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Separation and characteristics of different mucopolysaccharides from bovine trachea cartilage

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Bovine trachea cartilage may be used as a source of mucopolysaccharides (MPS) for medical and cosmetic purposes. The content of MPS in bovine cartilage ranged from 10 to 11%, on a dry weight basis. Crude MPS isolation involved hydrolysis of the tissue with papain at 60°C for 24 h at a tissue to enzyme solution ratio of 1:3 (w/v), separation of enzyme and some peptides with trichloroacetic acid and subsequent precipitation of MPS with ethanol. The MPS contained 54% of polysaccharides and about 27% of residual peptides. The crude MPS were purified and separated into four fractions by precipitation with ethanol at concentrations in reaction media of 20, 30, 40 and 66% (v/v). These fractions contributed 88, 16.5, 1.5 and 8%, respectively, to the hexosamines present in crude MPS. Total MPS recovery in all fractions was 71% of crude MPS weight. The main components of fractions I, II and IV were chondroitin sulphates, hyaluronic acid, and keratosulphates, respectively. However, fraction III contained 1.6% of total MPS of an unidentified polysaccharide. © 1997 Elsevier Science Ltd

INTRODUCTION

The principal components of all connective tissues are the cells, extracellular collagen, reticular or elastin fibres, and protein fibres containing mucopolysaccharldes. Mammalian tissues contain different mucopolysaccharides, e.g. hyaluronic acid, chondroitin, chondroitin sulphates A, B, C, heparin, heparin sulphates, keratosulphate and blood group substances. They are composed of uronic acid and sulphated or acetylated hexosamine residues. The molecular weights of mucopolysaccharides range from 30–8000 kDa. The mucopolysaccharides of connective tissues have an important role in a number of physiological processes, including calcification, control of electrolytes and water in extracellular fluids, wound healing, lubrication, and the maintenance of the stable transport medium of the eye (Brimacombe & Webber, 1964). The participation of mucopolysaccharides in a number of those roles was associated with their polyanionic nature, resulting from the presence of carboxyl and sulphate residues. The content and composition of mucopolysaccharides in connective tissues depend on the species, sex and age of the animals (Lindahl & Roden, 1972). The principal problem encountered in the isolation of pure mucopolysaccharides concerns removal of bound protein under conditions which do not significantly degrade the polysaccharide. The reviews of available methods were given by Brimacombe and Webber (1964) and Synowiecki and Grabowska (1987). Bovine trachea cartilage is a rich source of mucopolysaccharides containing, on a dry weight basis, about 10% of these compounds (Synowiecki & Shahidi, 1994).

MATERIALS AND METHODS

Fresh bovine trachea cartilages without fat and meat residues were cut into small pieces, packed in polyethylene bags and kept frozen at −18°C until use. Before processing, the frozen samples were ground using a meat grinder (Model KU-2, Predom Mesko, Skarzysko Kam., Poland) through a 5 mm grind plate. The mucopolysaccharides, from about 3 kg of cartilage, were isolated using the method of Synowiecki and Shahidi, (1994) with modifications given in Fig. 1. The individual MPS were separated according to the procedure of Meyer *et al.* (1953) by precipitation with ethanol in the presence of calcium ions (Fig. 2). The concentration of ethanol in crude mucopolysaccharide solutions ranged from 20 to 66% (v/v). The separated samples were washed three times with acetone and air-dried at +20°C.

ANALYSES

Moisture content was determined by oven-drying at 105°C to a constant weight (AOAC, 1990). The crude protein (P) in the samples was calculated from their total nitrogen content (AOAC, 1990) using the equation: $P = (N - N_{hexosamines}) \times 5.56[\%]$.

Hexosamines were determined according to the method of Elson and Morgan (1933) modified by Cessi, (1960). The analysis was performed after hydrolysis of the samples with 2M HCl at 105°C for 15 h. The total content of mucopolysaccharides (MPS) was calculated using the equation: $MPS\,[\%] = Hexosamine \times f$, where (f) is the percentage of molecular weight of glucosamine

