BIOSYNTHESIS OF CHONDROITIN SULFATE PROTEINS ISOLATION OF FOUR POOLS OF CHONDROITIN SULFATE PROTEINS DIFFERING IN THEIR SOLUBILITY AND LABELING RATES WITH RADIOSULFATE IN CALF RIB CARTILAGE

Tilman O. KLEINE
With technical assistance of
B. HEINRICH and K. GOETZ
Klinisch-chemisches Labor. der Univ.-Nerv.-Klinik, 335 Marburg/Lahn, Western Germany

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1. Introduction

Earlier experiments have shown a considerable metabolic and physicochemical heterogeneity of the proteoglycans in calf rib cartilage [1–8]. Although about 5% of total sulfopolysaccharides is represented by keratan sulfate and the bulk material (95%) consists of chondroitin-4-sulfate [1, 9] at least five proteoglycan fractions could be isolated. They differ besides metabolic heterogeneity in degree of sulfation and average chain lengths of the chondroitin sulfate side chains, as well as in amino acid composition of the peptide core and glycosamine content [3–5, 8]. The heterogeneity of the chondroitin sulfate proteins could be explained by mechanisms of their biosynthesis in the cartilage where 6 monosaccharide transferases and a sulfotransferase build up chondroitin sulfate side chains at preformed acceptor proteins, which very probably exist in pools of different size, composition and turnover rates [2, 8]. Furthermore, the novo-synthesized chondroitin sulfate proteins can be distinguished by extraction with solutions of guanidin of different molarity.

2. Experimental

4 g freshly prepared slices (rib cartilage of young calves 100 μm thick) were incubated for 20, 90 and 300 min with 320 μCi of carrier free $^{35}SO_4^{2-}$ under conditions described previously [3, 5, 10]. At the end of the incubation slices and medium (total volume 58 ml) were made 0.4 M with respect to guanidinium-chloride and brought to pH 5.8 by addition of 2 N acetic acid. The mixture was stirred in an ice-bath for 24 hr. The cartilage slices were filtered through a nylon net and washed twice with 2 ml 0.4 M guanidinium-chloride in 0.05 M acetate buffer, pH 5.8. The extracts were combined (*extract I*, fig. 1).

The cartilage slices were then stirred for 24 hr at 25° in 20 ml 4.0 M guanidinium-chloride in 0.05 M acetate buffer, pH 5.8, filtered and washed twice with 2 ml 4.0 M guanidinium-chloride, pH 5.8. The extracts were combined (*extract II*, fig. 1). The cartilage slices were stirred again for 20 hr at 60° in 16 ml 8.0 M guanidinium-chloride in 0.05 M acetate buffer, pH 5.8, filtered and washed twice with 3 ml 8.0 M guanidinium-chloride pH 5.8. The extracts were combined (*extract III*, fig. 1).

The insoluble residue (pellucid cartilage slices) (fig. 1) were dialysed against bidist. water at 4° to remove the salt and digested with polymer-bound papain (CM-cellulose bound papain, Merck AG, Darmstadt, 0.2–0.3 U/mg) as previously described [1, 10]. From the digest the *chondroitin sulfate peptides* were isolated by precipitation with cetylpyridinium-chloride [10] and fractionated on Ecteola-cellulose according to increasing chain length and degree of sulfation [1, 3, 8, 9, 10].

To isolate the crude proteoglycan fractions of the three extracts a portion of extract I, II and III was dialysed in dialysis tubes (Fa. Union Carbide, Chicago,

