DEGRADATION OF THE PEACH GUM POLYSACCHARIDE BY THE MIXTURE OF EXTRACELLULAR GLYCANOHYDROLASES OF *Aspergillus flavus*

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The polysaccharide of peach gum, prepared by a partial acid hydrolysis, was degraded by a mixture of extracellular glacanohydrolases of *Aspergillus flavus*. This way obtained polysaccharide differed from the original one especially by a higher content of D-mannose and a lower content of D-galactose bound by (1 → 3) glycosidic bonds. The obtained results are discussed from the standpoint of composition of the main and side chains of the native polysaccharide.

One of the approaches to solve the structure of polysaccharides is their depolymerization and isolation of fragments, which are characterized by chemical or physicochemical methods. The most suitable and the mildest general way to obtain fragments seems to be the hydrolysis of glycosidic bonds under catalysis of various specific enzymes. This procedure proved to be successful mainly with homopolysaccharides, especially of one type of glycosidic bonds. Plant gums have, however, very complex structures as far as components, bonds and branching are concerned and therefore, several authors[1] assumed that such polysaccharides, in the synthesis of which a highly organized complex system of enzymes had to be involved, would probably resist the action of degradative enzymes and fungi. Several attempts to degrade plant gums by enzymes have been reported; thus Lutz[2,3] observed that the fungus isolated from the stump of acacia trees was able to hydrolyze the cherry gum. This action exerted a change in consistence during three months. Aspinall and Baillie[4] found that the original gum tragacanth (*Astralgus gumifer*) was not attacked by commercially available pectinase, nor by hemicellulase preparations; nonetheless tragacanthic acid, obtained after a controlled hydrolysis, was accessible to the action of these enzymes. Nagel[5] attempted to degrade the caraya gum (*Sterculia urens*) by microorganisms and commercially available glycosidases, and ascertained that these preparations did not attack the gum to a measurable extent. Beauquesne[6] was unsuccessful with degradation of the *Sterculia fomentosa* gum by hepato-pancreatic enzyme complex of snail, *Penicillium erlichii* and some kinds of *Clostridium*. Dagal and Swanson[7] modified enzymatically the locust bean and guar gums by a specific hydrolysis of galactose units of the polymers. A 152 h hydrolysis freed over 95%

of galactose, 98% of arabinose and only 2·5% of mannose. The remaining polysaccharide contained mainly mannose. A more detailed study on microbial degradation of caraya gum appeared in 1973 (ref.[8]). The gum was degraded by a preparation of the fungus *Cephalosporium* sp., which caused an initial fragmentation of the polysaccharide, since the formation of the necessary enzymes was induced immediately at the beginning of the reaction. This fungus was, moreover, able to degrade a great portion of fragments of chains coming from the endo cleavage, since these fragments induced the formation of all enzymes inevitable for their degradation. A certain part of the polysaccharide was not attacked for this very reason that the fungus was unable to produce β-glucosiduronase in addition to other enzymes.

In our more detailed study of the structure of polysaccaride of the peach gum, the structural feature of which has already been described[9], the enzyme preparations of *Aspergillus flavus*[10], isolated in this Laboratory, were employed. The low-molecular products of enzyme hydrolysis of the degraded polysaccharide of the peach gum were desribed earlier[11,12]. This paper deals with the alterations of the peach gum polysaccharide, prepared by a partial hydrolysis, by catalytic effect of the mixture of extracellular enzymes of the above-mentioned microorganism.

