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PREPARATION AND FRACTIONATION OF MUCOPOLYSACCHARIDES

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Abstract—Techniques are described for the preparation and fractionation of polysaccharides. The breakdown of protein in connective tissues for the release of mucopolysaccharides is best accomplished by the use of proteolytic enzymes. Good recovery of polysaccharides is obtained with this method. Subsequent to proteolytic digestion of tissue, polysaccharides can be recovered from a mixture of amino acids, salts, fats and nucleic acids by subjecting the mixture to successive treatments with alkaline and acid alcohol followed by phenol extraction. Quaternary ammonium compounds may be used prior to alcohol precipitation. Polysaccharide mixtures can be fractionated by zone electrophoresis. The use of chromatographic procedures and of precipitation with different salt concentrations in the presence of quaternary ammonium compounds is also described.

THE connective tissue is made up of fibrous elements, cells and an amorphous intercellular substance generally called the ground substance. The ground substance contains polysaccharides.

In Table 1 the polysaccharides isolated from connective tissue are listed.

TABLE 1.

Polysaccharide	Monosaccharide composition
Non-sulphated	
Hyaluronic acid	N-acetylglucosamine glucuronic acid
Chondroitin	N-acetyl-galactosamine, glucuronic acid
Sulphated	
Chondroitinsulphate A	N-acetylgalactosamine, glucuronic acid
Chondroitinsulphate B	N-acetyl-galactosamine, iduronic acid
Chondroitinsulphate C	N-acetyl galactosamine, glucuronic acid
Heparitinsulphate	Glucosamine, glucuronic acid
Keratosulphate	N-acetylglucosamine, galactose

In order to determine the nature and the amount of mucopolysaccharides in a tissue specimen two main principles can be used:

(1) Direct acid hydrolysis of the tissue and analysis of the resulting mixture of monosaccharides.

(2) Extraction and fractionation of the polysaccharides and determination of their chemical and physical properties.

The first method does not give any detailed information regarding the polysaccharide pattern in the tissue. Several polysaccharides may exist with all or some of

their building stones in common. The second method will give a more detailed picture of the polysaccharide pattern of the tissue, but being more complicated it is liable to larger errors.

The ideal method for preparation and fractionation of the polysaccharides should allow all polysaccharides to be extracted quantitatively from any tissue and to be quantitatively separated into clear-cut fractions, each consisting of a single chemically and physically individual polysaccharide. Such a method has not to my knowledge been worked out yet.

Most of our knowledge of the polysaccharide pattern derives from the combination of the results obtained by different authors using different methods.

EXTRACTION OF POLYSACCHARIDES FROM TISSUES

At the beginning of this century extraction with alkali was the general method used for the isolation of the polysaccharides from tissues. This method was later on used in most of the early work done by the leading schools in this field like those of P. A. Levene, Karl Meyer, Erik Jorpes and H. Masamune.

The greatest disadvantage in the alkaline extraction is the possibility of degradation of the polysaccharide molecules. Moreover the possibility exists that not all polysaccharides are extracted.

Blix and Snellman[1] showed that the physical properties of CSA* prepared by the alkaline extraction method are different from those given by the polysaccharide extracted with milder methods. On the other hand the possibility exists that the larger molecules obtained by milder methods are complexes between proteins or peptides which cannot be regarded as part of the polysaccharide molecule itself.

Today most investigators agree that the use of proteolytic enzymes for the breakdown of tissue proteins in order to release the mucopolysaccharides gives the best recovery of polysaccharides. Moreover these methods do not seem to degrade the polysaccharides, provided they are not attacked by the enzymes used in the procedure.

As early as 1891 Schmiedeberg[2] used proteolytic enzymes for the preparation of polysaccharides. He used gastric juice. The amount of carbohydrate in the gastric juice was, however, fairly high, and thus contaminated the final product. Later on pepsin, both crystalline and purified, have been used. In order to obtain a satisfactory digestion with pepsin the procedure has to be carried out at an acid pH which may cause degradation of the polysaccharide. It cannot, for instance, be excluded that acid-labile bonds like the sulphaminic group in heparin[3] can be broken during this treatment. When trypsin, chymotrypsin or intestinal enzymes are used, the degradation due to the acid reaction is excluded.