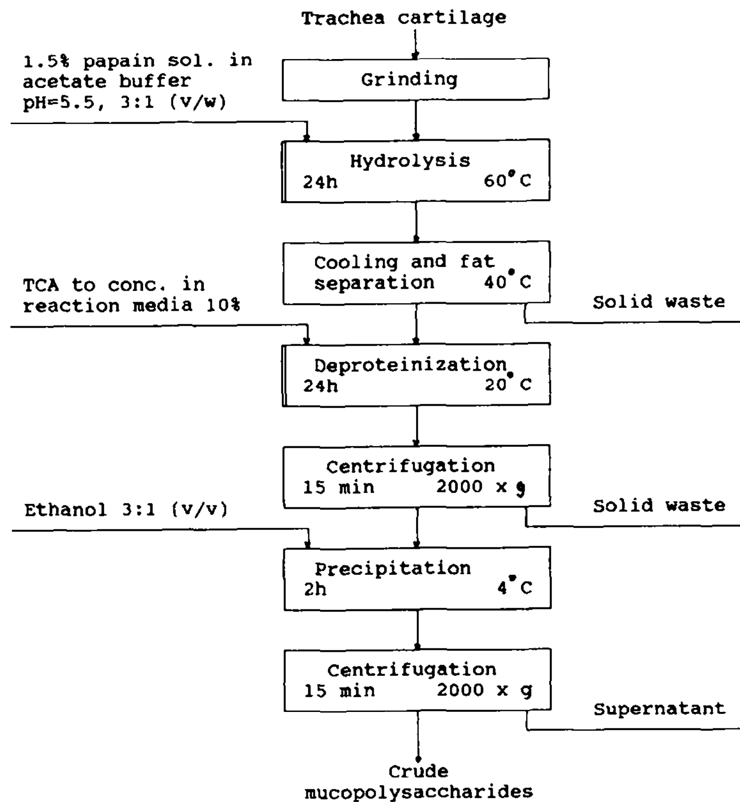

Fig. 1. Flowsheet for the isolation of crude mucopolysaccharides from bovine trachea cartilage.

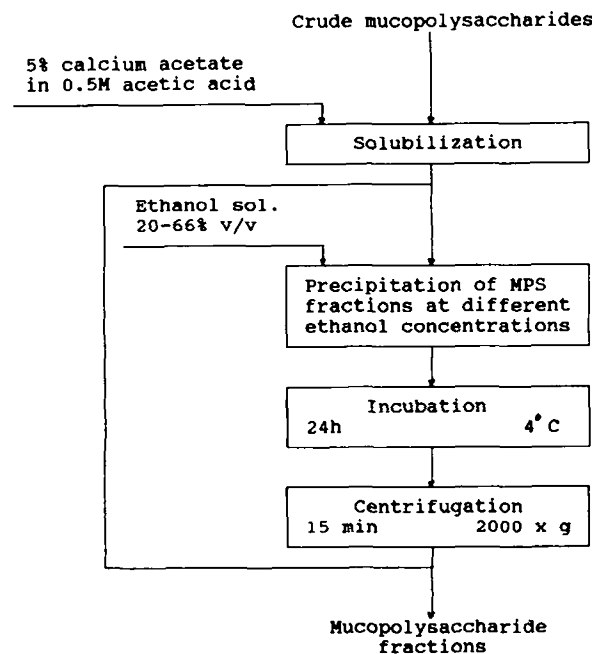

Fig. 2. Flowsheet for fractionation of crude mucopolysaccharides at different ethanol concentrations.

residue in a mole of disaccharide composed of *N*-acetylglucosamine and glucuronic acid (for hyaluronic acid) or the percentage of moles of sulphonated *N*-acetylgalactosamine in a mole of disaccharide composed of glucuronic acid and sulphonated *N*-acetylgalactosamine (monomer of chondroitin sulphates).

Hydroxyproline content was assayed according to ISO, (1978) after hydrolysis of the samples with 6M HCl at 105°C for 15 h. The SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out using 0.1 M sodium phosphate buffer (pH = 7.0) containing 0.1% (w/v) of sodium dodecylsulphate (SDS). The analysed samples were: crude mucopolysaccharides, MPS precipitated in 20% ethanol solution, standards of chondroitin sulphates A and C (Sigma Chemical Co., Ltd) and the protein residue in isolated MPS. The concentrations of the gels for MPS and protein analysis were 5 and 7.5%, respectively (Weber & Osborne, 1969). The samples for SDS-PAGE were dissolved at 100°C during 4 min in a 0.01M sodium phosphate buffer (pH = 7.0) containing 1% (w/v) of SDS and 5 mM of dithiothreitol (DTT). The concentrations of the sample solutions for analysis of MPS or residual proteins in MPS were 3 and 60 mg ml^{-1}, respectively. Disc electrophoresis was performed at a constant current of 5 mA per gel. The gels, after protein separation, were stained for 2 h in 0.25% Coomassie Blue R 250 and then destained in 7.5% acetic acid and 5% methanol (Weber & Osborne, 1969). The staining of MPS electrophoretograms was performed by Kreuger and Schwartz, (1987) using 0.025% Alcian Blue 8GX (Sigma Chemical Co.,Ltd) and ammoniacal silver solution. These gels were then destained in 10% acetic acid in 25% ethanol.

RESULTS AND DISCUSSION

The bovine trachea cartilage contained, on a dry basis, 10–11% of mucopolysaccharides and 49–55% of proteins. The collagen content calculated according to formula ($Hyp \times 7.46$) was 27% of total protein. Isolation of the crude mucopolysaccharides from cartilage involved the main operations, such as release of MPS by enzymic digestion, precipitation of the enzyme and products of protein hydrolysis in trichloroacetic acid (TCA) solution and precipitation of the MPS in ethanol (Fig. 1). For protection against the influence of sodium hydroxide on MPS and formation of MPS salts, the basic hydrolysis of residual proteins and subsequent dialysis were omitted. The recovery yield of crude MPS from bovine trachea cartilage was 97% of their amount in the tissue. However, the enzymic digestion was incapable of removing some of the peptides which were attached to the MPS molecules (Lindahl & Roden, 1972). Some peptides were also not precipitated in TCA solution. This caused the final product to contain 54.1% of mucopolysaccharides and 26.9% of proteins; the hexosamine nitrogen to total nitrogen ratio for the crude MPS was only 23.6%, considerably less than the ratio (31.2%) for MPS additionally treated with NaOH solution (Synowiecki & Shahidi, 1994).

