A novel keratan sulphate domain preferentially expressed on the large aggregating proteoglycan from human articular cartilage is recognized by the monoclonal antibody 3D12/H7

Dagmar-Christiane FISCHER, Hans-Dieter HAUBECK*, Kirsten EICH, Susanne KOLBE-BUSCH, Georg STÖCKER, Helmut Wolfgang STUHLSTATZ and Helmut GREILING

Institut für Klinische Chemie und Pathobiochemie, Pauwelsstr. 30, D-52057 Aachen, Germany

Monoclonal antibodies (mAbs) were prepared against aggrecan which has been isolated from human articular cartilage and purified by several chromatographic steps. One of these mAbs, the aggrecan-specific mAb 3D12/H7, was selected for further characterization. The data presented indicate that this mAb recognizes a novel domain of keratan sulphate chains from aggrecan: (1) immunochemical staining of aggrecan is abolished by treatment with keratanase/keratanase II, but not with keratanase or chondroitin sulphate lyase AC/ABC; (2) after chemical deglycosylation of aggrecan no staining of the core-protein was observed; (3) different immunochemical reactivity was observed against keratan sulphates from articular cartilage, intervertebral disc and cornea for the mAbs 3D12/H7 and 5D4. For further characterization of the epitope, reduced and ³H-labelled keratan

sulphate chains were prepared. In an IEF-gel-shift assay it was shown that the ³H-labelled oligosaccharides obtained after keratanase digestion of reduced and ³H-labelled keratan sulphate chains were recognized by the mAb 3D12/H7. Thus it can be concluded that the mAb 3D12/H7 recognizes an epitope in the linkage region present in, at least some, keratan sulphate chains of the large aggregating proteoglycan from human articular cartilage. Moreover, this domain seems to be expressed preferentially on those keratan sulphate chains which occur in the chondroitin sulphate-rich region of aggrecan, since the antibody does not recognize the keratan sulphate-rich region obtained after combined chondroitinase AC/ABC and trypsin digestion of aggrecan.

INTRODUCTION

Keratan sulphate (KS) is a glycosaminoglycan which was first isolated from cornea [1] but was later detected in cartilage as well as in several other tissues [2–6]. KS chains have been classified according to their linkage to the core-protein: KS-I chains are N-linked and occur in cornea [7–9] and on the cartilage proteoglycan fibromodulin [10], whereas the KS-II chains are O-linked [11] and occur on most other KS-containing proteoglycans. KS from brain tissue was shown to be O-linked from mannose to serine or threonine [3] and may therefore represent a third type.

The general structure of KS is based upon the repeating disaccharide (-3Gal β 1-4GlcNAc β 1-) in which most of the *N*-acetylglucosamine residues and some of the galactose residues are sulphated on C-6 [12]. Analysis of KS isolated from porcine and calf cornea revealed a domain structure in which the linkage region is followed by one or two unsulphated disaccharides, a constant number of monosulphated disaccharides and a stretch of 6–36 disulphated disaccharides [13]. Previous studies [14–16] on KS isolated from articular and non-articular cartilage have shown that the former (KS-II-A) contains α (1-3)-linked fucose and α (2-6)-linked *N*-acetylneuraminic acid, whereas that from non-articular cartilage (KS-II-B) contains neither of these features.

Monoclonal antibodies (mAbs) which might be helpful tools for a detailed analysis of the structure and distribution of KS have been raised in mice against skeletal KS from human [17] and bovine cartilage [18], as well as from porcine chondrocytes [19] (mAb MZ15 [19], mAbs 5D4 and 1B4 [17,18]). Detailed

analysis of the oligosaccharides recognized by these antibodies showed that all of them recognize disulphated domains in the KS chain [20–23], domains which seem common to all KS species analysed so far. None of the antibodies mentioned above allowed the discrimination between different KS types. Although these antibodies are useful for the detection of KS, they are not suitable for the selective determination of one KS species among others, e.g. the monitoring of cartilage-derived KS in serum during the course of chronic inflammatory or degenerative joint diseases. In contrast to these antibodies described previously, the mAb 3D12/H7 recognizes an epitope located in the linkage region of the KS chains. Moreover, this domain seems to be restricted to aggrecan from human articular cartilage since KS chains prepared from human non-articular cartilage or non-human cartilage tissues are recognized neither in an antigen-inhibition assay nor after immunoblotting.

