Vol. 92, No. 2, 1980 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
January 29, 1980 Pages 618-623

PRELIMINARY CHARACTERIZATION OF A XYLOSE ACCEPTOR PREPARED BY HYDROGEN FLUORIDE TREATMENT OF PROTEOGLYCAN CORE PROTEIN

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Received December 10, 1979

SUMMARY

Polysaccharide was removed from rat chondrosarcoma chondroitin sulfate proteoglycan by hyaluronidase digestion followed by treatment with 70% polyhydrogen fluoride (HF) in pyridine or by the Smith procedure. Both methods were greater than 95% effective in removing carbohydrate. However, the nearly complete removal of carbohydrate by either method resulted in a 50-fold decrease in immunoreactivity as determined by a quantitative radioimmune inhibition assay, suggesting that some structural alteration may have occurred. In contrast, core protein deglycosylated by the HF procedure was a better xylosyltransferase acceptor than that prepared by the Smith procedure. HF-treated core protein exhibited a higher acceptor capacity (V_{max} approximately 2.5-fold that for the Smith-treated core protein) although the K_m values were similar.

INTRODUCTION

Core protein of chondroitin sulfate proteoglycan (CSPG)[2] remains the least well-characterized component of cartilage matrix. Following removal of polysaccharide chains, it is difficult to identify core protein or ascertain whether native properties have been altered. We have available an enzymatic method for identifying core protein by measuring xylosyltransferase acceptor activity, as well as immunological methods using antibodies to core protein. These latter procedures can be used not only for identification, but to a limited extent can also provide information on structural changes which may result from various treatments of core protein.

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[2]Abbreviations used are: CSPG, chondroitin sulfate proteoglycan; MES, 2-(N-morpholino)ethane sulfonic acid; PGSD, Smith-degraded chondroitin sulfate proteoglycan; PGHF, HF-deglycosylated chondroitin sulfate proteoglycan.

0006-291X/80/020618-06$01.00/0

In recent work from this laboratory, a chemical method has been devised for deglycosylating CSPG for structural studies (1). Similar procedures have been used to deglycosylate glycoproteins (2,3). In the studies reported here, the method is described for removing polysaccharide from chondroitin sulfate proteoglycan and preparing core protein with high xylosyltransferase acceptor activity.

MATERIALS AND METHODS

UDP-[^{14}C]Xylose (278 Ci/mol) and [^{125}I]sodium iodide (carrier free) were purchased from New England Nuclear. Additional reagents were obtained from the following sources: UDP-xylose, MES and 6-aminocaproic acid from Calbiochem; polyhydrogen fluoride (70%) in pyridine from PRC, Inc., Gainsville, FL; anisole from Aldrich Chemical Company; testicular hyaluronidase (20,000 units/mg) from Leo Helsingborg Laboratories, Sweden; Sephadex G-100 from Pharmacia; guanidinium hydrochloride and cesium chloride (both sequanal grade) from Pierce Chemical Company; and benzamide from Matheson, Coleman and Bell.

Xylosyltransferase[3] Assay. The enzyme was obtained from rat chondrosarcoma tumors which were grown in Sprague-Dawley rats as described previously (4). The 100,000x*g* supernatant, prepared from fluid obtained after soaking tumor minces overnight in 0.05 M MES buffer, pH 6.5, containing 0.25 M KCl, 3 mM $MnCl_2$ and 12 mM $MgCl_2$ (5), was used as the enzyme source in the experiments presented here. The protein concentration of this preparation was 4.6 mg/ml, as determined by the method of Lowry *et al.* (6). Enzyme activity was assayed according to Method 2a (7). Briefly, the assay mixture contained 2.5 µmol of KCl, 0.15 µmol of $MnCl_2$, 0.6 µmol of $MgCl_2$, 0.75 µmol of KF, 1.5 nmol of UDP-[^{14}C]xylose (specific activity approximately 30 mCi/mmol), 230 µg of enzyme protein and various amounts of xylose acceptor (PGHF or PGSD) in a total volume of 80 µl. The mixtures were incubated for 60 min at 37°C, bovine serum albumin was added, and protein was precipitated with cold 10% trichloroacetic acid/4% phosphotungstic acid. Precipitated protein was recovered by centrifugation, washed three times with 5% trichloroacetic acid and dissolved in 0.1 ml of 1 N NaOH for liquid scintillation counting.