The crude mucopolysaccharides were separated into four fractions by precipitation from their solutions using ethanol at concentrations in reaction media of 20, 30, 40 and 66% (v/v). These fractions contributed 54, 11, 1.0 and 5%, respectively, to the mass of MPS present in the solution. However, total MPS recovery in all fractions together was about 71% of the crude mucopolysaccharides weight. The relatively small yield of precipitation was caused by oss of peptides released during protein hydrolysis, which were not precipitated by ethanol. This enhanced the mucopolysaccharide concentration in all fractions as compared with raw MPS (Table 2). The effectiveness of deproteinization was limited mainly by residual small peptides and amino acids directly attached to MPS molecules and resistant to enzymic hydrolysis. Their amounts in precipitated MPS depend on fraction number and ranged from 6.7 to 15.7 (Table 2). The first fraction of MPS contained 88.1% of the total hexosamines content in the MPS mixture. Moreover, fractions II, III and IV contributed 16.5, 1.5 and 8.2%, respectively, to the hexosamines present in crude MPS. These data show that almost all mucopolysaccharides were recovered from the solution during precipitation. According to Meyer *et al.* (1956) and Stuhlsatz and Greiling (1978) the main components of mucopolysaccharides from bovine

Table 1. Yield of crude mucopolysaccharides (MPS) and their fractions obtained from 1000 g of wet bovine trachea cartilage

Fraction	Percentage of ethanol in MPS sol.	Amount of crude isolate (g)	Calculated amount of MPS in isolate (g)	Recovery of MPS from crude isolate (%)
Crude MPS	—	68.8	37.2	100
I	20	37.4	32.9	88.1
II	30	7.5	6.2	16.6
III	40	0.7	0.6	1.6
IV	66	3.6	3.1	8.3

In column 4, the crude MPS is less than the sum of the amounts of MPS in 4 fractions isolated from it by about 14%. This was probably caused by different degrees of hexosamine degradation during acid hydrolysis of the MPS samples containing different amounts of proteins.

Table 2. Chemical composition (% on a dry weight basis) of bovine trachea cartilage, crude mucopolysaccharides (MPS) and MPS precipitated at concentrations of ethanol

Source	Total nitrogen	Hexosamines	MPS	Proteins
Trachea cartilage	9.1 ± 0.6	3.7 ± 0.1	10.8 ± 0.4	49.0 ± 3.8
Crude MPS	6.4 ± 0.5	19.3 ± 0.6	54.1 ± 1.9	26.9 ± 3.6
MPS precipitated at ethanol concentration (%):				
20	3.7 ± 0.0	31.3 ± 1.3	88.0 ± 3.7	6.7 ± 0.1
30	3.9 ± 0.1	29.4 ± 0.4	82.5 ± 1.0	8.7 ± 0.1
40	4.3 ± 0.1	30.3 ± 1.4	85.1 ± 3.9	10.4 ± 0.6
66	5.2 ± 0.1	30.7 ± 0.3	86.3 ± 0.8	15.7 ± 0.1

Results are mean values of data from three determinations ± standard deviation.

trachea cartilage are chondroitin sulphates A and C. Polyacrylamide gel electrophoresis (SDS-PAGE) of the samples and chondroitin sulphates standards confirmed that these aminosugars were main components of the first MPS fraction precipitated at an ethanol concentration of 20%.

The mucoproteins in cartilages are composed of chondroitin sulphates and keratosulphates bound to serine and threonine residues of proteins (Hopwod & Robinson, 1974). These subunits are attached to long fibrillar molecules of hyaluronic acid (Rosenberg *et al.*, 1979). The amount of hyaluronic acid separated from crude MPS (Fraction II) was about 16% of total MPS content in bovine trachea cartilage (Table 1). Precipitation of hyaluronic acid from bovine cornea at 30% ethanol concentration was also observed by Meyer *et al.* (1953). Concentration of ethanol (66%, v/v) used for precipitation of the fourth fraction of MPS shows that it was composed of keratosulphates (Stuhlsatz & Greiling, 1978). The ratio of keratosulphates to chondroitin sulphates in mucoproteins from bovine trachea cartilage was about 1:10 (Table 1). However, the small fraction III (1.6% of total MPS content) contained an unidentified mucopolysaccharide, probably hyaluronic acid with molecular weight different from that in fraction II.

In conclusion, bovine trachea cartilage may be used as a good source of MPS. Separation of crude MPS on hyaluronic acid, chondroitin sulphates and keratosulphates could be effectively achieved by precipitation at different ethanol concentrations. However, each MPS fraction contained 6.7 to 15.5% of residual proteins not removed during enzymic hydrolysis of cartilage.

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