EXPERIMENTAL

Materials

Sepharose Q HP (HR 16/10 column), Protein-G-Sepharose, DEAE-Sepharose, preformed gels for SDS/PAGE (8–25% T gradient gels, 20% T homogeneous gels, 20% T homogeneous gels with high-density), and preformed gels for isoelectric focusing (pH 4–6.5) were obtained from Pharmacia/LKB (Freiburg, Germany). BioGel P2 and BioGel P10 were obtained from Bio-Rad (Munich, Germany). Polyvinylidene difluoride (PVDF) blotting membranes (Immobilon N, Immobilon P) were purchased from

Abbreviations used: DAB, diaminobenzamidine-tetrahydrochloride; mAb, monoclonal antibody; KS, keratan sulphate; HexN, hexosamine; PVDF, polyvinylidene difluoride.

* To whom correspondence should be addressed.

Waters (Dreieich, Germany). Diaminobenzamidine-tetrahydrochloride (DAB) was from Serva (Heidelberg, Germany), *o*-phenylenediamine (tablets of 2 mg) and peroxidase-conjugated rabbit anti-(mouse Ig) serum were from DAKO (Copenhagen, Denmark). Peroxidase-conjugated goat antibodies against mouse IgG were from Dianova (Hamburg, Germany). Chondroitin sulphate lyase AC (EC 4.2.2.5), chondroitin sulphate lyase ABC (EC 4.2.2.4), KS hydrolyase (EC 3.2.1.103), KS endo- β -N-acetylglucosaminidase (keratanase II) and the KS-specific mAb 5D4 were purchased from Seikagaku (Tokyo, Japan). NaB^3H_4 (25 mCi; specific radioactivity 16 Ci/mmol) was purchased from Amersham (Braunschweig, Germany) and EN³HANCE for fluorography of IEF gels was obtained from Dupont (Brussels, Belgium). CHAPS and papain (10 mg/ml) were from Boehringer Mannheim (Mannheim, Germany). All remaining reagents were of analytical grade.

Methods

Determinations of the amino acid and hexosamine compositions were carried out after acid hydrolysis (3 M HCl, 15 h, 105 °C) on an AlphaPlus amino acid analyser (Pharmacia, Freiburg, Germany), the sulphate content was determined after acid hydrolysis (3 M HCl, 15 h, 105 °C) by ion-chromatography on an IONPAC AS4A column (4 mm $\times$ 250 mm; guard column 4 mm $\times$ 50 mm; Dionex, Idstein, Germany) with conductivity detection. Molecular-mass determinations were performed on a HEMA Bio linear column (8 mm $\times$ 600 mm) or on a HEMA Bio 40 column (8 mm $\times$ 600 mm) each equipped with a guard column (8 mm $\times$ 50 mm). The gel-filtration columns were eluted with 50 mM sodium phosphate (pH 6.7) containing 0.2 M NaCl at a flow rate of 0.5 ml/min. The elution profiles were monitored at 214 nm and/or with an on-line radioactivity monitor (B 506 C1; Berthold, Bad Wildbad, Germany); both columns were calibrated using glycosaminoglycans of known molecular-mass distribution [24]. Combined Alcian Blue/silver staining of SDS/polyacrylamide gels was carried out essentially as described by Möller et al. [25]. For estimation of the molecular-mass distribution of KS chains and derived oligosaccharides, glycosaminoglycans of known molecular-mass distribution were used [24]. The mAb 3D12/H7, obtained by immunization of BALB/c mice with aggrecan [26], was purified from cell culture supernatant by chromatography on Sepharose A/G (Pierce, Munich, Germany) as described by the manufacturer.

