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ENZYMIC CLEAVAGE OF THE α-LINKAGES IN AGAROSE, TO YIELD AGARO-OLIGOSACCHARIDES*

KWAN S. YOUNG, SHYAM S. BHATTACHARJEE, AND WILFRED YAPHE
Department of Microbiology and Immunology, McGill University, Montreal, Quebec H3A 2B4 (Canada)
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ABSTRACT

An α-agarase was isolated from a Gram-negative marine bacterium that liquefies agar. The enzyme was purified by chromatography on diethylaminoethyl-cellulose (HO^- form) buffered at pH 7.2. The purified enzyme specifically cleaves (1→3)-α-L-linkages in agarose, yielding a homologous series of agaro-oligosaccharides. Agarotetraitol and agarohexaitol were characterized.

INTRODUCTION

Agar, an extract of red algae, consists of a spectrum of polysaccharides having three extremes in structure, namely, neutral agarose, pyruvated agarose with little sulfation, and a sulfated galactan[1]. Agarose, the gelling component of agar, is composed of alternating residues of 3-*O*-linked β-D-galactopyranose and 4-*O*-linked 3,6-anhydro-α-L-galactopyranose; this sequence of sugar residues is also present in other polysaccharide fractions to a varying degree. The agar polysaccharides are degraded by hydrolytic enzymes extracted from marine bacteria. The cleavage of the (1→4)-β-D-linkage produces neoagaro-oligosaccharides having D-galactose at the reducing end and 3,6-anhydro-L-galactose at the non-reducing end, whereas the scission of the (1→3)-α-L-linkage forms agaro-oligosaccharides having D-galactose at the non-reducing end and 3,6-anhydro-L-galactose at the reducing end (Fig. 1). β-Agarase, cleaving the (1→4)-β-D-linkage, has been shown to be useful in elucidating the structural features of polysaccharides present in agar by the characterization of neoagaro-oligosaccharides[2]. Although there have been several reports on β-agarase from different bacteria[3–9], cleavage of the (1→3)-α-L-linkage by an α-agarase has been reported only in two instances[6,10]. Ishimatsu and Kibesaki[10] claimed that an enzymic hydrolysis of agar with a preparation of bacterial agarase produced agarobiose, indicating that the enzyme was an α-agarase. However, these authors reported that the disaccharide did not have the same chromatographic properties as agarobiose obtained by mild hydrolysis of agar with acid, and well-characterized by Araki[11].

*Dedicated to Dr. Elizabeth Percival.

More recently, Yaphe *et al.*[6] have reported on an α-agarase isolated from a Gram-negative bacterium. We now present a fuller account of the α-agarase with respect to characterization of the agaro-oligosaccharides.

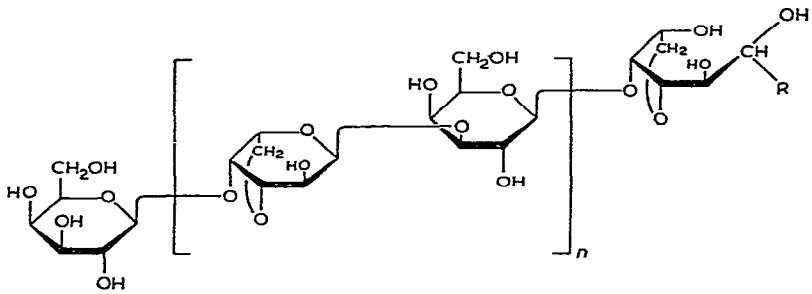

Fig. 1. Agaro-oligosaccharides (open-chain, hydrated, aldehydo form: R = OH): *A* (agarotetraitol: R = H, $n = 1$); *B* (agarohexaitol: R = H, $n = 2$).

RESULTS AND DISCUSSION

The cell-free culture medium of a Gram-negative bacterium that was found to liquefy agar contained agarase activity, as determined by the decrease in the viscosity of an agarose solution. The crude agarase preparation was obtained by precipitating the culture-supernatant with ammonium sulfate solution, and further purification was attained by chromatography on buffered diethylaminoethyl-(DEAE-)cellulose (Table I). The purified enzyme was stabilized by calcium ion and had optimum activity at pH 7.2. The enzyme did not hydrolyse neoagaro-biose and -tetraose.

TABLE I

PURIFICATION OF α-AGARASE

Step	*Volume (ml)*	*Units*	*Protein (mg)*	*Specific activity*[a]	*Yield (%)*
Culture medium	828	124,200	67.7	1835	100
Ammonium sulfate	10	93,650	17.4	5388	75.4
DEAE-Cellulose 0.1M $CaCl_2$	16	26,720	4.2	6423	21.5

[a]Specific activity: units per mg of protein.

