Carbohydrate Research, 62 (1978) 271–279

QUANTITATIVE β-ELIMINATION–REDUCTION OF *O*-GLYCOSYL LINKAGES IN CHONDROITIN SULFATES

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(Received February 28th, 1977; accepted for publication in revised form May 20th, 1977)

ABSTRACT

The chondroitin 4-sulfate–peptide from whale cartilage contains serine, xylose, and galactose in ~1:1:2 molar ratio. Deamination with nitrous acid showed that about 50% of the serine is at the amino terminus. Various conditions of β-elimination–reduction were employed with the preparation to provide quantitative data on the linkage region between protein and carbohydrate. The optimal conditions used, 0.4M sodium hydroxide in the presence of 0.3M sodium borohydride and 0.01M $PdCl_2 \cdot 2H_2O$ for 24 h at 25°, resulted in an increase of alanine content and concomitant decrease of serine and conversion of xylose into xylitol, all in equimolar amounts. Furthermore, substitution of both the terminal amino and carboxyl groups, and elimination–reduction, brought about cleavage of most of the linkages; over 90% of the amino acids originally present were lost after re-isolation of the polymer fraction. These results indicate that β-elimination–reduction alone, under the optimal conditions, allows the mode of linkage to be quantitatively determined as an *O*-xylosylserine linkage. Under these optimal conditions, the linkage region between protein and a chondroitin 4- and 6-sulfate hybrid (1:1) from bovine tracheal cartilage was determined to be Gal-Gal-Xyl-O-Ser, thus being similar to that found in chondroitin 4-sulfate–peptide.

INTRODUCTION

The carbohydrate–peptide linkages of glycosaminoglycans have been studied mainly by alkaline β-elimination–reduction, which shows that the majority of linkages are *O*-glycosyl bonds to serine[1–5] or to both serine and threonine[6,7]. However, the formation of alanine and 2-aminobutanoic acid does not fully account for the loss of serine and threonine.

In this paper, β-elimination–reduction of chondroitin 4-sulfate–peptide from whale cartilage is examined under various conditions, to establish optimal conditions under which the losses of serine and xylose involved in *O*-glycosyl linkages would be equivalent to the gains of alanine and xylitol. Under these optimal conditions the linkage between protein and a 1:1 chondroitin 4- and 6-sulfate hybrid[8] was also investigated.

EXPERIMENTAL

Materials. — Chondroitin 4-sulfate–peptide and chondroitin 4- and 6-sulfate hybrid–peptide were isolated by pronase digestion from whale nasal cartilage and bovine tracheal cartilage, respectively[8].

Analytical methods. — Hexosamine was determined by the Elson–Morgan reaction as modified by Gardell[9], or by using an amino acid analyzer (JEOL JLC-5AH), after hydrolysis in 3.0M hydrochloric acid for 15 h at 100°. Uronic acid was determined by the carbazole reaction[10], with D-glucurono-6,3-lactone as standard. Sulfate was determined by the method of Dodgson and Price[11]. Hexose was estimated by the anthrone reaction[12], with D-galactose as standard, and protein was determined by the method of Lowry *et al.*[13]. Amino acids were analyzed with an amino acid analyzer after hydrolysis in 6M hydrochloric acid for 22 h at 110° in a vacuum-sealed tube.

Gas–liquid chromatography. — For analysis of neutral sugars, the sample was hydrolyzed in 0.5M sulfuric acid for 4 h at 100° and neutralized with sodium hydroxide; arabinose was added as an internal standard, and the sample was passed through columns of AG-1 (X2, formate) and AG-50 (X8, H^+). The deionized hydrolyzate was trifluoroacetylated after reduction with sodium borohydride[14] and then analyzed by g.l.c. A Shimazu GC-1C gas chromatograph equipped with a glass column packed with 2% XF-1105 on Gas Chrom P (80–100 mesh) was used, with nitrogen (47 ml.min^{-1}) as the carrier gas at a column temperature of 140°.

For analysis of neutral sugars and alditol, after alkaline reduction, the sample was hydrolyzed, neutralized, arabinitol was added as an internal standard, and the sample was deionized as already described. This sample was acetylated with acetic anhydride–sodium acetate[15], and analyzed by g.l.c. with a Shimazu GC-4BM gas chromatograph equipped with a glass column packed with 3% ECNSS-M on Gas Chrom Q (80–100 mesh) with nitrogen (55 ml. min^{-1}) as the carrier gas at a column temperature of 190°.