Isolation of peptido-KS from aggrecan

Aggrecan (55 mg, calculated as GlcNAc), isolated and purified from human articular cartilage as described previously [26], was digested with chondroitin sulphate lyase AC/ABC (each 0.1 unit/mg; 37 °C, 12 h) prior to digestion with papain (1 mg of papain per mg of aggrecan, 65 °C, 12 h). The digest was purified by anion-exchange chromatography on a Sepharose Q HP column (16 mm $\times$ 100 mm), equilibrated with 0.02 M NaH_2PO_4 (pH 4.5) containing 0.05 M NaCl, 0.05 M LiCl and 0.05 % (w/v) CHAPS. Elution was performed with a linear gradient from 0.05 M NaCl to 3 M NaCl in the buffer described above, and the elution profile was monitored at 214 nm. Fractions were tested for the presence of KS peptides recognized by the mAb 3D12/H7 with an enzyme-immunoassay based on that described by Hoadley et al. [6]. Immunochemical active fractions were pooled and desalted by gel-permeation chromatography on a BioGel P10 column (16 mm $\times$ 740 mm; Bio-Rad, Munich, Germany) eluted with 10 % ethanol. Peptido-KS eluted in the void volume and was recovered after lyophilization.

Figure 1 Antigen inhibition assay with biotin-labelled aggrecan from human articular cartilage and peptido-KS from human intervertebral disc (A) or peptido-KS derived from human articular cartilage aggrecan (B)

(A) Increasing amounts of peptido-KS from human intervertebral disc (46 nmol/l to 47.4 $\mu\text{mol/l}$; calculated as GlcN) and a constant amount of biotin-labelled aggrecan (112 nmol/l; calculated as GlcN) were incubated with the immobilized mAb 3D12/H7 (●) or alternatively with the mAb 5D4 (○). Bound biotin-labelled antigen was detected by peroxidase-conjugated streptavidin. (B) Increasing amounts of peptido-KS from human articular cartilage aggrecan (3 nmol/l to 6.2 $\mu\text{mol/l}$; calculated as GlcN) and a constant amount of biotin-labelled chondroitinase AC/ABC-digested aggrecan (15.5 nmol/l; calculated as GlcN) were incubated with the immobilized mAb 3D12/H7. Bound biotin-labelled antigen was detected by peroxidase-conjugated streptavidin.

Preparation of reduced KS chains

The lyophilized peptido-KS was treated with either 0.1 M NaBH_4 (6 mg of KS-peptide) or NaB^3H_4 (2.7 mg of KS-peptide) in the presence of 0.05 M NaOH, essentially as described by Brown et al. [27]. After neutralization of the reaction mixture with 1 M acetic acid the reduced KS chains were recovered by chromatography on a BioGel P2 column (10 mm $\times$ 90 mm) eluted with 10 % ethanol, further purified by gel-chromatography on a BioGel P10 column (see above) and lyophilized.

Digestion with keratanases

Aliquots of the reduced KS chains or of the reduced and ^3H -labelled KS chains were digested with keratanase (0.4 unit/ μmol

Table 1 Comparison of the mAbs 3D12/H7 and 5D4 with respect to their reactivity towards different KS preparations

The antigens were tested in an antigen-inhibition assay as described in the legend to Figure 1. In general, the content of GlcN, determined by combined amino acid and HexN analysis after acid hydrolysis, was taken as a measure for the KS content. For evaluation in the inhibition assay the ratio of unlabelled to labelled antigen was varied between 400 and 0.1. For immunoblotting, 1 μ l (about 1 mg/ml) of the respective KS was submitted to SDS/PAGE (4–15% gels and 8–25% gels were used) and blotted on to nylon membrane (70 °C, 30 min). The membranes were further processed essentially as described in [26]; the intensity of the signal is given in arbitrary units. Abbreviation: n.d., not done due to the fact that only limited amounts of material were available; + + +, very strong signal; $\pm$, very faint signal (if any).

