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Note

Isolation of agarase from *Littorina mandshurica* by affinity chromatography on Biogel A

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All known agarases cleave the β-(1→4)-linkage between D-galactopyranose and 3,6-anhydro-L-galactopyranose residues in agarose and related polysaccharides to give oligosaccharides of the neoagarobiose [*O*-(3,6-anhydro-α-L-galactopyranosyl)-(1→3)-D-galactopyranose] series. Agarases of microbial origin[1–4] have been used to obtain structural information on agarose[5,6], porphyran[7,8], and several other red-seaweed polysaccharides[9,10]. Unfortunately, the isolation and purification of agarase is a complex procedure[11,12].

We have recently shown that an extract of the hepatopancreas of the mollusc *Littorina mandshurica* possesses agarase activity[13]. However, before using the enzyme in the structural analysis of polysaccharides, it was desirable to remove other polysaccharidases and glycosidases present in the crude extract[14]. This paper describes the selective purification of the enzyme by means of affinity chromatography.

The agarase activity was assayed by the dyed-substrate technique. The substrate was prepared by treatment of agar with Remazol Brilliant Blue R[15]. The dyed agar so obtained is water-soluble, unlike the agar-azur from Calbiochem, and affords fairly reproducible results.

Bio-Gels A, insoluble derivatives of agarose, which are widely used for gel-permeation chromatography of biopolymers, were tested as biospecific agarase-adsorbing materials. In preliminary experiments (*cf.* Ref. 16), agarase was found to be adsorbed on Bio-Gel A-5m at low temperatures and pH 5.6 (optimal for the enzyme action), and was further released at increased ionic strength, at higher temperatures, or by pH changes.

The chromatography of the enzyme was studied on small columns containing 15 ml of Bio-Gel. A solution of 100 mg of the crude enzyme preparation in a dilute buffer (pH 5.6) was applied to each column. The column was washed with the same buffer until the removal of the non-adsorbing proteins was complete. This fraction possessed high cellulase [(1→4)-β-D-glucan glucanohydrolase, E.C. 3.2.1.4] and xylanase [(1→4)-β-D-xylan xylanohydrolase, E.C. 3.2.1.8], but negligible agarase,

activities. Agarase was then eluted under different conditions, which are listed in Table I together with the results obtained.

TABLE I

AFFINITY CHROMATOGRAPHY OF AGARASE[a]

Enzyme	*Packing*	*Elution conditions*	*Solution volume (ml)*	*Protein content (mg)*	*Total activity (%)*	*Specific activity of lyophilized preparations (units)*
Crude preparation	—	—	1	52	100	0.5
Purified preparation	Bio-Gel A-5m	Buffer *A*, pH 5.6, 13°	120	0.4	45	—
		Buffer *A*, pH 5.6 or 6.0, 20°	130	1.0	250–300	25–50
		Buffer 0.1*A*, pH 7.5, 20°	130	0.7–1.0	100–250	42[b]
	Bio-Gel A-0.5m	Buffer *A*, pH 5.6 or 6.0, 20°	260	0.5–1.0	260–310	22–34

[a]The column size and the chromatographic conditions are described in the Experimental. [b]This is the best result obtained at pH 7.5, usually, the specific activities in parallel experiments are subject to considerable variations.

Table I shows that complete elution of the enzyme takes place only at 20°. Both Bio-Gels used (A-5m and A-0.5m) selectively adsorbed agarase, but the former binds the enzyme less strongly and therefore a smaller volume of buffer was required for elution. Dilute buffer (pH 7.5) as eluant did not always give reproducible results, and better yields were obtained with concentrated buffers (pH 5.6 or 6.0) at 20°. The yield of agarase was 2% with respect to protein, but its total activity was 300% of that of the starting preparation. This fact is probably attributable to removal of enzyme inhibitor during chromatography.

The solutions of the purified enzyme were stable during 5–8 h at room temperature or dialysis for 24 h at 5°. Lyophilization decreased the activity 3–4-fold (see Table I), but partial freeze-drying or cold storage of frozen solutions during 3 weeks at −15° caused no loss of activity. The optimal pH for the purified agarase is 6.0, whereas that of the starting preparation is 5.6. However, the kinetic curve of the agar hydrolysis by the purified enzyme is similar to that obtained for the crude preparation[15]. Therefore, the determinations of purified agarase were carried out at pH 6.0.

The chromatographic conditions found in the preliminary experiments were employed for preparative isolation of the enzyme on a larger column. After partial desalting by dialysis, with subsequent lyophilization of active fractions, an agarase preparation was obtained having a specific activity of 25 units, and being completely

devoid of cellulase and xylanase activities. The properties of the purified enzyme will be described elsewhere.