Preparation of Core Protein. CSPG was extracted from rat chondrosarcoma and purified through associative and dissociative gradients as described previously (8). PGSD was prepared directly from CSPG using the method of Baker *et al.* (7,9). CSPG for HF deglycosylation and for antigen preparation was treated with testicular hyaluronidase (10).

Deglycosylation of CSPG by HF in Pyridine. Thoroughly dry, hyaluronidase-treated CSPG (75-100 mg) was placed in a polyethylene vial, 1 ml of anisole scavenger (2) and 10 ml of 70% polyhydrogen fluoride in pyridine were added. The mixture was stirred for 8 hr at room temperature, followed by dilution with a large volume of water (ca. 100 ml) and extensive dialysis against water. The sample was concentrated and applied to a Sephadex G-100 column (2.2 x 120 cm) which was equilibrated and eluted with 0.2 M NaCl containing 0.02% sodium azide. The material in the excluded volume was pooled, dialyzed and lyophilized. This

[3]Xylosyltransferase refers to UDP-D-xylose:core protein β-D-xylosyltransferase. The activity of this enzyme is expressed as nmol [^{14}C]xylose incorporated into acceptor core protein.

material is referred to as PGHF. Since anhydrous HF and hydrofluoric acid are corrosive agents, precautions were taken to prevent inhalation and contact with skin and eyes, and the reagent was kept in polyethylene or teflon containers until neutralized.

Carbohydrate Analysis. Samples of PGHF, PGSD and hyaluronidase-treated CSPG were analyzed for carbohydrate as previously described (11). The sample (400-800 μg) was hydrolyzed with methanolic HCl, polytrimethylsilyl ether derivatives prepared and the sugars quantitated by gas-liquid chromatography on an OV-1 column using D-mannitol as an internal standard.

Radioimmune Assay for Core Protein. The hyaluronidase-treated rat chondrosarcoma CSPG was used as antigen to elicit antibodies in rabbits by an immunization procedure similar to that developed for chick CSPG (10). A radioactive antigen was prepared by labeling hyaluronidase-digested CSPG with [^{125}I] sodium iodide using the chloramine-T procedure (12). The radioimmune assay system utilized the ammonium sulfate technique of Farr (13), and the percentage of antibody-bound radioactivity and inhibition were calculated as described previously (10).

RESULTS

In the present study, polysaccharide was removed from rat chondrosarcoma chondroitin sulfate proteoglycan by hyaluronidase digestion followed by treatment with 70% HF in pyridine or by the previously described Smith procedure (7,9). As shown in Table I, the extent of chondroitin sulfate removal, as estimated by gas-liquid chromatography in terms of residual xylose, was greater than 95% effective and comparable for the two procedures. However, PGHF was

TABLE I

Characterization of Deglycosylated Proteoglycan

	CSPG[1]	PGHF	PGSD
Xylosyltransferase acceptor activity[2] (nmol/hr/mg acceptor/ mg enzyme)	0.17	7.3	3.6
Xylose content (μg/mg protein)	51	3	4
Radioimmune response (μg/50% inhibition)	2	115	100

[1]Hyaluronidase-digested chondroitin sulfate proteoglycan.

[2]Average of three separate acceptor preparations using 200 μg of acceptor per assay.