EXPERIMENTAL

Isolation and characterization of the mixture of extracellular glycanohydrolases of *Aspergillus flavus* was described[10]. For paper chromatography on paper Whatman No 1 the following solvent systems were used: ethyl acetate–pyridine–water 8 : 2 : 1 (S_1), ethyl acetate–pyridine–acetic acid–water 5 : 5 : 1 : 3 (S_2) and ethyl acetate–acetic acid–water 18 : 7 : 8 (S_3). Saccharides were detected with anilinium hydrogen phthalate[13] and alkaline solution of silver nitrate[14]. Electrophorese (Zeiss, Jena) apparatus was used for electrophoresis, Perkin–Elmer, model 141 spectrophotometer for measuring optical rotation. IR spectra of chloroform solutions were recorded with a Perkin–Elmer, model 457, instrument. The equivalent weight was determined by potentiometric titration with 0·1M-NaOH to pH 7·5 on an automatic Titrator TTT 1c (Radiometer, Copenhagen) apparatus. The molecular weight of polysaccharides was estimated by a light scattering method with a scattering photometer Brice-Phoenix, model 2000 (Phoenix Precision Intruments, Philadelphia, USA) at room temperature and monochromatic 546 nm light. Solvents and solutions were purified by a repeated pressure filtration through ultrafilters Millipore (Millipore, Bedford, USA), or Synpor (VChZ Synthesia, Uhřiněves, Czechoslovakia). The increment of the refractive index was determined with the interferometer Zeiss, Jena.

Monosaccharides were quantitatively determined with an automatic saccharide analyzer (Technicon USA), the reducing sugars during enzyme hydrolysis were estimated by the Somogyi method[15], absorption of light was measured with a Beckman DB-GT spectrophotometer at 530 nm. Mass spectra of the partially methylated alditol acetates were recorded with a JMS-100 (Jeol) spectrometer at an ionizing ion energy 23 eV, trap current 100 μA and ionizing chamber temperature 150—180°C. Gas chromatography/mass spectrometry on a JGC-20K apparatus proceeded at an inlet He pressure 0·1 MPa, 220° injection port temperature and 170—210°C (4° per min) column temperature. For gas chromatography Hewlett–Packard, model 5711 A instrument equipped with a flame-ionizing detector (carrier gas N_2, 30 ml per min, 170°C) was used.

Preparation of Degraded Starting Polysaccharide

Polysaccharide of peach gum (2% aqueous solution) prepared according to[9] was hydrolyzed with 0·5M-H_2SO_4 at 100°C for 20 min, the solution filtered through a sintered glass filter S_1 and the polysaccharide precipitated with ethanol at a 1 : 3 ratio was washed with a dilute aqueous ethanol (60% solution), dissolved in water and freeze dried.

Enzyme Hydrolysis of the Degraded Starting Polysaccharide

The polysaccharide (5 g) was dissolved in a 0·1M acetate buffer of pH 4·7 (250 ml) containing 0·1M-NaCl and the solution topped with toluene was incubated with a mixture of extracellular glycanohydrolases of *Aspergillus flavus* at 30°C for 5 days. The increases in reducing groups and products of hydrolysis were monitored during incubation by paper chromatography. Hydrolysis was ceased by inactivation of the enzyme by a 10 min boiling of the solution. The residual polysaccharide was precipitated from the solution concentrated to 50 ml with acidified ethanol (1% HCl) in a 1 : 3 ratio. The precipitate was centrifuged, washed with dilute ethanol (60%), dissolved in water and percolated through a column packed with Dowex 50WX4 (H^+) and Dowex 1X8 (CH_3COO^-); freeze drying afforded 1·55 g of the polysaccharide.

Total Hydrolysis of Polysaccharides

The polysaccharide (10 mg of the lyophilizate) was hydrolyzed with 0·5M-H_2SO_4 (10 ml) in a fused test tube at 105°C for 24 h, the acid neutralized with barium carbonate, the excess of Ba^{2+} ions removed by passing through a Dowex 50W X 4 (H^+) column and acid saccharides separated from the neutral ones using a Dowex 1 X 8 (CH_3COO^-) ion exchanger. Neutral saccharides were eluted with water, the acidic ones with 4M acetic acid. The thickened solutions freed from acid were thereafter chromatographed in the given solvent systems.