The oligosaccharides in an enzymic hydrolysate of agarose were reduced with sodium borohydride and then hydrolysed with 0.5M sulfuric acid. Chromatographic analysis of the resulting acid hydrolysate showed the presence of 3,6-anhydro-galactitol and galactose, but no galactitol, suggesting that the oligosaccharides in the enzymic hydrolysate have a 3,6-anhydrogalactose residue at the reducing end. Under the condition of acid hydrolysis, 3,6-anhydrogalactose is decomposed. The enzymic hydrolysate yielded a ^{13}C-n.m.r. spectrum containing a signal at 91.42 p.p.m.

assigned to C-1 of the 3,6-anhydrogalactose residue at the reducing end of the agaro-oligosaccharides[12]; the spectrum did not contain any signal in the region of 93.8 and 97.8 p.p.m. due to C-1 of α- and β-galactose residues, respectively, thus confirming that the purified enzyme preparation specifically cleaves the (1→3)-α-L-linkage in agarose, yielding oligosaccharides having 3,6-anhydrogalactose as reducing-end residue.

The oligosaccharides present in the hydrolysate of agarose with α-agarase streak on thin-layer chromatography (t.l.c.) with solvents used to separate neoagaro-oligosaccharides[13]. However, a good resolution of the products was obtained either by t.l.c. in which cellulose was impregnated with freshly prepared 0.1M sodium bisulfite, or by chromatography of the reduced (borohydride) oligosaccharides. T.l.c. of the oligosaccharide alditols showed the presence of five components (*A–E*) having R_{GAL} values 1.08, 0.63, 0.39, 0.23, and 0.13, respectively, in solvent *A*. A plot of R_m value against increasing molecular weight gave a straight line, indicating that the oligosaccharides belong to a homologous series[14].

The borohydride-reduced enzymic hydrolysate was fractionated by chromatography on Sephadex G-25 and by cellulose t.l.c. Chromatographically pure, oligosaccharide alditols *A* and *B* were isolated for further characterization. G.l.c. analysis of acid hydrolysates of compounds *A* and *B* showed the presence of galactose and 3,6-anhydrogalactitol in both compounds. Treatment of acid hydrolysates of compounds *A* and *B* with sodium borohydride yielded 3,6-anhydrogalactitol and galactitol. On the basis of the area ratios of the g.l.c. peaks due to galactitol and 3,6-anhydrogalactitol, an estimate of the degree of polymerisation (d.p.) of the oligosaccharides was obtained (Table II); compound *A* was identified as agarotetraitol, and *B* as agarohexaitol (Fig. 1). The ^{13}C-n.m.r. spectrum of compound *A* contains four signals assigned to C-1 of a β-D-galactopyranosyl group at the non-reducing end (103.63 or 103.54 p.p.m.), and C-1 of 3-*O*-linked β-D-galactopyranosyl (103.63 or 103.54 p.p.m.), 4-*O*-linked 3,6-anhydro-α-L-galactopyranosyl (99.47 p.p.m.), and 4-*O*-linked 3,6-anhydro-L-galactitol (64.32 p.p.m.) residues[12]. Compound *B* yielded a ^{13}C-n.m.r. spectrum similar to that of compound *A*, but with different relative intensities of the

TABLE II

G.L.C. ANALYSIS OF ACID HYDROLYSIS PRODUCTS[a] OF AGARO-OLIGOSACCHARIDE ALDITOLS

Agaro-oligosaccharide alditol	*3,6-Anhydrogalactitol/galactitol ratio*	
	Peak-area ratio	*Molar ratio*[b]
Agarobiitol	5:7.0	1:1.0
Compound *A*	1:2.7	1:1.9
Compound *B*	1:3.8	1:2.7

[a]The acid hydrolysis products were treated with $NaBH_4$, converting galactose into galactitol; under the condition of acid hydrolysis, 3,6-anhydrogalactose decomposed. The products were converted into trimethylsilyl derivatives for g.l.c. analysis. [b]On the basis of the data obtained with agarobiitol, the peak-area ratios for compounds *A* and *B* were converted into molar ratio values.

C-1 signals. It has been demonstrated earlier that the integrated intensities of C-1 signals can be used for determination of the d.p. of agaro- and neoagaro-oligosaccharides[12]. Based on the ^{13}C-n.m.r. data, compounds *A* and *B* are characterized as agarotetraitol and agarohexaitol, respectively, confirming the evidence provided by g.l.c.

Analysis of the oligosaccharides proves that the α-agarase specifically cleaves (1→3)-α-L-linkages in agarose, yielding a homologous series of agaro-oligosaccharides.

EXPERIMENTAL

General methods. — Whatman No. 1 paper was used for paper chromatography. Microcrystalline cellulose (Camag D.S.O.) or cellulosepulver MN 300 (Macharey, Nagel and Co.) were used for t.l.c. The solvent systems were: (*A*) 1-butanol–ethanol–water (3:2:2), and (*B*) 1-butanol–acetic acid–water (4:1:2). Spots were revealed by the naphthoresorcinol[15], aniline hydrogen phthalate, or ammonical silver nitrate reagents. Good fractionation of agarase hydrolysates (α-agarase) was obtained by t.l c. on cellulose impregnated with freshly prepared 0.1M sodium bisulfite buffer[16] (pH 4.7). The buffer was freshly prepared by dissolving 9.5 g of sodium pyrosulfite and 8.8 g of sodium acetate trihydrate in 1 liter of distilled water. The pH was adjusted to 4.7 with acetic acid, and the t.l.c. plate was sprayed with the buffer and allowed to air-dry before the hydrolysate was applied. The plate was developed in solvent *B*. G.l.c. was performed on an 8-ft glass column containing 5% of XE-60 on Chromosorb-Q (100–200 mesh), by using the trimethylsilyl derivatives.