Treatment with nitrous acid[16]. — The sample (25 mg) was dissolved in 1.5 ml of 5% sodium nitrite and mixed with 1.5 ml of 33% acetic acid. After 20 h at room temperature, the solution was diluted to 20 ml with water and dialyzed, and then the product was precipitated with ethanol in the presence of 5% calcium acetate and 0.5M acetic acid.

N-*Acetylation of amino-terminal groups.* — The sample (40 mg) was dissolved in 3 ml of 2.5M sodium acetate and mixed with 10 µl of acetic anhydride at the temperature of an ice bath[3]. After stirring for 10 min, the same amount of acetic anhydride was added and stirring was continued for 1 h, and then 25 ml of water was added to the mixture. After dialysis, the product was conventionally precipitated with ethanol as the calcium salt.

Blocking of carboxyl groups. — Free carboxyl groups were converted into amides by using EDC [3-(3-dimethylaminopropyl)1-ethyl-carbodiimide] essentially by the method of Danishefsky and Siskovic[17]: the sample (50 mg), EDC (50 mg)

and dimethyl L-glutamate hydrochloride (50 mg) were dissolved in water (1 ml) and the pH was adjusted to 4.7 with 0.1M hydrochloric acid. The mixture was stirred at room temperature, and the pH was maintained for 4 h at 4.7 by adding 0.1M hydrochloric acid. The mixture was diluted with 10 ml of water and dialyzed exhaustively. The polysaccharide derivative was recovered by precipitation with ethanol in the presence of calcium acetate and acetic acid.

Treatment with alkali and alkaline reduction. — A solution of the sample (5–10 mg/ml) was mixed with (*a*) 1.5M sodium hydroxide (0.5 ml), (*b*) 0.9M sodium borohydride in 1.2M sodium hydroxide (0.5 ml), (*c*) 3.0M sodium borohydride in 1.2M sodium hydroxide (0.5 ml), or (*d*) 1.2M sodium borohydride in 1.6M sodium hydroxide (0.5 ml) and 0.04M $PdCl_2 \cdot 2H_2O$ (0.5 ml). The mixtures were kept under nitrogen at different temperatures for various periods. The $PdCl_2 \cdot 2H_2O$ was prepared from commercial palladium chloride according to the procedure of Tanaka and Pigman[18].

Gel chromatography. — For isolation of the polysaccharide component after alkaline reduction, the mixture and washings were applied to a column (2.2 × 120 cm) of Sephadex G-75 that was eluted with M sodium chloride. Fractions showing a positive carbazole reaction were collected, and dialyzed, and the product was precipitated with ethanol.

RESULTS

Composition of chondroitin 4-sulfate–peptide. — The chondroitin 4-sulfate preparation obtained from whale cartilage by pronase digestion contained equimolar amounts of 2-amino-2-deoxy-D-galactose, D-glucuronic acid, and sulfate[8], together with small but significant proportions of protein and neutral sugars.

The amino acid and neutral-sugar compositions of the preparation are shown in Table I. The principal amino acids were serine, glycine, and glutamic acid in the molar ratio of 1.0:1.2:0.5. Analysis for neutral sugars showed the presence of xylose and galactose in a molar ratio of 1.0:2.0; The amount of xylose was approximately equivalent to that of serine.

Determination and protection of the amino-terminal amino acid. — After treatment with nitrous acid, analytical data showed that about 50% of the serine had been selectively decomposed (Table I), and the data indicated that the serine is located at the amino terminus.

When the product isolated after *N*-acetylation was treated with nitrous acid, there was no decomposition of the amino acid, showing that all of the amino groups were completely acetylated.

Optimal conditions for β-elimination–reduction of chondroitin 4-sulfate–peptide. — In the presence of reductant, treatment with alkali resulted in the formation of alanine and a selective decrease in serine, indicating that β-elimination–reduction occurred at the *O*-glycosyl linkage between serine and carbohydrate of chondroitin 4-sulfate from both whale cartilage and from bovine nasal cartilage[1]. The ease of decomposition