The digestion has been carried out in different ways. Some authors only digest for a short time and thus have the tissue liquefied. This does not necessarily mean that all proteins are broken down in fragments small enough not to interfere with the subsequent purification procedures. In such cases some additional treatment like alkali extraction[4] or a preliminary treatment with pepsin is necessary.[5]

A better method of digestion with trypsin and chymotrypsin is to add small amounts of the enzymes frequently. The course of the digestion can be followed by

*The following symbols are used:—CSA, Chondroitinsulphuric acid; CPC, Alkylpyridinum chloride; CTAB, Cetyltrimethylammonium bromide.

determination of the ratio of amino-nitrogen to total nitrogen. Crude glycerol extract of acetone dried and powdered pig pancreas seems to be much more effective in digesting several tissues than crystalline trypsin and chymotrypsin. The digestion is still better if a glycerol extract of intestinal mucosa is added.[6] This is obviously due to the presence of several peptidases which depolymerize the proteins far better than the pure pancreatic enzymes. This point is very important when the polysaccharides have to be recovered from the solution by means of alcohol precipitation.

It can be argued that the addition of the crude extract may contaminate the final product. However, the amount of extract needed is small and its carbohydrate content rather low. In an experiment where cornea was digested with a total of 150 ml of pancreatic extract, 6·9 g of polysaccharide was obtained: the amount of carbohydrate due to pancreatic extract being only of the order of a few milligrams.[6]

The use of proteolytic enzymes from plants has been used by several workers. Bromelin, ficin and papain have been used. These enzymes are capable of breaking down tissue proteins rapidly and to small fragments.

There are several techniques, the best of which seems to be that of Scott[7]. He makes use of the fact that papin is a very heat-stable enzyme and thus carries out the digestion at 65 °C. This has two advantages: first no preservative has to be added; secondly the digestion proceeds at a much faster rate.

Before subjecting a tissue to proteolytic digestion it is of importance to prepare the tissue in a suitable way. The tissue should be finely minced or ground and the fats extracted, in order to facilitate the penetration of the enzyme solution into the tissue. A preliminary heat coagulation is of importance in the case of tryptic digestion, since the denatured proteins are more easily attacked by the enzyme.

PURIFICATION OF TISSUE EXTRACTS

After the proteolytic digestion of the tissue the investigator is left with a solution containing amino acids, peptides, salts, fats and nucleic acids or their breakdown products, and in most cases only a few per cent of polysaccharides.

In order to recover the polysaccharides in a reasonable pure form the mixture has to be subjected to some further purification procedure. Most protein and peptide precipitating agents have been used to reach this goal. Unfortunately they have a tendency to precipitate polysaccharides together with the impurities.

A method frequently used for the removal of protein impurities has been the so-called Sevag[8] technique. This method which was originally described by K. A. H. Mörner[9] consists of denaturating the proteins by shaking the solution with chloroform and amyl alcohol.

Schiller *et al.*[4] precipitated the proteins with trichloracetic acid after a combined alkali extraction tryptic digestion procedure. The same precipitant has been used by Balazs[10] after peptic digestion.

The simplest way to dispose of most of the impurities is to precipitate the polysaccharides either by alcohol or by quaternary ammonium compounds.

Alcohol precipitation yields a product which is often largely contaminated with proteins or protein residues, depending on the organ from which the polysaccharide has been isolated. Dialysis to remove split products is not always to be recommended, since, depending on the quality of the dialysis membranes, some losses of polysaccharides may occur. Further purification is frequently required. Protein adsorbants

such as Fullers earth[11] or Lloyds reagent have been used. However, these adsorbents have a tendency to adsorb considerable amounts of carbohydrate-containing material together with proteins.