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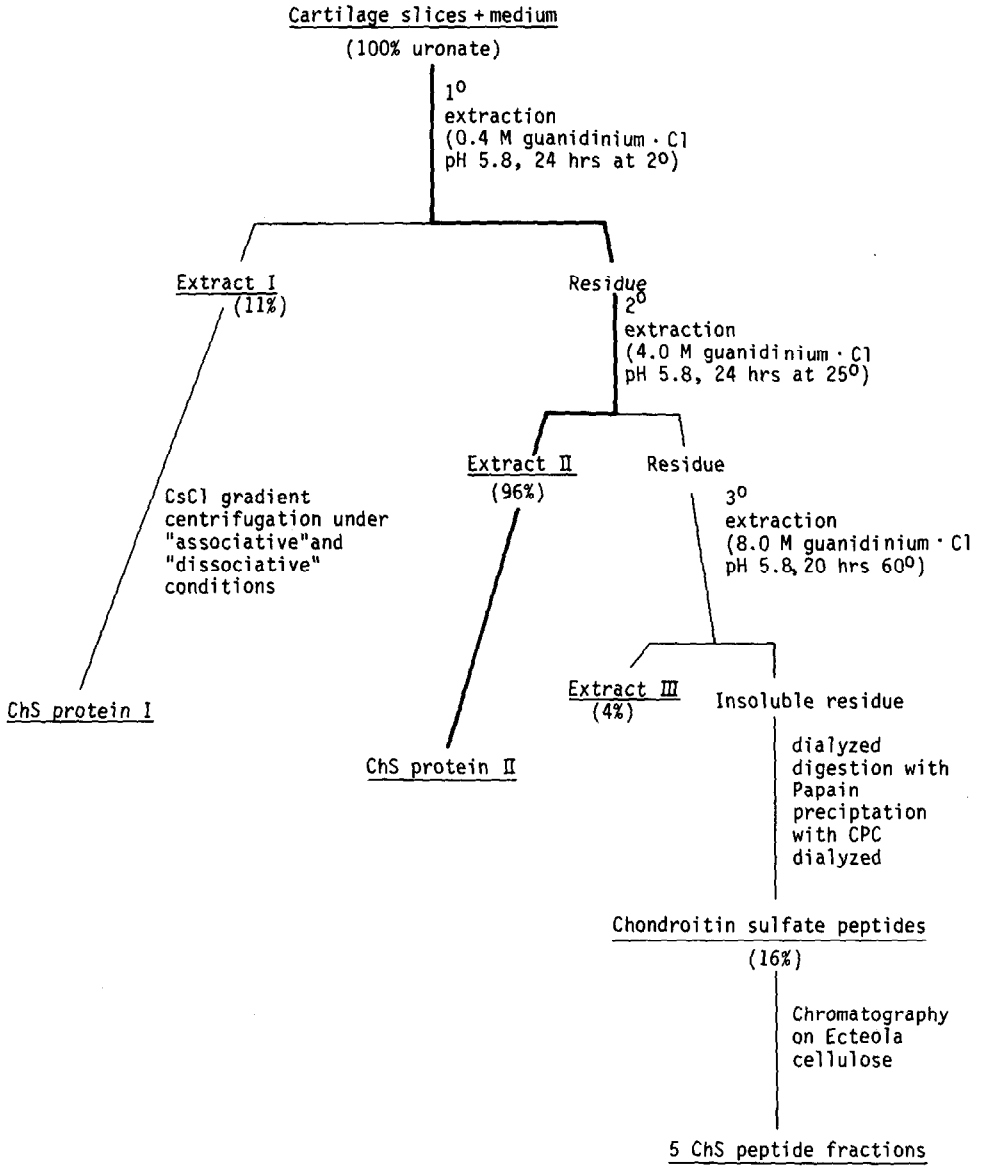

Fig. 1. Fractionation scheme. For further details see text.

Ill., USA) with internal mixing against bidistilled water, changed daily, for 7 days at 4°.

The chondroitin sulfate proteins I and II were isolated from the first and the second extract, resp., applying CsCl gradient centrifugation under associative (0.4 M guanidinium-chloride in 0.05 M acetate buffer pH 5.8, ρ = 1.69) and dissociative (4.0 M guanidinium-chloride in 0.05 M acetate buffer pH 5.8, ρ = 1.50) conditions (cf. [5]).

Analysis of the components (uronic acid, galactosamine, glucosamine, sulfate esters, galactose, xylose), chondroitin sulfate chain length and determination of

absolute radioactivity were carried out as described previously [1, 5, 10].

3. Results and discussion

As shown in fig. 1 at least 80% of the proteoglycans could be solubilized in calf rib cartilage with 0.4 and 4.0 M guanidinium-chloride yielding *extracts I* and *II*. Under stronger conditions (8.0 M guanidinium-chloride, 60°) 4% of total proteoglycans went into solution. These proteoglycans are particle bound, probably fixed to collagen fibrils. 16% of total uronate still remained insoluble and could be solubilized as *chondroitin sulfate peptides* after digestion with papain (chondroitin sulfate peptides of the insoluble residue, see fig. 1).