Source of KS	Immunoblot		Antigen-inhibition assay	
	5D4	3D12/H7	5D4	3D12/H7
Bovine tracheal cartilage	+ + +	$\pm$	n.d.	n.d.
Bovine nasalseptum cartilage	+ + +	—	Yes	n.d.
Bovine cornea	+ + +	$\pm$	Yes	No
Human intervertebral disc	+ + +	—	Yes	No
Human aorta	+ + +	—	Yes	n.d.
Human articular cartilage	+ + +	+ + +	Yes	Yes

Figure 2 Anion-exchange chromatography of aggrecan after digestion with chondroitinase AC/ABC and papain

Aggrecan (1 mg/ml, calculated as GlcN) was digested with chondroitinase AC/ABC (5 units each; 37 °C, overnight) followed by digestion with papain (1 mg of papain/mg of aggrecan; 65 °C, overnight). For isolation of peptido-KS a Sepharose-Q HP column (16 mm $\times$ 100 mm; Pharmacia/LKB, Freiburg, Germany) was equilibrated with 20 mM NaH_2PO_4 , 50 mM NaCl, 50 mM LiCl, 0.05% CHAPS (pH 4.5) and the sample was applied with a flow rate of 1 ml/min. Elution of bound KS peptides was performed with a linear gradient (3 ml/min) of 50 mM NaCl to 3 M NaCl in the buffer described above. Elution profile was monitored at 210 nm (dotted line) and 280 nm (continuous line). In addition, aliquots of fractions (3 ml each) were analysed with the KS-specific mAb 3D12/H7. Immunochemical active fractions (indicated by the bar) were pooled and further characterized.

of GlcN) in 10 mM sodium acetate, pH 6.5 (4 h, 37 °C). Oligosaccharides were recovered by gel chromatography on a BioGel P10 column, the elution profile was monitored for immuno-

Figure 3 Gel-permeation chromatography of aggrecan-derived KS peptides

Purified peptido-KS was submitted to high-performance gel-permeation chromatography on a HEMA Bio linear column (8 mm $\times$ 600 mm) equipped with a precolumn (8 mm $\times$ 50 mm) and equilibrated with 25 mM NaH_2PO_4 , 25 mM Na_2HPO_4 , 0.2 M NaCl (pH 6.7). The column was calibrated with glycosaminoglycans of known molecular-mass distribution. Aliquots (20 μ l) of the sample were submitted to chromatography with a flow rate of 0.5 ml/min. The elution profile was monitored at 214 nm, V_0 and V_t are indicated.

chemically active oligosaccharides by an enzyme-immunoassay and for radiolabelled material using a scintillation counter. An aliquot of the reduced and ^3H -labelled keratanase-derived oligosaccharides was further digested with keratanase II (0.1 unit/ μ mol of GlcN, 4 h, 37 °C) in the buffer mentioned above. In an additional experiment oligosaccharides obtained after keratanase digestion of reduced KS chains were further purified by absorption to DEAE-Sephacel (microscale batch procedure) in the presence of 0.05 M Tris/HCl (pH 6.8). Contaminations were washed off with 0.75 M NaCl in the buffer mentioned above, bound oligosaccharides were desorbed by washing the gel with 3 M NaCl in 0.05 M Tris/HCl (pH 6.8) and desalted on BioGel P2. Oligosaccharides were eluted with 10% ethanol and recovered after lyophilization.

