in this context that other monoclonal antibodies that recognise epitopes within chondroitin sulphate chains may enable special or more unique sequences to be investigated[11].

ACKNOWLEDGMENTS

We are grateful to Dr. Bruce Caterson (University of N. Carolina, Chapel Hill, NC, USA) for the generous supply of monoclonal antibodies and shark cartilage proteoglycan. We also thank Dr Stephen Carney (Lilly Research Centre, Windlesham, UK) for Capillary Zone Electrophoresis analysis. This work was carried out with the support of the Arthritis and Rheumatism Council, UK.

REFERENCES

1 H. Muir and T.E. Hardingham, in W.J. Whelan (Ed.), *Biochemistry of Carbohydrates*, 1975, pp 153–222.
2 S. Arnott and A.K. Mitra, in S. Arnott, D.A. Rees, and E.R. Morris (Eds.), *Molecular Biophysics of the Extracellular Matrix*, Humana Press, New Jersey, 1984, pp 41–67.
3 J.E. Scott, Y. Chen, and A. Brass, *Eur. J. Biochem.*, 209 (1992) 675–680.
4 B. Caterson, F. Mahmoodian, J.M. Sorrell, T.E. Hardingham, M.T. Bayliss, S.L. Carney, A. Ratcliffe, and H. Muir, *J. Cell Sci.*, 97 (1990) 411–417.
5 T.E. Hardingham and A.J. Fosang, *FASEB J.*, 6 (1992) 861–870.
6 T.E. Hardingham, A.J. Fosang, and R.J.F. Ewins, *Trans. Ann. Meeting Orthop. Res. Soc.*, 16 (1991) 4.
7 T.E. Hardingham, *Biochem. J.*, 177 (1979) 237–247.
8 A. Ratcliffe, M. Doherty, R.N. Maini, and T.E. Hardingham, *Ann. Rheum. Dis.*, 47 (1988) 826–832.
9 R.W. Farndale, C.A. Sayers, and A.J. Barrett, *Conn. Tiss. Res.*, 9 (1982) 247–248.
10 S.L. Carney and D.J. Osborn, *Anal. Biochem.*, 195 (1992) 132–140.
11 P.K. Hazell, C. Dent, J.A. Fairclough, M.T. Bayliss, and T.E. Hardingham, *Trans. Ann. Meeting Orthop. Res. Soc.*, 18 (1992) 711.
12 T. Yamagata, S. Hidehiko, O. Habuchi, and S. Suzuki, *J. Biol. Chem.*, 243 (1968) 1523–1535.
13 K. Hiyama and S. Okada, *J. Biol. Chem.*, 250 (1975) 1824–1828.
14 T.R. Oegama, V.C. Hascall, and D.D. Dziewiatkowski, *J. Biol. Chem.*, 250, (1975) 6151–6159.

15 Y. Oike, K. Kimata, T. Shinomura, K. Nakazawa, and S. Suzuki, *Biochem. J.*, 191 (1980) 193–207.
16 D. Heinegard, *Chim. Scripta*, 4 (1973) 199–201.
17 T.E. Hardingham, *Biochem. Soc. Trans.*, 9 (1981) 489–497.
18 V.C. Hascall, R.L. Riolo, J. Hayward, Jr., and C.C. Reynolds, *J. Biol. Chem.*, 247 (1975) 4521–4528.
19 B. Caterson, J.E. Christner, J.R. Baker, and J.R. Couchman, *Fed. Proc.*, 44 (1985) 386–393.
20 C.F. Thurston, T.E. Hardingham, and H. Muir, *Biochem. J.* 145 (1975) 397–400.
21 B. Caterson, C. Hughes, B. Johnstone and J.S. Mort, in K. Kuettner, R. Schleyerbach, J.G. Peyron, V.C. Hascall (Eds.), *Articular Cartilage and Osteoarthritis*, Raven Press Ltd., New York, (1992) Ch. 29, pp 415–427.
22 B. Caterson, J. Griffin, F. Mahmoodian, and J.M. Sorrell, *Biochem. Soc. Trans.*, 18 (1990) 820–823.
23 M.J. Seibel, W. Macaulay, R. Jelsma, F. Saed-Nejad, and A. Ratcliffe, *Arch. Biochem. Biophys.*, 296 (1992) 410–418.