Methylation, Hydrolysis, Reduction and Acetylation of Polysaccharides

The polysaccharide was methylated with dimethyl sulfate in the presence of sodium hydroxide according to[16] and with methyl iodide and silver oxide according to[17]. A part of the methylated product (35 mg) was hydrolyzed with 90% formic acid (12 ml) at 100°C for 1 h and concentrated. Sulfuric acid (0·13M, 18 ml) was added and the hydrolysis continued at 100°C for additional 10 h. The acid was neutralized with barium carbonate, and the acid constituents in the filtrate were separated from neutral ones with a Dowex 1X8 (CH_3COO^-) packed column. Methylated saccharides (4 ml of aqueous solution) were reduced with sodium tetrahydroborate (150 mg) for 2 h under stirring. The solution was then acidified to pH 3·5 with Dowex 50WX4 (H^+), filtered and thickened to dryness; boric acid was removed by evaporation with methanol (3 × 25 ml). The reduced product was acetylated with acetic anhydride–pyridine 1 : 1 (4 ml) at 100°C for 1 h. The excess of reagents was removed by distillation with toluene and the product was extracted with dichloromethane and concentrated. Characteristic peaks of ions (m/z) of the partially methylated alditol acetates in mass spectrum: 1,5-O-acetyl-2,3,4,6-tetra-O-methyl-D-mannitol (87, 101, 117, 129, 145, 161, 205), 1,5-di-O-acetyl-2,3,4,6-tetra-O-methyl-D-galactitol (87, 101, 117, 129, 145, 161, 205), 1,2,5-tri-O-acetyl-3,4,6-tri-O-methyl-D-mannitol (71, 87, 99, 101, 129, 145, 161, 189), 1,3,5-tri-O-acetyl-3,4,6-tri-O-methyl-D-galactitol (87, 99, 101, 117, 129, 143, 159, 161, 173, 189, 201, 233), 1,5,6-tri-O-acetyl-2,3,4-tri-O-methyl-D-galactitol (87, 99, 101, 117, 129, 159, 161, 173, 189, 233), 1,2,5,6-tetra-O-acetyl-3,4-di-O-methyl-D-mannitol (43, 87, 99, 129, 189), 1,3,5,6-tetra-O-acetyl-2,4-di-O-methyl-D-galactitol (87, 117, 129, 189, 201, 233).

RESULTS

Polysaccharide of peach gum is an acid heteropolysaccharide consisting of D-glucuronic, 4-O-methyl-D-glucuronic acids, D-galactose, D-mannose, D-xylose, L-arabinose and a trace amount of L-rhamnose. Results of partial acid hydrolysis of the polysaccharide, during which a series of acid oligosaccharides was obtained, together with the results of methylation analysis and periodate oxidation suggested that D-galactose units were bound in the main chain to a great extent by (1 → 6) glycosidic bonds, whereas side chain consisted mainly of pentoses, *cf.*[9]. Partial acid hydrolysis afforded a degraded polysaccharide containing mainly D-galactose, D-mannose, D-glucuronic and 4-O-methyl-D-glucuronic acids and virtually neither D-xylose nor L-arabinose. This polysaccharide was, according to sedimentation in ultracentrifuge and free electrophoresis in borate buffer of pH 9·3, homogeneous. Its characteristic data are listed in Table I.

The reduction ability of the solution increased and low-molecular products were concurrently detected by paper chromatography when incubating the degraded polysaccharide with a mixture of extracellular enzymes. Monosaccharides and neutral oligosaccharides related in a considerable measure to D-galactose and partly to D-mannose and also a little amount of aldobiouronic acids were the identified constituents. Structures of four neutral oligosaccharides have already been elucidated and published[11,12]. The polysaccharide isolated from the hydrolysate was again homogeneous according to the methods used; its characterization is given in Table I. The molecular weight of the polysaccharide considerably decreased during the enzyme hydrolysis. The equivalent weight of the enzyme degraded polysaccharide also dropped, what is reasonable, since a great amount of neutral saccharides and only small amounts of aldobiouronic acids were cleaved during the hydrolysis. Decrease of the methoxyl group content is in accord with the representation of D-glucuronic

TABLE I
Characteristic Data of Polysaccharides

Polysaccharide	Mol. weight	Equivalent weight	—OCH_3, %	$[\alpha]_D^{25a}$	Molar ratios					
					D-GlcA	4-O-Me-D-GlcA	D-Man	D-Gal	L-Ara	D-Xyl
Starting	16 400	802	1·50	+16·9°	0·63	0·36	0·35	3·56	traces	traces
Enzyme degraded	5 600	584	0·70	+ 9·2°	0·86	0·13	0·71	1·77	—	—

[a] Water.

and 4-O-methyl-D-glucuronic acids in the starting and enzyme degraded polysaccharides. Specific optical rotation values of both polysaccharides indicate β-glycosic bonds.