In our laboratory the following general scheme has been used for the isolation of the polysaccharide (Table 2). Two consecutive precipitations with alcohol, one of alkaline (pH about 9–10), the other of an acid reaction (pH about 2–3), purify the product to a great extent. The extraction of the alcohol-precipitated material with phenol has two advantages and one disadvantage. First the removal of the proteolytic-split products is fairly good and secondly the losses at that stage can be easily demonstrated by precipitating the phenolic solution with alcohol and analysing the precipitate. The disadvantage is that 90% phenol is a very unpleasant solvent to work with, especially on a large scale work.

TABLE 2. PREPARATION OF POLYSACCHARIDES

Freeze-dried organ —→ digestion with proteolytic enzymes —→
—→ Precipitation with ethanol at pH 11 —→ precipitate dissolved at pH 2 and reprecipitated with ethanol —→
—→ Precipitate dissolved solution neutralized and freeze-dried —→
—→ Extraction with 90% phenol —→
—→ Phenol-insoluble part dissolved in water —→ acidified and precipitated with ethanol —→
—→ Dissolved neutralized and precipitated with ethanol (or freeze-dried)

The polysaccharides prepared according to this method generally contain between 5–10 per cent protein as an impurity. This impurity, however, does not interfere with the subsequent fractionation procedure.

Scott[12] has recently described a method for deproteinizing mucopolysaccharide solutions, based on his extensive work on the use of quaternary ammonium compounds for the fractionation of acidic polysaccharides. In this method the solution is treated with a quaternary ammonium compound at an alkaline pH. Both polysaccharides and proteins are thus precipitated. The formation of complexes between polysaccharides and proteins is prevented by the alkaline reaction and by the competition between the quaternary ammonium compound and the proteins. At a faintly acid pH the complexes between the proteins and the quaternary ammonium compound are dissolved, while those of the polysaccharides are not. They can, however, be dissolved by salt solutions from which the polysaccharides can be recovered by alcohol precipitations.

FRACTIONATION OF POLYSACCHARIDE MIXTURES

The most commonly used method for the fractionation of polysaccharide mixture has been the fractional precipitation with organic solvents from aqueous solutions containing different metal ions.

A fractionation scheme, based on the addition of increasing amounts of ethanol to the polysaccharide solution in the presence of calcium ion, has been worked out by Meyer *et al.*[5], and has been used in most of their outstanding work in this field. By their method the known mucopolysaccharides of connective tissue have been separated,

and some of them have also been characterized by their behaviour in the fractionation scheme.

Similar schemes using barium instead of calcium have been used by Smith and Gallop[13] and in our laboratory.[14]

The fractionation with alcohol has certain drawbacks. It is difficult to apply on a microscale, a point of great importance both in metabolic work, and in work where changes of the mucopolysaccharide pattern in diseased organs have to be studied. Moreover the fractions are not always clear cut.

A new approach to the problem was made in 1950 when zone electrophoresis applied to the fractionation of polysaccharides was introduced by Gardell *et al.*[15]. Kieselguhr was used as the supporting medium, and the electrophoresis was performed in an acetate buffer. The supporting medium was packed as a slab which, after completion of the electrophoresis, was cut at right angle to the direction of movement. The polysaccharides were then eluted from each cut and their concentration was determined.

This method was later on used by Boström and Gardell[16] in their work on the metabolism of sulphated mucopolysaccharides in the skin and later on by Dorfman and his group working on the metabolism of the carbon skeletons of these compounds.[4]

Recently electrophoretic separations have been carried out on cellulose columns, which are said to give better results and are easier to handle.[17] For smaller amounts of material Rienits[18] used paper electrophoresis and detected the polysaccharides by means of their metachromatic staining properties.

Fuller and Northcote[19] used silk or glass paper. On these materials other reagents for the detection of polysaccharides like α-naphthol and sulphuric acid can be used, thus allowing polysaccharides other than those with metachromatic properties to be detected.

Paper chromatographic methods have been used by Kerby[20]. However, polysaccharides do not give any discrete spots on paper chromatograms.