Isoelectric-focusing gel-shift assay

Oligosaccharides obtained by keratanase digestion of reduced KS chains, as well as those incubated with the mAb 3D12/H7, were submitted to isoelectric focusing (pH 4–6.5 gradient gel). The gels were either stained with silver or submitted to diffusion blot on to PVDF membrane. ^3H -labelled KS-derived oligosaccharides were detected by fluorography. For specific detection of the mAb 3D12/H7 membranes were incubated with a peroxidase-conjugated goat anti-(mouse IgG) serum (Dianova, Hamburg, Germany) after unspecific binding sites on the membrane had been blocked (2% BLOTTO in 0.02 M Tris/0.3 M NaCl, pH 7.5). Visualization of bound antibody was performed with DAB and H_2O_2 as described previously [26].

Table 2 Amino acid and HexN composition of aggrecan, the purified peptido-KS and the KS chains obtained after reductive β -elimination

The amino acid content is given in mol/1000 mol; the ratios of HexN/protein, GlcN/protein, GalN/protein, GlcN:GalN and SO_4^{2-} :GlcN are given as molar ratios. Abbreviation: n.d., not detectable.

Residue	Content (mol/1000 mol)		
	Aggrecan	Peptido-KS	KS chains
Asp	104	49	
Thr	66	66	
Ser	95	101	
Glu	141	147	
Pro	106	350	
Gly	117	61	
Ala	50	50	
Val	41	74	
Met	n.d.	15	
Ile	26	n.d.	
Leu	78	20	
Tyr	38	n.d.	
Phe	43	29	
His	38	n.d.	
Lys	16	19	
Arg	41	19	
HexN:protein*	0.69	1.88	40.0†
GlcN:protein	0.25	1.73	
GalN:protein	0.44	0.15	
GlcN:GalN	0.56	11.77	12.9
SO_4^{2-} :GlcN		1.5	

* Protein is determined as sum of amino acids.

† After reductive β -elimination and gel chromatography mostly basic amino acids were detectable.

RESULTS AND DISCUSSION

The aim of the present study was not only to isolate and characterize the domain recognized by the KS-specific mAb 3D12/H7 but also to get a more detailed knowledge of the fine structure and microheterogeneity of KS chains present on aggrecan. The novel antibody 3D12/H7 recognizes aggrecan and aggrecan after enzymic degradation of the chondroitin sulphate chains. The mAb shows no reactivity to keratanase/keratanase II-digested aggrecan nor to aggrecan, which was chemically deglycosylated with trifluoromethanesulphonic acid (results not shown). Moreover, previous experiments have shown that the mAb 3D12/H7 does not react with KS-containing peptides from other tissues (tracheal cartilage, intervertebral disc, aorta) or other species (bovine, human), regardless of whether inhibition assays or diffusion blots were performed (Figure 1 and Table 1). Therefore, the epitope recognized by this mAb must be different from that recognized by the well-characterized mAb 5D4. Moreover, there is evidence that this epitope is specifically expressed on KS chains present in aggrecan from human articular cartilage.

Aggrecan which has been isolated and purified previously from human articular cartilage [26] was sequentially digested with chondroitin sulphate lyase AC/ABC and papain. The aggrecan fragments were purified by anion-exchange chromatography on a Sepharose Q HP column (Figure 2) and fractions containing immunochemically active KS peptides were desalted on BioGel P10 prior to further characterization. By high-performance gel chromatography on a HEMA Bio linear column it was shown that the KS peptides isolated had a molecular mass of approximately 20 kDa (Figure 3). Analysis of the sulphate-,

Figure 4 Gel-permeation chromatography of reduced and ^3H -labelled KS chains

Reduced and ^3H -labelled KS chains (A) as well as oligosaccharides obtained after keratanase digestion (B) or after combined keratanase/keratanase II digestion (C) were submitted to high-performance gel-permeation chromatography on a HEMA Bio 40 column (8 mm $\times$ 600 mm) equipped with a precolumn (8 mm $\times$ 50 mm). The column was equilibrated and calibrated as described in the legend to Figure 3. The elution profiles were monitored with an on-line radioactivity monitor, V_0 and V_t are indicated.

