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Separation of Mucopolysaccharides on a Cellulose Column

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A column technique is described for the separation of mucopolysaccharides by fractional elution with alcohol.

The method has been applied on amounts from 10 mg up to 4 g of material.

The separation of mucopolysaccharides from nucleus pulposus and from cornea as well as the separation of chondroitinsulfuric acid and heparin monosulfuric acid is described.

Fractional precipitation from an aqueous solution with organic solvents is the most commonly used technique for the separation of the components of a mixture of polysaccharides. Sometimes clearcut separations can be obtained by varying the pH and the ionic strength and by adding metal ions such as calcium, barium or lead. In the majority of cases, however, and especially if the polysaccharides are closely related, the separation of the different types of polysaccharides is a tedious procedure. In spite of repeated precipitations and washings it is seldom possible to achieve complete separations. Moreover, the fractional precipitation methods can only be applied on a rather large scale, a drawback, which makes such methods of very limited value in metabolic studies in which frequently minute amounts of material have to be separated in pure form.

In such cases electrophoretic methods have been used (Boström and Gardell [1], Dorfman *et al.*[2]). However, these methods can be used only in such cases where a distinct difference in electrophoretic mobility exists, *e. g.* between hyaluronic acid and chondroitinsulfuric acid.

The use of partition methods is restricted mainly because of lack of suitable pairs of liquids. In a few cases this difficulty has been overcome by the use of "carriers" such as fatty amines [3–5]. The paperchromatographic separation of heparin and chondroitinsulfuric acid has also been reported [6].

Fractionation can also be obtained by a column technique, in which the column is used mainly as a carrier for the material to be separated. These are selectively brought into solution and eluted from the column by stepwise or

gradual changes in the composition of the eluting solvent. This principle has been used by Desreux and Ghauysen [7] for the separation of nucleic acids and by Zahn and Stahl [8] and recently by Zittle and Della Monica [9] for the separation of proteins.

In this work closely related acid mucopolysaccharides have been separated on a cellulose column by means of fractional or gradient elution. Ethanol of varying concentrations together with barium acetate has been used as the eluting solvent. The method has been applied on a micro scale as well as on preparative scale.

EXPERIMENTAL

Preparation of the column. Columns of different sizes are prepared from Whatman cellulose powder *. A suitable quantity of the powder for the preparation of a 20 cm long column is mixed with water to a rather thin slurry. This mixture is poured into a suction flask care being taken that the flask is not filled to more than one fifth of its total volume. The flask is connected to a water pump and gently rotated during evacuation. In this way most of the air present in the cellulose is removed, a procedure which greatly facilitates the even packing of the column.

The slurry is then poured into a chromatographic tube and allowed to settle to constant height. About one bed volume of water is then passed through the column with air pressure of 30—40 cm Hg. After washing with water under hydrostatic pressure of about 2 m until no carbohydrate can be detected in the effluent, the column is equilibrated with 80 % ethanol containing 0.3 % barium acetate.

Preparation of the sample. The polysaccharide mixture to be separated is dissolved in a 0.3 % aqueous solution of barium acetate. A solution containing about 10 % of the polysaccharide mixture is prepared.

Addition of the sample to the column. About 0.5 ml of the polysaccharide solution per cm^2 of cross section area of the column is added on top of the latter and allowed to drain in slowly. The walls of the tube are then washed with a small amount of 0.3 % barium acetate.

Elution of the material. The elution of the material from the column can be accomplished either by the use of ethanol in stepwise decreasing concentrations or by an arrangement where it is possible to decrease the ethanol concentration continuously. In the latter procedure the effluent is collected by means of a suitable fraction collector.

In both cases the column is first washed with at least three bed volumes of 80 % ethanol containing 0.3 % barium acetate in order to ascertain complete precipitation of the material on top of the column. With this washing little or no carbohydrates appears in the effluent.

In order to find the best conditions for a stepwise elution, *i. e.* the concentration of alcohol and the volume in each step, it is advisable to make a preliminary run. In such a run a suitable difference in ethanol concentration in the elution solvents between each step would be 5 %. A volume of three to four bed volumes is sufficient.

In the case of a continuous decrease of the ethanol concentration a suitable gradient is also chosen on the basis of a preliminary run either stepwise or made with an arbitrarily chosen gradient.

The concentrations and volumes as well as the concentration gradients used in actual experiments are given below.

The flow rate through the column must not exceed 5—10 ml per h per cm^2 cross section area of the column.

Analyses of the fractions. The fractions can be treated according to the purpose of the experiment. In general a qualitative or quantitative carbohydrate reaction is performed on the fractions.

