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A simple method for the separation of glucosaminoglycans and galactosaminoglycans

Macroscale procedures used for the fractionation of glycosaminoglycans of other connective tissues are not suitable for the separation of corneal glucosaminoglycans and galactosaminoglycans, especially in experiments in which only a very small amount of tissue is available for analysis. Fractionation methods involving more procedure steps are not only too complicated to be performed on a microscale but affect the overall recovery of glycosaminoglycans in the sample analysed.

The use of cetylpyridinium chloride (CPC) for the fractionation of glycosaminoglycans reduces the procedure to a single step. The technique was developed by SCOTT[1] and has since been applied, with several modifications, to the fractionation of connective tissue glycosaminoglycans[2-6]. The method is based on the stepwise elution of the CPC-glycosaminoglycan complex from a cellulose column with a set of specific eluants. In this paper a very simple modification of the method is described which permits the fractionation of glycosaminoglycans from milligram quantities of corneal tissue.

Material and methods

A piece of the fresh cornea, weighing 10 to 20 mg, was dehydrated in 10 ml of dry acetone overnight and then dried *in vacuo* at 60° to constant weight. Per 1 mg of the dry weight were added 25 to 50 μl of 0.05 *M* Tris–HCl buffer (pH 7.2), containing cysteine hydrochloride and disodium ethylenediaminotetraacetate both in 0.005 *M* concentration, and 1 μl of the papain suspension (3.4.4.10, PAP 5607, Worthington Biochemical Corp., N.J., U.S.A.; content of the protein *ca.* 20 mg per ml). The mixture was incubated at 65° for 6 h.

Columns, size 4 × 40 mm, were prepared from cellulose powder Whatman CF 11 by the free sedimentation of a cellulose suspension in water, and washed with 5 ml of 1 % CPC before fractionation. Cellulose powder was purified before use as described by ŠVEJCAR AND ROBERTSON[5]. 100 μl of the digest were applied to the top of the column and washed into the cellulose with 0.1 ml of 1 % CPC. The elution started with 1 ml of 1 % CPC, followed by 1 ml of 0.05 % CPC, and 1 ml of 1.5 *M* KCl in 0.05 % CPC. The eluants were taken separately and directly into centrifuge tubes provided with tight stoppers. Kimax test tubes, size 10 × 100 mm, with teflon lined screw caps, were found to be very suitable for this purpose. The 1 % CPC and 0.05 % CPC fractions were mixed with three volumes of cold-saturated sodium acetate solution in 95 % ethanol, and left in a refrigerator overnight. The 1.5 *M* KCl fractions were mixed with 0.5 ml of 1 % CPC and 3 ml of water, and left at room temperature for 1 h. In all cases, the reprecipitated glycosaminoglycans were collected by centrifugation at 3000 r.p.m. for 1 h.

The supernatants were removed carefully and the precipitates were drained free of excess fluid. The sediments were hydrolysed with 0.5 ml of 8 *N* HCl in closed test tubes at 95–100° for 3 h. The hydrolysates were evaporated to dryness at 40° in a vacuum desiccator connected to an oil pump *via* a column filled with sodium hydroxide pellets and silica gel. 1 ml of water and 0.5 ml of acetylacetone reagent

(a freshly prepared solution of 0.5 ml of acetylacetone, analytical grade, in 25 ml of 0.5 *N* sodium carbonate) was added to each test tube. After mixing, the closed test tubes were placed in a boiling water bath for 20 min and cooled. 2.5 ml of ethanol and 0.5 ml of Ehrlich reagent (0.8 g of *p*-dimethylaminobenzaldehyde dissolved in 30 ml of ethanol and 30 ml of 37 % HCl added) was added to the contents of the test tubes. The mixture was warmed at 60° for 5 min. The colour developed was measured photocolorimetrically at 530 nm. Standards containing 10 and 20 μg of glucosamine, respectively, in 1 ml of water, and a blank (1 ml of water) were treated in a similar way.

The separation of glucosamine and galactosamine on Dowex 50 exchange resin was carried out according to the method of ANTONOPOULOS[7]. Total hexosamine in the papain digest of corneal tissue was determined as described by BOAS[8].

Results and discussion

By means of the technique described, several parallel fractionations of bovine corneal glycosaminoglycans were performed in order to demonstrate the reproducibility of the procedure. The results obtained are shown in Table I. It is evident

TABLE I

RECOVERY OF HEXOSAMINES FROM BOVINE CORNEAL GLYCOSAMINOGLYCANS AFTER ELUTION FROM A MICROCOLUMN

An amount of papain digest containing a total of 24.4 μg of hexosamine (determined according to BOAS[8]) was applied to the column in each experiment.

Experiment No.	*Hexosamines (μg per fraction)*			*Recovery (%)*
	1% CPC	*0.05% CPC*	*1.5 M KCl–0.05% CPC*	
1	15.3	0.3	8.3	98.8
2	12.5	0.4	8.9	89.3
3	14.6	0.0	11.3	107.0
4	12.5	0.2	8.3	87.0
5	13.9	0.0	11.1	103.2
6	13.9	0.1	9.0	95.3
7	13.3	0.0	9.0	92.2
8	13.9	0.0	9.4	96.4
Mean ± S.E.	13.7 ± 0.4	0.1 ± 0.0	9.4 ± 0.4	94.9 ± 2.4
% of total hexosamine eluted	59.1	0.4	40.5	
% of glucosamine[a]	100.0	—	9.1	
% of galactosamine[a]	0.0	—	90.9	

[a] Values based on Dowex 50 ion-exchange resin column chromatography.

that the hexosamine is present mainly in the 1 % CPC and 1.5 *M* KCl–0.05 % CPC fractions. The middle fractions eluted with 0.05 % CPC contain a trace of hexosamine only. The recovery of hexosamine was about 95 % on average. The purity of the peak fractions was verified by subsequent fractionation of the hexosamines on Dowex 50 exchange resin. It was found that the hexosamine of the 1 % CPC fraction is pure glucosamine, and the hexosamine of the 1.5 *M* KCl–0.05 % CPC fraction predominantly galactosamine.

The microfractionation technique described allows the separation of small

amounts of corneal glycosaminoglycans into glucosaminoglycan and galactosaminoglycan fractions with a satisfactory reproducibility and recovery of the polysaccharides analysed. The method is very satisfactory for the analysis of small quantities of corneal tissue. The chemical composition of the glycosaminoglycans in the two principal fractions is very similar to that found by other authors for bovine corneal glycosaminoglycans[9,10]. The minor amount of glucosamine in the galactosaminoglycan fraction can be explained by existence of certain forms of glucosaminoglycan in the bovine cornea which cannot be separated by the CPC technique from galactosaminoglycan. The glucosamine portion varies a little from sample to sample. The precipitation of glycosaminoglycans of the 1% CPC fraction at the outset is a very important purification step as it removes the remainder of glycoproteins and proteins, present in the papain digest, which are soluble in the supernatant.

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