amino acid- and hexosamine (HexN) contents revealed a molar ratio of HexN to protein of 1.88, of GlcN to GalN of 12, and of sulphate to GlcN of 1.5 (Table 2). KS chains prepared by reductive β -elimination with either NaB^3H_4 or NaBH_4 in the presence of 0.05 M NaOH were isolated by gel-permeation chromatography on BioGel P10 and eluted in the void volume ($0 \leq K_{av} \leq 0.09$). The reduced KS chains, recognized by the mAb 3D12/H7, were submitted to HPLC on a HEMA Bio 40 column calibrated with glycosaminoglycan chains of known molecular-mass distribution [24]. By this, for the KS chains a molecular-mass distribution from 12 kDa to 8.4 kDa was estimated (Figure 4A). After digestion with keratanase the ^3H -labelled fragments were recovered by chromatography on a BioGel P10 column ($0.1 \leq K_{av} \leq 0.2$). SDS/PAGE (20% high-density gel) and combined Alcian Blue/silver staining revealed the presence of at least six oligosaccharides (Figure 5). Analysis of this oligosaccharide mixture by gel chromatography on HEMA Bio 40 showed a molecular-mass distribution from 6.7 kDa to 1.9 kDa (Figure

Figure 5 SDS/PAGE and combined Alcian Blue/silver staining of reduced, reduced and keratanase-digested, and reduced and keratanase II-digested KS chains

Reduced KS chains (lane 1), as well as reduced and keratanase-digested (lane 2) and reduced and keratanase II-digested (lane 3) KS chains, were submitted to SDS/PAGE on a 20% T homogeneous gel with high density. For estimation of the molecular-mass distribution the same glycosaminoglycan chains used for calibration of the HEMA Bio 40 column were taken. The gel was stained with Alcian Blue and silver essentially as described by Möller et al. [25].

Figure 6 Isoelectric focusing of KS-derived oligosaccharides before and after incubation with the mAb 3D12/H7

Reduced and ^3H -labelled KS chains were digested with keratanase or keratanase II and aliquots of the oligosaccharide mixtures were incubated with the mAb 3D12/H7 prior to isoelectric focusing on a pH 4–6.5 gel. Part of the oligosaccharide mixture was purified further by ion-exchange chromatography on DEAE-Sephacel (microscale batch procedure) to remove the enzyme contaminations (C). The gel was either stained with silver (A and C) or submitted to a diffusion blot on to PVDF membranes. The membranes were used for fluorography (lane 2, A) or incubated with a peroxidase-conjugated goat anti-(mouse IgG) serum (B). (A) and (B): lane 1, protein standard for isoelectric focusing; lane 2, reduced and ^3H -labelled KS chains after digestion with keratanase (fluorography); lane 3, reduced and ^3H -labelled KS chains after digestion with keratanase and incubation with the mAb 3D12/H7; lane 4, mAb 3D12/H7. (C) Lane 1, reduced and ^3H -labelled KS chains after digestion with keratanase and further purification; lane 2, reduced and ^3H -labelled KS chains after digestion with keratanase, further purification, and incubation with the mAb 3D12/H7; lane 3, mAb 3D12/H7. (A) Silver stain; (B) immunoblot; (C) silver stain.

4B). After subsequent digestion of these keratanase-derived oligosaccharides with keratanase II all fragments eluted at V_t (Figure 4C). Since these oligosaccharides were too small to be detected in an enzyme-immunoassay or after SDS/PAGE and diffusion blot, an isoelectric-focusing gel-shift assay was de-

Figure 7 Proposed structure of the KS domain recognized by the mAb 3D12/H7

The proposed substitution of the linkage region oligosaccharide with three sulphate- and two fucose-residues is concluded from the molecular mass of the smallest oligosaccharide recognized by the mAb 3D12/H7 and the known specificity of keratanase and keratanase II. Two possible positions for the two fucose residues are shown. Whereas substitution of the second GlcNAc with fucose has been shown by NMR analysis [34,35], the second fucose residue might be attached either to the first or third GlcNAc residue.