By increasing the incubation period these four proteoglycan fractions are labeled with $^{35}SO_4^{2-}$ in a different manner (fig. 2): after 20 min of incubation with $^{35}SO_4^{2-}$ the specific radioactivity of sulfate ester groups in proteoglycans of extract I and III and in the insoluble residue are 5 to 8 times higher than those of the bulk material (extract II). After longer incubation times the easily dissoluble proteoglycans of extract I show the lowest labeled sulfate ester groups and chondroitin sulfate peptides of the insoluble residue the highest labeled ones. The bulk material (proteoglycans of extract II) is labeled linearly with increasing incubation time only after a lag phase of about 30 min. The specific radioactivity of the sparingly soluble proteoglycans in extract III exhibits a labeling plateau after 300 min of incubation indicating a saturation

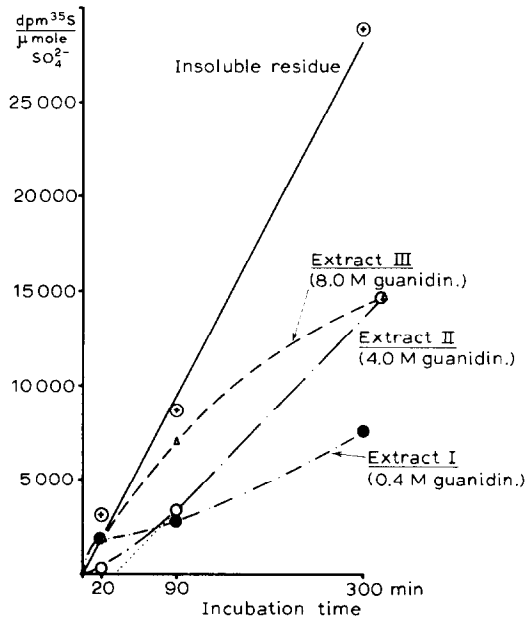

Fig. 2. $^{35}SO_4$ Labeling of four pools of chondroitin sulfate proteins in calf rib cartilage. For further details see text.

level of sulfate ester groups. Nevertheless, during whole incubation time labeling rate of sulfate esters increases linearly in the chondroitin sulfate peptides of the insoluble residue. Therefore, these structure bound chondroitin sulfate peptides could represent the synthesizing pool of proteoglycans in cartilage cells.

After short incubation with $^{35}SO_4^{2-}$ (20 min) the separation of the chondroitin sulfate peptide of the insoluble residue on Ecteola cellulose yields 5 sub-

Ecteola fractions no.	Uronic acid (UA) %	Degree of sulfation SO_4^{2-}/UA*	Specific radioactivity of sulfate esters dpm/µmole SO_4^{2-}	Chain length UA:Gal:Xyl*
I 0.05 HCl plus	8	0.24	110	2.5:0.09:1
II 0.50 NaCl	1.5	–	6600	28 :2 :–
III 0.75 NaCl	12	1.09	4950	12.2:1.8 :1
IV 1.00 NaCl	64	1.05	2460	24.2:1.8 :1
V 2.00 NaCl	14	1.22	16410	35.4:2.0 :1

Fig. 3. Metabolic and chemical heterogeneity of chondroitin sulfate peptides in the insoluble residue of calf rib cartilage after a 20 min incubation with $^{35}SO_4^{2-}$. For further details see fig. 1 and text.

* Molar ratio.

Purified product	Amino sugar composition		Degree of sulfation SO_4^{2-}/	ChS chain length
	Glucosamine (%)	Galactosamine (%)	Uronate*	UA:Gal:Xyl*
ChS-protein I	3.8	96.2	0.96	12.5:1.9:1
ChS-protein II	5.2	94.8	0.94	25.2:2.0:1

Fig. 4. Chemical characterization of the sulfopolysaccharide portion in purified ChS-proteins from calf rib cartilage. The ChS-proteins I and II are purified applying CsCl gradient centrifugation of the extract I and II. For further details see fig. 1 and text.

* Molar ratio.

fractions differing considerably in specific radioactivity of sulfate esters (fig. 3): shorter and longer ChS chains show a 3 to 7 times higher labeling rate than the medium chained chondroitin sulfate peptides of the bulk material.

Considering the chondroitin sulfate peptides of the insoluble residue as the immediately synthesized product of the cartilage cells, these findings indicate a metabolic and chemical heterogeneity of the chondroitin sulfate proteins during their biosynthesis in the cell. Similar findings have been reported by Hardingham and Muir [11] recently. Since specific radioactivity of sulfate ester groups of the immediately synthesized insoluble proteoglycans are considerably higher labeled than those of the soluble ones (see figs. 1 and 3), a transport of the newly synthesized product out of the cartilage cell must occur as has been shown autoradiographically in chondrocytes [12] and in cartilage cells of mice [13].