* Whatman cellulose powder standard grade obtained from W. and R. Balston Ltd, England.

In the present investigation the carbazole [10] and the cysteine [11] reactions of Dische were carried out. Column chromatography of aminosugars [12] and neutral monosaccharides [13] were also used.

RESULTS AND DISCUSSION

The fractionation of the polysaccharides from nucleus pulposus by means of stepwise elution

The polysaccharides were prepared as described earlier [14,15]. Fifty mg of the polysaccharide preparation was dissolved in about 0.5 ml of 0.3 % barium acetate solution and applied on top of a column 1 × 20 cm prepared according to the principles given above. The polysaccharides were then eluted with solutions of decreasing alcohol concentrations. All solvents contained 0.3 % barium acetate. Fifty ml of each solution was used. The composition of the elution solvents as well as the aminosugar analysis of the fractions are given in Table 1.

Table 1. Fractionation of the nucleus pulposus polysaccharides by means of stepwise elution.

Per cent EtOH in elution solvent	Glucosamine-HCl			Galactosamine-HCl		
	mg	Per cent of total hexosamine	Per cent recovery	mg	Per cent of total hexosamine	Per cent recovery
80	0.0	0	0	0.0	0	0
50	6.02	99.2	10.2	0.05	0.8	0.6
45	17.30	95.4	29.3	0.84	4.6	1.0
40	12.9	92.6	21.9	1.03	7.4	1.3
35	4.82	89.1	8.2	0.59	10.9	0.7
30	4.70	81.0	8.0	1.10	19.0	1.3
25	1.60	50.0	2.7	1.60	50.0	2.0
20	0.82	16.1	1.4	4.26	83.9	5.2
15	0.60	3.3	1.0	17.80	96.7	21.8
10	0.60	2.2	1.0	27.08	97.8	33.2

In another experiment 4 g of the same material was fractionated on a column 6.5 × 23 cm. The elution was carried out with 80, 50, 35, 25 and 20 % of ethanol containing 0.3 % of barium acetate, and finally with 0.3 % barium acetate solution. Two litres of each of the solvents was used. The fraction eluted with 35 % ethanol and that eluted with barium acetate solution were concentrated *in vacuo* to about 50 ml each, made faintly acid with acetic acid and precipitated with alcohol. The precipitates were dissolved in 50 ml of water and reprecipitated with alcohol. The analysis of these fractions are given in Table 2.

From these experiments it is evident that the mucopolysaccharides from nucleus pulposus can be fractionated into at least two components, one appear-

Table 2. Analysis of the two main fractions from the nucleus pulposus polysaccharides (air dry substance).

	Fraction I eluted with 35 % ethanol	Fraction II eluted with barium acetate solution
Weight	1.33 g	2.14 g
Ash	24.3 %	31.9 %
Nitrogen	2.60 »	2.00 »
Micro Kjeldahl		
Sulfur in ester sulfates	5.45	4.83
Glucosamine-HCl	21.1 » (59.1 of the total glucosamine)	0.3 »
Galactosamine-HCl	0.3 »	22.8 » (82.4 % of the total galactosamine)
Galactose	16.8 »	—
Fucose	2.3 »	—

ing at an ethanol concentration of about 10—15 %. Most of the glucosamine was found in the first component and correspondingly most of the galactosamine was found in the second component.

Fractionation of the polysaccharides from nucleus pulposus by means of gradient elution

The arrangement used in order to obtain the concentration gradient was described by Lindberg and Wickberg [16].

A column 1 × 20 cm was used, prepared as described above. After the application of the polysaccharide mixture (145.5 mg dissolved in about 1.5 ml of the barium acetate solution) the column was first washed with 60 ml of 80 % ethanol containing barium acetate in the concentration described above. The column was then connected to the gradient arrangement. Vessel I, closest to the column, was filled with 350 ml of 80 % ethanol containing 0.3 % barium acetate, and vessel II was filled with 0.3 % barium acetate solution alone to hydrostatic equilibrium with the solvent in vessel I. The ratio between cross section area of the vessels was vessel I / vessel II = 2.7. The effluent was collected in 5—7 ml fractions. Each fraction was analysed with Dische's carbazole method as modified by Gurin and Hood [10] and by the cysteine method of Dische and Shettles [11]. 0.5 ml aliquots were used for each test.

In Fig. 1 the results of these analyses are plotted against the fraction numbers. The cysteine reaction showed a peak for hexoses and methyl pentoses with a maximum in fraction 25 and after this peak practically no colour was found. The carbazole reaction, on the other hand, showed several peaks one of which coincides with the peak given by the cysteine reaction. In the later part of the curve a large unsymmetrical peak, probably composed of several small peaks, is visible.