TABLE I

AMINO ACID AND NEUTRAL SUGAR COMPOSITION OF CHONDROITIN 4-SULFATE–PEPTIDE, BEFORE AND AFTER VARIOUS TREATMENTS

Component	*Before treatment (μmol/g)*	*After treatment with nitrous acid (μmol/g)*	*Re-isolated after alkaline reduction[a] (μmol/g)*
Asp	8.2	7.7	1.1
Thr	5.1	4.2	0
Ser	30.5	15.6	2.9
Glu	14.2	12.5	1.7
Pro	5.3	4.4	0
Gly	35.3	31.0	3.0
Ala	6.3	5.5	0.8
Leu	6.0	3.5	0
Tyr	2.1	tr[b]	0
Phe	2.2	2.1	tr[b]
Xylose	26.4		tr[b]
Xylitol	0		23.6
Galactose	52.3		53.8

[a] *N*-Acetylchondroitin 4-sulfate–peptide was used. [b] tr = trace.

TABLE II

β-ELIMINATION AND β-ELIMINATION–REDUCTION OF CHONDROITIN 4-SULFATE–PEPTIDE UNDER VARIOUS CONDITIONS

Sample	*Temp. (°)*	*Period (h)*	*Concn. of NaOH (M)*	*Reductant*	*Ser decomposed (%)*	*Net conversion of Ser to Ala (%)*
Chondroitin 4-sulfate–peptide	4	24	0.5	—	37	—
Chondroitin 4-sulfate–peptide	4	48	0.5	—	43	—
Chondroitin 4-sulfate–peptide	4	72	0.5	—	52	—
Chondroitin 4-sulfate–peptide	25	24	0.5	—	47	—
N-Ac-Chondroitin 4-sulfate–peptide	4	24	0.5	—	77	—
N-Ac-Chondroitin 4-sulfate–peptide	25	24	0.4	0.3M $NaBH_4$	77	32
N-Ac-Chondroitin 4-sulfate–peptide	25	24	0.4	1.0M $NaBH_4$	78	53
N-Ac-Chondroitin 4-sulfate–peptide	45	24	0.4	1.0M $NaBH_4$	79	50
N-Ac-Chondroitin 4-sulfate–peptide	25	24	0.4	0.3M $NaBH_4$ 0.01M $PdCl_2$	77	98
N-Ac-Chondroitin 4-sulfate–peptide	45	24	0.4		83	101
Chondroitin 4-sulfate–peptide–Glu	25	24	0.4		67	95
N-Ac-Chondroitin 4-sulfate–peptide–Glu	4	24	0.4	0.01M $PdCl_2$ 0.3M $NaBH_4$	87	95
N-Ac-Chondroitin 4-sulfate–peptide–Glu	25	24	0.4		90	98
N-Ac-Chondroitin 4-sulfate–peptide–Glu	45	24	0.4		89	97
Chondroitin 4-sulfate–peptide	25	24	0.4		47	101

of serine in the chondroitin 4-sulfate–peptide was influenced by the temperature of reaction, as shown in Table II.

It is noteworthy that *N*-acetylation of the chondroitin 4-sulfate–peptide caused a dramatic loss in serine, even during 24 h at 4°. In this case, the amount of serine decomposed increased by 77% (23.1 μmol/g), corresponding to about 90% of the xylose originally present.

The effects of reductants on β-elimination–reduction of *N*-acetylchondroitin 4-sulfate–peptide were studied under the various conditions shown in Table II. In the presence of 0.3M sodium borohydride alone, the formation of alanine corresponded to only 32% of the serine lost. Increasing the concentration of sodium borohydride to 1.0M brought about a greater conversion into alanine, of the serine lost up to 53%, whereas raising the temperature to 45° gave no significant increase in reduction.

When $PdCl_2 \cdot 2H_2O$ was added to 0.3M sodium borohydride in 0.4M sodium hydroxide at 25° for 24 h, the formation of alanine rose to equal the amount of serine decomposed.

After conversion of the free carboxyl groups of the chondroitin 4-sulfate–peptide into amides with dimethyl glutamate in the presence of EDC, the molar ratio of glutamic acid to 2-amino-2-deoxygalactose in the product was approximately 0.4:1.0. The proportion of alkali-sensitive serine residues increased from 47 to 67% during 24 h at 25°.

Substitution of both the amino and carboxyl groups caused the decomposition of about 90% (27 μmol/g) of the serine, which accounted for almost all of the xylose (26.7 μmol/g) present in the preparation. The β-elimination–reduction of this derivative at different temperatures showed that the amounts of serine decomposed are constant, and the conversion into alanine of the serine lost is usually >95%.