Figure 8 SDS/PAGE and diffusion blot of the KS-rich region before and after digestion with papain

The KS-rich region, prepared as described previously [26], was submitted to SDS/PAGE on a 20% T homogeneous gel with high density and diffusion blot on to PVDF membrane before (lane 1) and after digestion with papain (lane 2). Detection of KS-containing peptides was performed with either mAb 5D4 (A) or mAb 3D12/H7 (B) and a peroxidase-conjugated goat anti-(mouse IgG) antibody.

veloped. The reduced KS chains, as well as the oligosaccharides obtained after keratanase or combined keratanase/keratanase II digestion, were incubated with the mAb 3D12/H7 and the reaction mixtures were submitted to IEF on pH 4–6.5 gradient gels (Figure 6). In addition, an aliquot of the oligosaccharides obtained after keratanase digestion was further purified by anion-exchange chromatography prior to incubation with the mAb 3D12/H7 and subsequent submission to IEF on pH 4–6.5 gradient gels (Figure 6C). Since the isoelectric points (pI) from antibody (about pH 6; Figures 6A and 6B, lane 4; Figure 6C, lane 3) and oligosaccharides (between pH 4 and 5; Figure 6A, lane 2; Figure 6C, lane 1) differ, the interaction of antigen and antibody should result in a shift, e.g. a new band at a distinct pI. The gel was either silver-stained (Figures 6A and 6C) or submitted to diffusion blot on to PVDF membrane (Immobilon P). The blotted and ^3H -labelled oligosaccharides were detected after fluorography (Figure 6A, lane 2). The mAb 3D12/H7 was detected after incubation of the membrane with a peroxidase-conjugated goat anti-(mouse IgG) antibody (Figure 6B). By this, it could be shown that the antibody recognizes the reduced KS chain as well as the reduced and ^3H -labelled oligosaccharides obtained by keratanase digestion. It might be noted that the staining intensity of the immune complexes is lower than expected. This is most probably due to an incomplete transfer to

the membrane and/or incomplete immobilization of the immune complex on the membrane. In contrast, the ^3H -labelled oligosaccharides obtained after keratanase II digestion were not recognized (results not shown). Since the ^3H label was introduced only into the linkage region, all ^3H -labelled fragments recognized by the mAb 3D12/H7 must contain the linkage region oligosaccharide. The molecular mass of the smallest keratanase-derived oligosaccharide, together with the known specificity of the keratanase [16,28–30], suggests that the oligosaccharide shown in Figure 7 is the smallest one recognized by the mAb 3D12/H7. The substitution of the oligosaccharide with two fucose- and three sulphate-residues is concluded from the molecular mass, the published data about the specificity of keratanase and keratanase II, and the known data about the KS structure [14,16,27–35]. Whereas substitution of the second GlcNAc with fucose has been shown by NMR analysis [34,35], the second fucose residue might be attached either to the first or third GlcNAc residue. The occurrence of several ^3H -labelled oligosaccharides after keratanase digestion of the ^3H -labelled KS points towards a microheterogeneity within the KS chains; the position of the first keratanase-cleavable linkage may be variable. Moreover, the domain described seemed to be restricted to the KS chains present in the chondroitin sulphate-rich region of aggrecan, since the KS-rich region, obtained after combined chondroitinase AC/ABC and trypsin digestion, showed only very faint reactivity towards the mAb 3D12/H7 (Figure 8). However, to clarify this point further studies are required. Nevertheless, this antibody might be useful not only for a more detailed analysis of KS structure and distribution but also for monitoring the release of cartilage-specific KS during the course of chronic inflammatory and degenerative joint diseases.

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