It is claimed [11] that the cysteine reaction is specific for hexoses and methyl pentoses, and that no colour is given by uronic acids. The carbazole reaction on the other hand is known to give much stronger colour for uronic acids than

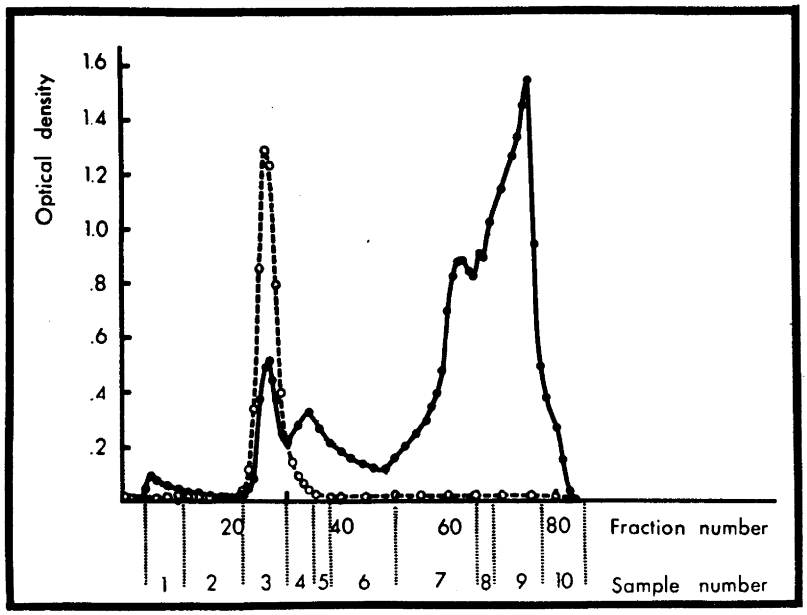

Fig. 1. Gradient elution from a cellulose column 1 × 20 cm of 145.5 mg of the polysaccharide mixture from nucleus pulposus. The fractions are analysed with the cysteine method of Dische and Shettels [11] and the carbazol method as modified by Gurin and Hood [10]. The fractions are pooled in ten samples as shown by the dotted lines.

for the hexoses. It is thus evident that in the peak given by the cysteine reaction a polysaccharide containing hexose — and methyl pentose — is present whilst the other peaks indicate uronic acid-containing substances.

The fractions were pooled as indicated in the figure and each of the ten samples thus obtained were evaporated to dryness and dissolved in 2 ml of water. 0.5 ml of this solution was hydrolyzed with an equal volume of concentrated hydrochloric acid for 8 h on a boiling water bath and the aminosugars determined as described by Gardell [12]. The result is given in Table 3.

Although two main components can be distinguished, the purity of the components obtained by gradient elution is not the same as that obtained by stepwise elution.

In the method of Zahn and Stahl [8] and in that of Zittle and Della Monica [9], the mixture to be separated was precipitated on the supporting medium suspended in the precipitating solvent. As this principle might give better conditions for the separation it was also tested in this case. Five g of Hyflo-Super Cel or cellulose powder was suspended in 50 ml of 80 % ethanol containing 0.3 % barium acetate, and 50 mg of the nucleus pulposus polysaccharides dissolved in about 5 ml of 0.3 % barium acetate solution was added. After thorough mixing the mixture was poured on a glass filter funnel and eluted with the aid of the same gradient as used above. In both cases the separation was very poor.

Table 3. Aminosugar analysis of the fractions obtained by gradient elution of the nucleus pulposus polysaccharides. The fractions are pooled as indicated in Fig. 1.

Sample	Glucosamine		Galactosamine	
	mg	Per cent of total hexosamine	mg	Per cent of total hexosamine
1	0		0	
2	trace		0	
3	9.05	91.1	0.88	8.9
4	1.18	89.4	0.14	10.6
5	0.31	88.6	0.04	11.4
6	0.37	36.6	0.64	63.4
7	0.36	5.8	5.86	94.2
8	0.06	1.8	3.25	98.2
9	0.19	2.6	7.21	97.4
10	trace	—	1.40	98—100

Table 4. Amino sugar analysis of the fractions obtained by fractional precipitation of the nucleus pulposus polysaccharides (analysis of air dry substances).