EXPERIMENTAL

General methods. — Crude agarase from *Littorina mandshurica* was obtained as described earlier[13]; the protein content was 52%, and the specific activity was 0.5 unit. Bio-Gels A-5m (200–400 mesh) and A-0.5m (100–200 mesh) were purchased from BioRad Labs., Los Angeles, California. Chromatographic buffer solutions from 0.1M citric acid and 0.2M Na_2HPO_4 were twice diluted with water (solutions *A*). More-dilute solutions of concentrations 2, 5, and 10 times lower (0.5*A*, 0.2*A*, and 0.1*A*, respectively) were also used. pH-Determinations were made with a TTT-1 (Radiometer, Copenhagen) instrument. Spectrophotometric measurements were performed on a SF-4A (LOMO, Leningrad) spectrophotometer. Protein was determined by the method of Lowry *et al.*[17] with bovine serum albumin as standard.

Preparation of dyed agar. — Difco Bacto-Agar (50 g) was dissolved in water (1000 ml) at 100° and cooled to 60°. A solution of Remazol Brilliant Blue R (Calbiochem) (5 g) in water (200 ml) was heated to 60° and added to the agar solution. Dry Na_2SO_4 (120 g) was added to the mixture during 45 min with continuous stirring, followed by an aqueous solution of $Na_3PO_4 \cdot 12H_2O$ (6 g in 60 ml) heated to 60°. Stirring was continued for another 4 h (60°), and the mixture was left overnight at room temperature, then heated to 100° to dissolve the gel, cooled again, and frozen. After thawing on the cloth, the gel was dissolved in distilled water at 100°, and the freezing–thawing procedure was repeated 6 times. The purified gel was dissolved on heating in water (1.5 l), and the solution was cooled to 50° and poured with stirring into acetone (3 l) at 50°. The precipitate was filtered off, reprecipitated twice with acetone from aqueous solution, washed with acetone on the filter, and dried (yield, 37.8 g). According to the spectrophotometric data, the polysaccharide contained 1 dye residue per 54 monosaccharide residues.

Determination of enzyme activities. — (*a*) *Agarase.* To 1 ml of a 0.5% aqueous solution of dyed agar (prepared by heating a polysaccharide suspension in water to 100° with subsequent cooling to 40°), an accurately measured volume of the enzyme solution was added, and the mixture was diluted with buffer to a total volume of 2 ml. The mixture was incubated at 37° for 3 h and then treated with 4 ml of cold acetone. After centrifugation, the absorbance of the supernatant solution was measured at 590 nm against water (the blank value with respect to water is 0).

To determine the specific activity, the incubation under the above-described conditions (pH 5.6 for crude preparation and 6.0 for purified enzyme) was carried out for 50 min (the linear part of the kinetic curve). Specific activity is defined as the amount of the dye (μg) released from the dyed agar by 1 mg of the protein during 1 min.

(*b*) Cellulase activity was determined by the method of Pettersson and Porath[18], and xylanase activity by the method of Hashimoto *et al.*[19].

Conditions for affinity chromatography. — Bio-Gel A (15 ml) in 0.1*A* buffer (pH 5.6) was placed in a column (1.5 × 15 cm) having a water-jacket. The column was cooled to 5°, and a solution of the crude enzyme preparation (100 mg) in the same buffer (1 ml) was applied to the column. Then the column was washed with the same buffer until the effluent ceased to show absorbance at 280 nm (a fraction of 65 ml was obtained containing 98–99% of the starting protein, which had high cellulase and xylanase, but no agarase, activities). The column was warmed to 13° or 20° and washed with the appropriate buffer to complete removal of the protein. For determination of agarase activity, the pH of the eluate was adjusted to 6.0. The agarase solutions were dialysed against distilled water (5°, 24 h) and lyophilized, but 0.1*A* buffer solutions were lyophilized without dialysis. Data for protein determination and the corresponding enzymatic activities are listed in Table I.

Preparative-scale isolation of agarase. — A solution of the crude enzyme preparation (500 mg) in the 0.1*A* buffer (pH 5.6, 5 ml) was applied to a column (3.3 × 15 cm) containing 50 ml of Bio-Gel A-5m at 5°. After elution with the same buffer, a fraction (350 ml) was obtained which contained 99% of the applied protein and had no agarase activity. The elution was continued at 20° with buffer *A* (pH 6.05, 800 ml) to give a fraction containing 366% of the starting agarase activity. Neither cellulase nor xylanase activities were detected in this fraction. After dialysis (5°, 24 h) and lyophilization, the dry residue was obtained (3.5 mg of protein, 91% of the starting agarase activity) having specific activity of 25 units.

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