Partition analysis, which has made so many important contributions to the analysis of proteins, has also been tried in the separation of mucopolysaccharides. Countercurrent distribution for the separation of commercial heparin samples into several fractions was used by O'Keeffe *et al.*[21] and Wolfrom *et al.*[22] The solvents were amyl alcohol and in order to have the heparin soluble in this solvent aliphatic amines were used as carriers. By using solvents containing aliphatic amines Dorfman and his group succeeded in resolving a mixture of chondroitin sulphuric acid and hyaluronic acid in its components on a column of kieselguhr.[23] The amounts which can be separated are rather small compared to the volumes of solvents necessary and the separations are not always very clear cut.

In 1955 we tried to use chromatography on a cellulose column using the same solvents as were used by Kerby for paper chromatography. It was then found that either the polysaccharide did not move at all on the column or it moved with the solvent front. These experiments led us to work out a method, the principle of which is very simple.[24]

A cellulose column is used and the column is saturated with a solvent which is known to precipitate all polysaccharides in the mixture to be separated. We used 80% ethanol containing 0·3% barium acetate for that purpose. The polysaccharide mixture dissolved in 0·3% barium acetate is then added to the column and the column

is washed with the 80% ethanol 0·3% barium acetate mixture. The polysaccharides are then eluted by using decreasing concentrations of alcohol in the eluting solvent. Similar methods have been used by Desreux and Ghauysen[25] for the separation of nucleic acids and by Zahn and Stahl[26] and Zittle and Della Monica[27] for the separation of proteins.

The possibilities of the method are best illustrated by the following example in which the polysaccharides from nucleus pulposus were fractionated.

From analysis of the unfractionated polysaccharide it was suspected that, besides chondroitin sulphuric acid detected in nucleus pulposus by Malmgren and Sylvén[28], the mixture also contained a sulphated polysaccharide composed of glucosamin, galactose and sulphate thus resembling keratosulphate (Table 3). When fractionated on a cellulose column two main fractions were obtained (Table 4). A continuous gradient was also tested in these fractionation experiments. Two peaks were obtained with widely different properties.

TABLE 3. ANALYSIS OF THE POLYSACCHARIDE MIXTURE OBTAINED FROM NUCLEUS PULPOSUS

	I	II
Nitrogen (%)	3·2	3·3
Ester sulphates (%)	4·9	5·9
Uronic acid (%)	15·7	20·1
Glucosamine (%)	8·8	10·6
Galactosamine (%)	14·8	14·7
Galactose (%)	8·0	12·7
Fucose (%)	—	2·1

TABLE 4. FRACTIONATION OF THE POLYSACCHARIDES FROM NUCLEUS PULPOSUS WITH ALCOHOL AND BARIUM ACETATE ON A CELLULOSE COLUMN

	Fraction eluted with 35% ethanol and 0·3% barium acetate	Fraction eluted with 0·3% barium acetate
Ash (%)	24·3	31·9
Nitrogen	2·6	2·0
Ester sulphates (%)	5·5	4·8
Glucosamine (%)	17·5	0·25
Galactosamine (%)	0·25	18·9
Galactose (%)	16·8	—
Fucose (%)	2·3	—

This is not chromatography in the generally accepted meaning of the term. The cellulose is only to be regarded as a supporting medium on which the polysaccharide is precipitated in a fine layer, and as the composition of the eluting solvent is changed the substances are dissolved and eluted. Thus there is no sorption step and no true adsorption equilibrium.

When Scott[7a] in 1955 made an attempt to improve the chromatographic method of Kerby he found that quaternary ammonium compounds gave insoluble precipitates with acidic polysaccharides.

Stacey[29] simultaneously found that CSA and heparin could be precipitated by cetyltrimethylammonium bromide and Terayama[30] found that cellulose sulphate could also be precipitated with quaternary ammonium compounds.