Fraction number	Per cent ethanol	Weight mg	Glucosamine-HCl			Galactosamine-HCl		
			Per cent of substance	Per cent of total hexosamine	Per cent recovery	Per cent of substance	Per cent of total hexosamine	Per cent recovery
1	11	21.5	1.5	6.0	0.5	23.6	94.0	6.1
2	14	144.1	0.7	2.8	1.7	24.5	97.2	42.4
3	20	19.6	2.9	9.5	1.0	27.7	90.5	6.5
4	28	26.4	20.8	86.3	9.1	3.6	14.7	1.2
5	33	62.8	18.9	86.3	19.7	3.0	14.7	2.3
6	37	21.2	22.1	89.5	7.8	2.6	10.5	0.7
7	45	28.0	20.4	89.5	9.5	2.4	10.5	0.8
8	10 volumes of ethanol	11.7	25.0	92.9	4.9	1.9	7.1	0.2

Fractional precipitation of the nucleus pulposus polysaccharides

In order to compare the efficiency of the column technique with that of the conventional fractional precipitation methods an alcohol fractionation of the nucleus pulposus polysaccharides was performed.

511 mg of the polysaccharide fraction was dissolved in 50 ml of a 3 % solution of barium acetate and fractionated at $\pm$ 0 °C with ethanol. The solution to be fractionated was constantly stirred and the alcohol was added very slowly until a turbidity appeared. The stirring was then continued for half an hour and the precipitate was then centrifuged in a Spinco centrifuge at 30 000 *g*, washed and dissolved in water and reprecipitated with several

volumes of alcohol from a faintly acid solution. The supernatant from the high speed centrifugation and the washings were combined and the fractionation continued on this solution. The result of the fractionation is given in Table 4.

Although it is possible to obtain the galactosamine containing portion in a rather pure state the glucosamine-containing one is not obtained with the same purity as with the column technique. The yield of the pure fractions is also much smaller.

Fractionation of a polysaccharide mixture from bovine cornea

As the cornea is known to contain different mucopolysaccharides [17–19] a fractionation experiment was also carried out on a polysaccharide fraction from this tissue.

The polysaccharides were prepared by proteolytic digestion of bovine corneas followed by extraction with 90 % phenol as described earlier [14,15]. A preparation was obtained which contained 2.99 % N and 18.3 and 13.2 % of glucosamine and galactosamine, respectively, calculated as the hydrochlorides. This polysaccharide was fractionated on a micro scale.

11.2 mg was dissolved in about 0.15 ml of the 0.3 % barium acetate solution and fractionated on a column 0.5 × 7 cm. The carbohydrate content of the fractions was determined by the cysteine reaction [11] and by the carbazole [10] reaction. 0.5 ml aliquots were taken for each determination. The rest of the fractions were evaporated to dryness and hydrolyzed with 0.5 ml of 6 N hydrochloric acid for the determination of their aminosugar composition. The result of the fractionation is given in Table 5.

Table 5. Analysis of the fractions obtained by stepwise elution of the polysaccharide mixture from bovine cornea. 6 ml of each solvent was used. D_{396} and D_{426} represent the optical densities given by the fractions in the cysteine reaction [11] at 396 mμ and 426 mμ, respectively. D_{530} represents the optical density given by the carbazole reaction [10] at 530 mμ.

Per cent EtOH in elution solvent	D_{396}	D_{426}	$D_{396} - D_{426}$	D_{530}	Glucosamine-HCl in per cent of total hexosamine	Galactosamine-HCl in per cent of total hexosamine
80	0.142	0.112	0.030	0.026	—	—
60	0.114	0.093	0.021	0.008	?	?
50	0.260	0.225	0.035	0.032	100	trace
45	0.235	0.206	0.029	0.052	96.4	3.6
40	0.290	0.255	0.035	0.071	87.1	12.9
35	0.105	0.082	0.023	0.342	71.9	28.1
30	0.033	0.019	0.014	0.242	6.8	93.2
25	0.013	0.005	0.008	0.118	trace	100
20	0.016	0.007	0.009	0.100	8.6	91.4
15	0.014	0.006	0.008	0.098	9.3	90.7
10	0.006	0.002	0.004	0.021	trace	trace
5	—	—	—	—	—	—
0	—	—	—	—	—	—

Fractionation of chondroitinsulfuric acid and heparin monosulfuric acid

These two polysaccharides are isomers with almost the same electrophoretic mobility and can be fractionated by means of fractional precipitation of their barium salts with alcohol [20]. They can also be completely separated using the column technique described above.

10 mg of heparin monosulfuric acid prepared from ox lung and 10 mg of chondroitinsulfuric acid from costal cartilage prepared according to Boström and Månsson [21] were mixed and the mixture was fractionated on a column 1 × 20 cm. In the fraction eluted with 20 % ethanol only galactosamine was found and correspondingly only glucosamine was found in the fraction eluted with 10 per cent ethanol.

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