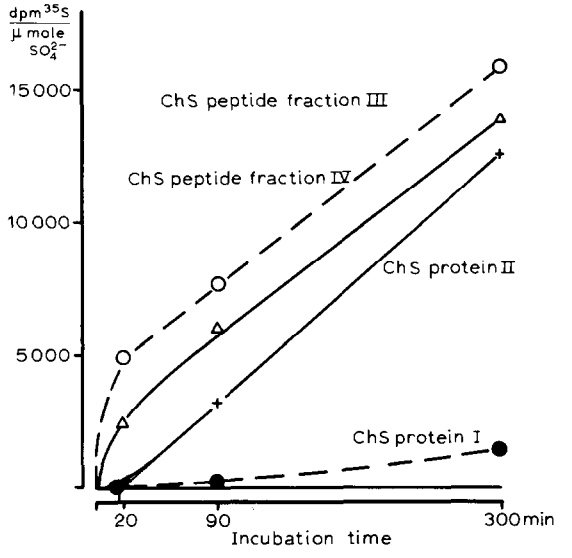

Fig. 5. $^{35}SO_4$-Labeling of soluble purified chondroitin sulfate proteins and chondroitin sulfate peptides of the insoluble residue in calf rib cartilage. Soluble ChS proteins I and II are purified by CsCl gradient centrifugation. ChS peptides of the insoluble residue are isolated by chromatography, for further details see figs. 1, 3 and text.

These findings can be further supported: after applying gradient centrifugation under associative and dissociative conditions [4, 5] a chondroitin sulfate protein is isolated from extract I and II showing an average chain length similar to two chondroitin sulfate peptides fractions of the insoluble residue (see fraction III and IV of fig. 3 and fig. 4). The specific radioactivity of the sulfate ester groups in both purified chondroitin sulfate proteins grows up in a different manner during increasing incubation time with $^{35}SO_4^{2-}$ (fig. 5); those of the chondroitin sulfate protein I increases almost linearly, those of the second one after a lag phase of 20 min. However, labeling rates of their sulfate ester groups are always lower than those of the corresponding chondroitin sulfate peptide fractions III and IV of the insoluble residue (see fig. 5).

The present results show that after incubation of cartilage slices with $^{35}SO_4^{2-}$ for 20, 90 and 300 min, four pools of metabolically and chemically different proteoglycans can be isolated applying dissociative extraction procedures with increasing concentrations of guanidinium-chloride. Indications exist that in the cartilage slices the de novo-synthesized insoluble chondroitin sulfate proteins are metabolically and chemically heterogeneous.

Acknowledgement

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References

[1] T.O. Kleine and H. Hilz, Hoppe-Seyler's Z. Physiol. Chem. 349 (1968) 1027.
[2] T.O. Kleine and H. Hilz, Fed. Exptl. Biol. Soc. (Madrid 1969) Abstr. VI, p. 258.
[3] T.O. Kleine and H. Hilz, in: Chemistry and molecular biology of the intercellular matrix, Vol. II, ed. E.A. Balazs (Academic Press, London 1970) p. 907.
[4] T.O. Kleine, H.J. Kirsig and H. Hilz, Z. Klin. Chem. Klin. Biochem. 9 (1971) 74.
[5] T.O. Kleine, H.J. Kirsig and H. Hilz, Hoppe-Seyler's Z. Physiol. Chem. 352 (1971) 479.
[6] T.O. Kleine, Fed. Exptl. Biol. Soc. (Varna 1971) Abstr. VII, p. 221.
[7] T.O. Kleine, Scand. J. Clin. Lab. Invest. 29 (1972) 20.
[8] T.O. Kleine, Naturwissenschaften 59 (1972) 64.
[9] T.O. Kleine and H. Hilz, Hoppe-Seyler's Z. Physiol. Chem. 349 (1968) 1250.
[10] T.O. Kleine, H.J. Kirsig and H. Hilz, Hoppe-Seyler's Z. Physiol. Chem. 349 (1968) 1037.
[11] T.H. Hardingham and H. Muir, Biochem. J. 126 (1972) 791.
[12] H. Rohr and S. Walter, Acta Anat. 64 (1966) 223.
[13] F.K. Thorp and A. Dorfman, J. Cell Biol. 18 (1963) 13.