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Note

Isolation of neoagarobiose and neoagarotetraose from agarose digested by *Pseudomonas elongata*

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Agarolytic enzymes are, as yet, an ill-defined group of enzymes that are able to digest the polysaccharides of agar. Thus far, these enzymes have been found in a wide range of organisms, including bacteria[1], algae[2], and also, in the animal world, among molluscs[3]. Although the number of agarolytic organisms described is small, few of them have yet been studied[4–7]. The first step in the study of agarolytic enzymes is to determine which of the glycosidic linkages are broken in polysaccharides of agar. Agarose, a polysaccharide that can be obtained pure from agar, is the substrate of choice for such an investigation. Agarose has been described essentially[8] as a neutral, linear molecule of (1→3)-linked β-D-galactopyranose residues alternating with (1→4)-linked 3,6-anhydro-α-L-galactopyranose residues. Thus, an agarolytic system may attack either the β-D-(1→4) bonds or the α-L-(1→3) bonds of agarose. Both types of agarolytic system have been described[9,10].

The agarolytic systems that cleave β-D-(1→4) bonds give oligosaccharides belonging to the neoagarobiose series. These neoagaro-oligosaccharides are characterized by having residues of D-galactose at their reducing ends. In contrast, attack at the α-L-(1→3) bonds will produce oligosaccharides belonging to the agarobiose homologous series. This series is characterized by having 3,6-anhydro-L-galactose at the reducing end. This last series can be obtained by mild hydrolysis of agarose[11], whereas the neoagaro-oligosaccharides have only been obtained by enzyme action.

The purpose of this work was to establish the type of bond in agarose that is attacked by the agarolytic system of *Pseudomonas elongata*.

RESULTS AND DISCUSSION

The enzymes isolated from *P. elongata* were found to digest agarose. Chromatographic analysis of the resulting solution gave a number of spots, three of which co-chromatographed with neoagarotetraose, neoagarohexaose, and neoagaro-octaose. No free monosaccharides were detected. Controls in the absence of *P. elongata* extract were devoid of oligosaccharides.

Reduction of the products caused no decrease in the proportion of 3,6-anhydrogalactose, as determined with the Roe–Papadopoulos reagents, indicating that the 3,6-anhydrogalactose residues are in the inner part of all of the oligosaccharides. Reduced 3,6-anhydrogalactose does not react with the detecting reagents.

After resolution on charcoal–Celite, three oligosaccharides were obtained; each was further purified on 3MM paper. The first oligosaccharide had the chromatographic mobility of neogarobiose, the second that of neoagarotetraose, and the third that of neoagarohexaose. The amount of neoagarobiose was very small, and this probably accounts for its lack of detection before fractionation of the mixture.

Quantitative determination of the proportion of galactose in a hydrolyzate of each of these oligosaccharides gave values of 1, 2, and 3 moles, respectively, per mole of original compound, based on its reducing power. The proportion of 3,6-anhydro-L-galactose also corresponded to 1, 2, and 3 moles per mole for the respective oligosaccharides. Thus, it was established that the three oligosaccharides separated are a di-, a tetra-, and a hexa-saccharide, respectively. Reduction of each of these oligosaccharides caused no decrease in the proportion of 3,6-anhydrogalactose, again confirming that this residue is not present at the reducing end of any of the molecules. Table I summarizes these results.

TABLE I

SOME PROPERTIES OF THE OLIGOSACCHARIDES PRODUCED BY *Pseudomonas elongata*

Oligosaccharide	*Galactose A*	*3,6-Anhydro-L-galactose*		*Degree of polymerization A+B*
		Reduced oligosaccharide	*Non-reduced oligosaccharide B*	
1	1.10	0.85	0.90	2.00
2	1.80	1.90	1.95	3.75
3	2.70	3.10	3.20	5.90
Agarobiose	0.90	0.00	0.85	1.75

Chromatographic analysis of the hydrolyzates of the reduced oligosaccharides **1** and **2** showed only galactitol for **1**, and galactose and galactitol for **2**. Thus oligosaccharide **1** has D-galactose at the reducing end and 3,6-anhydro-L-galactose at the non-reducing end, in agreement with the structure reported by Araki for neoagarobiose[12].