In order to determine the sugar component linked to serine, gas–liquid chromatography of neutral sugars as acetyl derivatives was performed, after treatment of *N*-acetylchondroitin 4-sulfate–peptide with 0.4M sodium hydroxide in the presence of 0.3M sodium borohydride and 0.01M $PdCl_2 \cdot 2H_2O$ for 24 h at 25°.

TABLE III

DIFFERENCES IN AMOUNTS OF AMINO ACIDS AND NEUTRAL SUGARS IN *N*-ACETYLCHONDROITIN 4-SULFATE–PEPTIDE BEFORE AND AFTER β-ELIMINATION–REDUCTION

Component	*Before treatment (μmol/g)*	*After treatment (μmol/g)*	*Difference (μmol/g)*
Ser	30.0	6.9	−23.1
Ala	6.1	28.7	+22.6
Xylose	26.4	tr	−26.4
Xylitol	0	23.8	+23.8
Galactose	52.3	54.0	+ 1.7

The results shown in Table III indicate that alkaline reduction resulted in the formation of xylitol as the only alditol, concomitant with loss of most of the xylose, whereas the galactose was not affected. The amount of xylitol formed corresponded to >90% of the xylose content.

In the present instance, the loss of serine was approximately equivalent to the gain of alanine or of xylitol.

Amino acid and neutral-sugar compositions of the polymer fraction re-isolated after β-elimination–reduction of N-*acetylchondroitin 4-sulfate–peptide.* — After treatment of *N*-acetylchondroitin 4-sulfate–peptide with 0.4M sodium hydroxide in the presence of 0.3M sodium borohydride and 0.01M $PdCl_2 \cdot 2H_2O$ for 24 h at 25°, the polymer fraction was recovered by gel filtration on Sephadex G-75. This fraction contained only 8% of the amino acids originally present, and did not contain an especially high proportion of any one amino acid (see Table I). On the other hand, the polymer fraction contained all the galactose and xylitol corresponding to the xylose originally present, and the analytical values (Table I) were in good agreement with the results (Table III) obtained by direct analysis of *N*-acetylchondroitin 4-sulfate–peptide after β-elimination–reduction, indicating that the galactose and xylitol residues were still linked to the carbohydrate chain of chondroitin 4-sulfate after alkaline reduction.

The carbohydrate–peptide linkage of chondroitin 4- and 6-sulfate hybrid–peptide. — The amino acid and neutral-sugar compositions of chondroitin 4- and 6-sulfate hybrid–peptide, containing equimolar amounts of the 4- and 6-sulfated 2-amino-2-deoxy-galactose residues, are shown in Table IV. The principal amino acids were

TABLE IV

AMINO ACIDS AND NEUTRAL SUGARS OF CHONDROITIN 4- AND 6-SULFATE HYBRID–PEPTIDE BEFORE AND AFTER β-ELIMINATION–REDUCTION

Component	*Before treatment (μmol/g)*	*After treatment (μmol/g)*
Asp	4.5	5.6
Thr	2.3	1.4
Ser	17.2	9.5
Glu	11.5	11.4
Pro	3.9	4.2
Gly	20.6	18.9
Ala	3.9	11.3
Val	2.5	tr
Leu	2.4	1.8
Phe	2.5	2.3
Xylose	15.6	8.1
Xylitol	0	7.8
Galactose	30.2	32.0

serine, glycine, and glutamic acid, and the only neutral sugars found were xylose and galactose, the molar ratio of Xyl:Gal:Ser being 1.0:1.9:1.1.

Alkaline reduction of the hybrid–peptide under the optimal conditions described here resulted in a decrease of serine by 45% of the amount originally present. In this case, the loss of serine was approximately equimolar to the loss of xylose and to the gain of alanine or of xylitol. On the other hand, the content of other amino acids and galactose remained unaffected.

After *N*-acetylation of the chondroitin 4- and 6-sulfate hybrid–peptide, the product showed an increase of alanine (12.8 μmol/g) with concurrent decomposition of 73% (12.6 μmol/g) of the serine, under the same conditions of alkaline reduction.

DISCUSSION

The chondroitin 4-sulfate–peptide preparation obtained after pronase digestion from whale cartilage contained a small but significant proportion of protein, and the molar ratio of serine, xylose, and galactose was approximately 1:1:2.

Alkaline treatment of this preparation resulted in decrease of only 47% of the serine. This would be as expected if the serine residue linked to the polysaccharide is at the amino- or carboxyl-terminal position.