To ascertain the character of the enzyme effect, the starting and enzyme degraded polysaccharides were methylated, hydrolyzed, reduced and acetylated. The products, partially methylated alditol acetates, were identified by gas chromatography (Table II) and mass spectrometry. As demonstrated, in the starting polysaccharide the substantial portion of D-galactose forms nonreducing terminal groups represented by the tetramethyl derivative. The predominant part of D-galactose is bound in the polysaccharide by (1 → 6) and (1 → 3) glycosidic bonds. As indicated by the presence of dimethyl derivative, only a small portion of D-galactose was involved in branching. The enzyme degraded polysaccharide showed, when compared with the starting one, a considerably lowered portion of (1 → 3) bound D-galactose. This great decrease probably indicates that D-galactose bound in this way came from side chains, where it is mostly accessible towards an attack of β-galactosidase present in a dominant amount in the mixture of enzymes employed for hydrolysis. Decrease of the terminal nonreducing units content was proportional to the overall decrease of D-galactose in the polysaccharide.

D-Mannose is bound in the starting polysaccharide in the backbone mainly by a (1 → 2) glycosidic bonds, whereas only small amounts of it were detected at branching points and as nonreducing terminal groups. The content of D-mannose bound by (1 → 2) glycosidic bonds notably increased in the enzyme degraded polysaccharide. So did the nonreducing terminal units and those representing the branching sites.

TABLE II

Partially Methylated Alditol Acetates Identified by Gas Chromatography

Component	R_t	Polymer[a]	
		starting	enzyme degraded
1,5-Di-O-Ac-2,3,4,6-tetra-O-Me-D-mannitol	1·00	1·10	4·72
1,5-Di-O-Ac-2,3,4,6-tetra-O-Me-D-galactitol	1·15	32·28	23·61
1,2,5-Tri-O-Ac-3,4,6-tri-O-Me-D-mannitol	1·61	8·58	25·23
1,3,5-Tri-O-Ac-2,4,6-tri-O-Me-D-galactitol	1·80	18·41	10·77
1,5,6-Tri-O-Ac-2,3,4-tri-O-Me-D-galactitol	1·95	33·67	29·22
1,2,5,6-Tetra-O-Ac-3,4-di-O-Me-D-mannitol	2·88	1·10	3·32
1,3,5,6-Tetra-O-Ac-2,4-di-O-Me-D-galactitol	3·10	4·84	3·09

[a] Relative molar composition expressed in % of total peak area.

The greater amount of the tetramethyl derivative of D-galactose when compared with the dimethyl derivative can be rationalized by the fact that D-galactose, bound as nonreducing terminal units to uronic acids (which are present in the chain and form branching loci[18]), was cleaved during methylation due to β-elimination of uronic acids[19] and was permethylated. It is admitted that a small amount of D-galactose could be split off as a result of alkaline hydrolysis at the beginning of methylation and was fully methylated thus increasing the portion of the tetramethyl derivative.

It could be finally stated that the assumption on the origin of the trisaccharide O-β-D-galactopyranosyl-(1 → 3)-O-β-D-galactopyranosyl-(1 → 6)-D-galactose[11] proved to be right, when mostly galactose units bound by (1 → 3) glycosidic bonds in side chains were split off during hydrolysis. The increased amount of D-mannose in the enzyme degraded polysaccharide indicates that this sugar appeared in the core of the native polysaccharide. Therefore, it is virtually unaccessible towards an attack of enzymes even with such a considerably degraded polysaccharide as was the above-mentioned starting polysaccharide, contrary to the fact that the employed mixture of extracellular enzymes displayed also mannosidase activity[10].

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