That polyanions could be precipitated in this way with amines and quaternary amines had been observed earlier. Kuhn[31] found in 1940 that proteins could be precipitated by these compounds and Powney in 1943[32] showed that hexametaphosphoric acid could also be precipitated in a similar way. Also nucleic acids can be precipitated by quatenary ammonium compounds.[33]

Scott found, when working with cetylpyridinum chloride and cetyltrimethyl ammonium bromide and various polysaccharides, that the complexes formed could be dissolved by salts. The salt concentration required varied for one type of amine with the type of polysaccharide. Thus the hyaluronic acid complex needed a concentration of about 0·2 N $MgCl_2$ or KCl to be dissolved. CSA needed about 1 N and heparin needed about 1·2 N KCl or 2 N $MgCl_2$.

Another factor which influences the precipitation is the pH. If the pH is brought to such low values around 2 that the carboxyl groups of hyaluronic acid are not dissociated no complex will be formed.

The properties of the precipitates in salt solutions can be used for the fractionation of acidic polysaccharides in two ways:

(1) The polysaccharides and the quaternary ammonium compound can be mixed in salt solution of such a concentration as to prevent any of the polysaccharides present from being precipitated. By diluting the solution with water the complexes precipitate at their critical salt concentration.

(2) The precipitation of all polyanions in the solution can be accomplished in very dilute salt concentration and the precipitate is then extracted with salt solutions of increasing concentrations.

When working with a batch-wise procedure the first principle is the best. Scott *et al.*[34] used this method for the fractionation of heparin, and Ricketts[35] used the principle in the fractionation of dextran sulphate. It has also been used by Schiller[36] in the fractionation of heparin monosulphate.

A great many complications have to be dealt with if the second principle is used. The precipitate has to be divided into small particles and the extraction has to be continued for a long period of time. And the possibility still exists that more soluble particles are trapped in particles consisting of less soluble material.

However, this principle can be used in a column procedure similar to that used for alcohol fractionation. In this case the cellulose column is saturated with the quaternary ammonium compound. An aqueous solution of the polysaccharides is introduced at the top of the column and allowed to drain into it. The column is then washed with a fresh portion of the precipitating solvent. By then eluting the column by solvents of increasing salt concentrations the polysaccharides are eluted (Fig. 1).[37]

Although neutral polysaccharides do not form complexes with CPC and CTAB at neutrality they can form complexes if the pH is brought to about 12, where the hydroxyl groups are dissociated. In this case only CTAB can be used since CPC is decomposed at alkaline reaction.[38, 39] Another possibility is to add borate and the resulting acid, polysaccharide can then form a precipitate with a quaternary ammonium compound.[38,39]

Recently a new principle has been used in the fractionation of acidic polysaccharides. The modified cellulose ion exchange materials introduced by Sober and Peterson[40] for fractionation of proteins have been used by Ringertz and Reichard[41, 42] for separation of several mucopolysaccharides.

The separation is carried out on *Ecteola*[43] at an acid reaction, i.e. pH about 2 with increasing concentrations of sodium chloride.

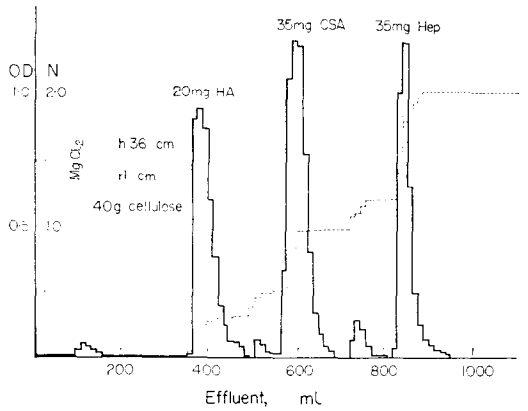

FIG. 1. Separation of polysaccharides on a cellulose column by means of cetylpyridinium chloride and magnesium chloride (Borelius, Gardell and Scott[37]).

It has for many years been suggested that changes in the polysaccharide composition of connective tissue takes place in different diseases. However, such changes have only been demonstrated in very few cases. It is very likely that with the recent developments in technique a new approach to these questions can be made with greater success particularly if the methods can be applied on a microscale.

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