By treatment with nitrous acid, about 50% of the serine in the preparation was selectively removed. After acetylation, the re-isolated product was unaffected by nitrous acid, indicating that the free amino groups had been completely substituted, and 77% of the serine was rendered labile to alkali. This amount corresponded to 90% of the xylose present.

Activation of free carboxyl groups in the chondroitin 4-sulfate–peptide with EDC, followed by their conversion into amides with glutamate dimethyl ester, would be expected to affect not only the free carboxyl groups of the serine but also those of the uronic acids in chondroitin 4-sulfate. The molar ratio of glutamate to 2-amino-2-deoxygalactose in the re-isolated derivative was 0.4, which was much higher than that reported for the chondroitin 4-sulfate–glycine derivative[17]. The decomposition of the serine by alkali increased from 47 to 67% by substitution of free carboxyl groups, indicating that some of the serine residues occupied the carboxyl-terminal position, but this substitution had less effect on β-elimination than did acetylation of the free amino groups of the serine in the chondroitin 4-sulfate–peptide preparation.

Substituting both the amino and carboxyl groups resulted in decomposition of about 90% of the serine, which corresponded to almost all of the xylose present. Thus the β-elimination of xylosylserine in the linkage region was completely achieved even after 24 h at 4°, although performing the reaction at a higher temperature or for a longer period would have been a better way to decompose the serine, had the amino or carboxyl groups of the serine been free.

The use of sodium borohydride with alkali was introduced by Schiffman *et al.*[19] for blood-group substances for isolation of reduced oligosaccharides, and Iyer and Carlson[20] established optimal conditions (0.05M sodium hydroxide in the presence

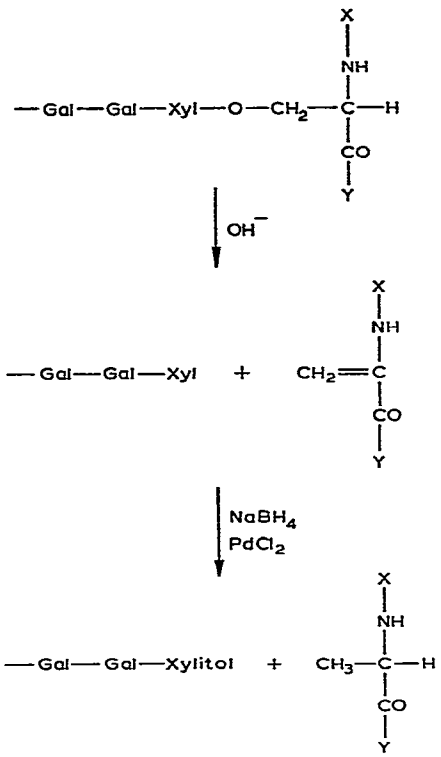

Fig. 1. Quantitative β-elimination–reduction of *O*-glycosyl linkages to serine in chondroitin 4-sulfate–peptide. When X was H, X was converted into acetyl. When Y was OH, Y was converted into amide with EDC and glutamate.

of M sodium borohydride, at 50°) for minimal degradation of the oligosaccharides released. A similar procedure, however, failed to demonstrate *O*-glycosyl linkages to threonine in bovine submaxillary mucin[21], and the method was improved by using $PdCl_2$ in sodium borohydride solution[18].

Quantitative reduction of dehydroalanine and xylose (resulting from β-elimination to alanine and xylitol) could not be achieved by sodium borohydride alone, even after increasing the concentration of the reductant to 1M and raising the temperature to 45°, and it was first accomplished by the addition of $PdCl_2$. These results indicate that the use of sodium borohydride alone is insufficient to bring about reduction, and that it is necessary to add $PdCl_2$ for quantitative β-elimination–reduction of the chondroitin 4-sulfate–peptide, as shown in Fig. 1.

The chondroitin 4- and 6-sulfate hybrid–peptide from bovine tracheal cartilage contained galactose and xylose in the molar ratio of 2:1. Alkaline reduction under the optimal conditions produced alanine in an amount equivalent to the decrease of serine. Consequently, it was proved that this hybrid is linked through an O-glycosyl bond between Gal-Gal-Xyl and serine to peptide, as in chondroitin 4- and 6-sulfates.

From these results, it is suggested that the polysaccharide–peptide linkage-

regions have the same structure in the chondroitin sulfates, regardless of differences in position of the sulfate groups.

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