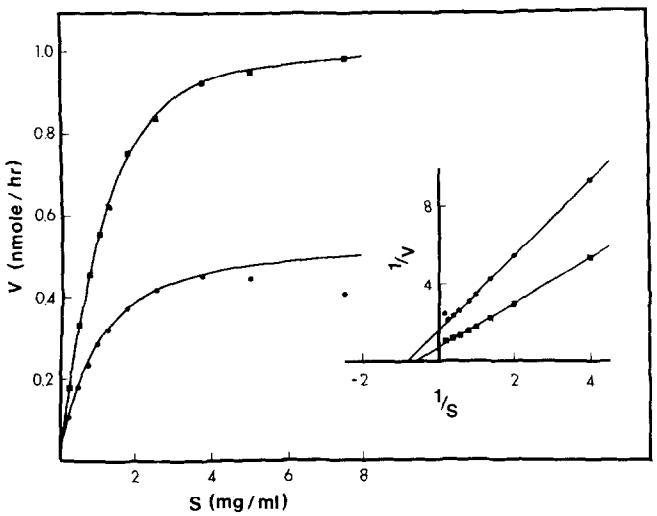

Fig. 1. Effect of acceptor concentration on the xylosyltransferase reaction. Conditions are as described in the text. (■-■, PGHF; ●-●, PGSD) Incorporation into endogenous acceptors was 0.011 nmol/hr and has been subtracted to give the values shown above. All values represent the average of two determinations.

found to be a significantly better xylose acceptor than PGSD, while the hyaluronidase-treated CSPG exhibited no acceptor activity.

The effect of PGHF and PGSD concentration on the xylosyltransferase reaction was examined and is shown in Fig. 1. Apparent K_m values of 1.85±0.11 mg/ml for PGHF and 1.15±0.05 mg/ml for PGSD were calculated using a BASIC program to fit the data to a rectangular hyperbolic function by a least squares procedure that assumes the velocities to have equal variances over the experimenta range. The V_{max} values were 1.57±0.06 nmol/hr for PGHF and 0.61±0.01 nmol/hr for PGSD, indicating that PGHF has a higher acceptor capacity than PGSD, although the K_m value is somewhat greater for PGSD. This finding may reflect an increase in accessibility of acceptor serine residues following HF treatment even though both procedures yield comparable reductions in xylose content.

In additional studies, the level of each core protein preparation required to yield 50% inhibition in a quantitative radioimmune assay with antisera prepared against hyaluronidase-digested proteoglycan was determined (Table I). Both deglycosylation procedures appear to result in some alteration of structure, as suggested by the 50-fold decrease in immunoreactivity.

DISCUSSION

Preparation of acceptor substrates for xylosyltransferase for enzymatic studies as well as structural and amino acid sequence studies on core protein require the prior deglycosylation of CSPG. In the past this was accomplished by removal of the chondroitin sulfate chains by periodate oxidation followed by acid cleavage (Smith procedure). Here we present evidence that treatment of CSPG with polyhydrogen fluoride in pyridine also results in complete removal of chondroitin sulfate chains, and furthermore, is simpler and more efficient than the Smith procedure.

An interesting finding of these studies is the difference in the kinetic properties of xylosyltransferase with the two acceptors. The PGHF yielded a V_{max} value approximately two and one-half times that with PGSD, while the K_m value was slightly lower with PGSD than with PGHF. Thus, xylosyltransferase may have an enhanced affinity for PGSD, although the catalytic efficiency of the enzyme is greater with PGHF. An increase in accessibility of acceptor serine residues may result from a conformational change induced in the core protein by HF treatment, rather than carbohydrate removal *per se*. Alternatively, a cleavage may have occurred during HF treatment of CSPG, yielding a portion of core protein more enriched in acceptor serine residues. Previous studies (7,9) have also shown that the K_m increases with reduction in size of protein acceptor. Studies currently are in progress to determine the size and composition of acceptor proteins obtained by HF-deglycosylation and Smith degradation.

These results suggest that HF-pyridine treatment is a promising procedure for deglycosylating proteoglycan for structural studies as well as preparing a xylosyltransferase core protein substrate with high xylose acceptor activity. Furthermore, this procedure may be used to prepare protein acceptors for other glycosyltransferases.

ACKNOWLEDGEMENTS

This work was supported by USPHS Grants AM-19622, HD-04583, Research Career Development Award K04-AM00603, and Established Investigatorship of the American Heart Association and training grants GM-07197 (C.C.) and GM-07183 (L.P.).

The authors wish to thank Dr. Glyn Dawson for performing the carbohydrate analyses and Dr. John Westley for the computer program used to determine kinetic values and for helpful discussion during the preparation of this manuscript.

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