Preparation and purification of α-agarase. — Marine bacterium, strain GJ1B, was received from Dr. Galen E. Jones as a mixed culture which was collected at Great Bay–Little Bay estuarine complex, New Hampshire, U.S.A. The bacterium was separated from a non-agar decomposer on an appropriate medium and then lyophilized. GJ1B is a Gram-negative, rod-shaped, unipolar flagellated, non-pigmented bacterium which bores a hole through 5 mm of agar in a petri plate in 24 h. The inoculum was prepared by subculturing 3 times at 24-h intervals in a medium composed of 0.2% of washed agar, 2.5% of NaCl, 0.5% of $MgSO_4 \cdot 7H_2O$, 0.02% of $CaCl_2 \cdot 2H_2O$, 0.01% of KCl, 0.2% of $NaNO_3$, 0.01% of Na_2HPO_4, and 0.003% of $FeSO_4 \cdot 7H_2O$. The culture medium was incubated on a rotary shaker at 25°; 5 ml of the inoculum was transferred to flasks containing 200 ml of the above medium, and incubated under the same conditions for inoculum preparation. The cell-free culture medium obtained after centrifugation of the culture contained the agarase activity. The crude agarase preparation was purified by precipitation with ammonium sulfate (to a final concentration of 70% saturation). The precipitate was collected by centrifugation and dissolved in a small volume of tris(hydroxymethyl)aminomethane (Tris)-HCl buffer (pH 7.2, 0.01M). The enzyme solution was dialysed against the buffer containing 0.1M $CaCl_2$, and later against the same buffer without $CaCl_2$. The dialysed enzyme preparation was kept frozen when not in use. The α-agarase

preparation was further purified by chromatography on DEAE-cellulose (HO^- form) equilibrated with 0.01M Tris buffer at pH 7.2 (Table I), and eluted successively with 0.025M NaCl, 0.01M $CaCl_2$, and 0.1M $CaCl_2$. The enzyme was eluted with the 0.1M $CaCl_2$.

Measurement of agarase activity. — Qualitative determination of agarase activity was done by the iodine method. Equal volumes of enzyme preparation and 0.2% of washed agar in 0.01M Tris buffer (pH 7.2) were mixed and incubated in a water bath at 42°. At various intervals, one drop of I_2/KI solution was mixed with one drop of enzymic hydrolysate on a spot plate. The disappearance of a blue-black color (achroic point) indicated partial hydrolysis of the agar.

Quantitative estimation of agarase activity was effected by measuring the change in specific viscosity of an agar solution at 42° in 0.01M Tris-HCl buffer (pH 7.2).

A unit of enzyme activity is defined as that amount of enzyme which will halve the specific viscosity of 5 ml of 0.8% washed-agar solution in 100 min at 42°. In order to measure the enzyme activity accurately, the concentration of enzyme was such that it halved the specific viscosity in 10 to 30 min. For α-agarase, a linear relationship was found between the enzyme dilutions and the units of activity.

Preparation of agarose hydrolysate. — Agarose (1 g) was dissolved in Tris buffer (pH 7.2; 0.01M, 100 ml) at 100°. Purified agarase (~50 units) was added to the solution at 42°, and the mixture incubated until the achroic point was reached. The hydrolysate was allowed to cool from 42 to 25°, and then incubated overnight after addition of more enzyme (~20 units). Ethanol (2 vol.) was added to the final hydrolysate, and the mixture was centrifuged. The pellet was discarded, and the ethanol removed under reduced pressure to afford a mixture of agaro-oligosaccharides.

Isolation of agarotetraitol (compound A) and agarohexaitol (compound B). — A solution of sodium borohydride (100 mg) in water (2 ml) was added to the mixture of agaro-oligosaccharides (0.5 g in 1.5 ml of water) and allowed to react overnight at 4°. The excess of borohydride was decomposed by addition of dilute acid, the water was evaporated, and boric acid was removed by repeated evaporation of methanol from the residue. The mixture of agaro-oligosaccharide alditols was dissolved in a small volume of distilled water and fractionated by column chromatography on Sephadex G-25 (Pharmacia Fine Chemicals). Compounds *A* and *B* were isolated, and further purified by t.l.c.

Hydrolysis of oligosaccharides. — The oligosaccharide alditols were hydrolysed in 0.5M H_2SO_4 at 100° for 4 h. The hydrolysate was cooled, and the acid neutralized with barium carbonate. The precipitate was removed by centrifugation, and the supernatant solution was evaporated to dryness.

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