Mild, acid hydrolysis of oligosaccharide **2** and reduced oligosaccharide **2**, as reported by Araki[13] for neoagarotetraose, gave rise to agarobiose, further confirming its constitution as neoagarotetraose.

Oligosaccharide **3** was identified as the hexasaccharide neoagarohexaose on the basis of its chromatographic mobility, degree of polymerization, and position of D-galactose in the reducing end of the molecule.

Thus, the enzyme complex of *P. elongata* attacks the β-D-(1→4) bonds of the agarose molecule with production of a mixture of neoagaro-oligosaccharides.

EXPERIMENTAL

Enzyme production. — *Pseudomonas elongata* strain 11044, an agar-digesting microorganism obtained from the American Type Culture Collection, was maintained by slant cultures in Difco Nutritive Agar containing 3% of sodium chloride. After 48–72 h of incubation at 30°, each culture was suspended in a sterile solution of 3% sodium chloride and used for inoculation of 200 ml of soft-agar medium composed of 0.2% agar, 0.25% peptone, 0.2% K_2HPO_4, and 3% sodium chloride. These cultures were incubated for about 5–7 days at 30° until the agarolytic activity reached about 6 units per ml. The soft-agar cultures were then centrifuged and the supernatants used as a crude enzyme preparation.

Test for agarase activity. — The culture supernatants (0.1 ml) were incubated for 60 min with addition of 0.05 ml of 0.1M phosphate buffer (pH 7), and 0.1 ml of 1% agarose, in a final volume of 0.3 ml. Incubations were performed for 60 min at 40° and the reducing power was measured.

Agarose digestion. — The crude enzyme-preparation was adjusted to pH 7 and 2 volumes of 1% agarose were added. The mixture was incubated for 72 h at 40° in the presence of toluene. Ethanol (2 volumes) was then added and the mixture was centrifuged. The pellet was discarded and the ethanol removed under vacuum to afford a water-soluble oligosaccharide preparation from the digestion.

Isolation of oligosaccharides. — The oligosaccharide preparation was adsorbed on a charcoal–Celite column[14], which was then washed with 250 ml of distilled water. The oligosaccharides were then eluted with a linear gradient of ethanol. The mixing chamber contained 250 ml of water and the reservoir 250 ml of 55.6% (w/v) ethanol. Three oligosaccharides were obtained relatively pure from this column and no attempts were made to isolate the higher oligosaccharides that also were present in the mixture. The oligosaccharides isolated were further purified by paper chromatography (solvent *2*). Reducing power was measured by the method of Somogyi[15] and Nelson[16] and total sugars by a modification of the phenol–sulfuric acid method[17].

Enzyme unit. — One enzyme unit was defined as the amount of enzyme that is able to produce 0.1 μmole of products per h under the conditions of the test for agarase activity.

Reduction of oligosaccharides. — Aliquots of 0.05–0.20 μmoles of oligosaccharides were reduced for 1 h with 0.20 ml of a 0.125% solution of sodium borohydride. The excess borohydride was decomposed by addition of dilute acetic acid. When necessary, boric acid was removed by repeated evaporation of methanol from the residue. 3,6-Anhydro-L-galactose was determined by the method of Roe and Papadopoulos[18].

Hydrolysis of oligosaccharides. — Reduced and non-reduced oligosaccharides were hydrolyzed with M hydrochloric acid for 12 h in sealed tubes in a boiling water-

bath. Hydrolyzates were evaporated under vacuum, diluted with distilled water, and desalted with Amberlite MB-3 resin. The samples were spotted on paper chromatograms that were developed overnight in solvent *3*. When desired, the chromatographic areas corresponding to galactose were detected by co-chromatography with D-galactose (solvent *1*) and then cut off and eluted. Galactose was determined by the phenol–sulfuric acid method with appropriate correction by controls for the paper.

Chromatographic methods. — The following solvent-systems were used: 6:4:3 (v/v) butanol–pyridine–water (solvent *1*); 1:1:1 (v/v) butanol–pyridine–water (solvent *2*); 7:7:2 (v/v) heptanol–ethanol–water (solvent *3*). Spots were revealed by the alkaline silver nitrate method[19].

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