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DEGRADATION OF AGAROSE GELS AND SOLUTIONS BY BACTERIAL AGARASE

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ABSTRACT

Agarase, prepared by affinity chromatography of a culture filtrate of an agar-degrading *Pseudomonas*-like bacterium, solubilized 72% of beaded agarose gel after incubation for 48 h. The gel structure was maintained but, in combination with mechanical disintegration, 95% of the gel could be solubilized. The molecular-sieving properties of the partially degraded agarose gels were changed. The molecular weight selectivity curve for dextran was not parallel to that of the original gel. The molecular weight distribution of the carbohydrate chains in agarose and partially degraded agarose was measured by gel filtration of melted samples at 60° on Sepharose CL-4B. It was found that degradation of agarose gel is a two-step reaction. The first step is a rapid hydrolysis of very long polysaccharide chains to chains having a molecular weight of 10^4 to 10^5, indicating restrictions due to the gel structure. The second step is a slower hydrolysis of these chains into soluble oligosaccharides. Independently of the degree of degradation, some very large polysaccharide chains are left, and these chains may be of importance for maintaining gel structure. Enzymic degradation of melted agarose resulted in a rapid depolymerization to soluble oligosaccharides, mainly neoagarotetraose and neoagarohexaose.

INTRODUCTION

Agarose is the relatively neutral constituent of agar and it has the greatest gelling ability[1]. It is an unbranched polysaccharide having an alternating sequence of 3-linked β-D-galactopyranosyl and 4-linked 3,6-anhydro-α-L-galactopyranosyl residues[2]. To a small extent, some galactopyranose residues are replaced by 4,6-*O*-(1-carboxyethylidene)-D-galactopyranose residues, or by methylated or sulphated sugars[3].

A model for the three-dimensional structure of the polysaccharides in the gel network has been proposed by Arnott *et al.*[4], in which the chains form double helices that are, in turn, arranged in bundles. As a given polysaccharide chain can participate in many different double helices, a continuous gel network is formed. The average width of the bundles has been estimated[5,6] to be 7 to 11 helices, corresponding to ~50 Å.

Very little is known about the mechanism of enzymic degradation of agarose gels, although they are good substrates for agar-degrading bacteria. The normal way of detecting the action of such bacteria is to look for holes in the gel around the bacterial colonies. This shows that bacterial enzymes have solubilized the agar gel close to the bacterial cell colonies. Other observed effects of agarase are a clearing effect on the gel and the inability of the gel to bind iodine after agarase treatment[7].

The pronounced hysteresis in setting and melting of agarose gels makes them special enzyme substrates. In the temperature interval from ~45° to 90°, agarose can exist as a sol with the polysaccharide chains in random coils, or as a gel with a specific three-dimensional structure. Thus, it is possible to study how agarase degrades different forms of the substrate. The arrangement of the polysaccharide chains in dense, water-insoluble[8] bundles of double helices partially transforms the soluble enzyme–substrate system into a two-phase system similar to cellulase–cellulose.

The agarase used in this investigation was obtained from culture filtrates of an agar-degrading *Pseudomonas*-like bacterium, which has cell-bound agarase during exponential growth[9]. Enzyme is released into the medium in the stationary growth phase, and can be concentrated and purified by affinity chromatography on divinyl sulphone cross-linked agarose[10]. This agarase has strong gel-degrading properties. Holes were formed in a 1 % agarose plate by drops of concentrated enzyme. A practical application of the enzyme is that glioma cells cultured in agarose can be freed from gel residues[11].

We now describe the enzymic action of the partially purified agarase on different substrates and with different conditions of incubation. The hydrolysis of melted agarose and of agarose gels is related to the molecular weight distribution of the residual polysaccharide chains, and a model for the degradation of agarose gels is proposed.

EXPERIMENTAL

Materials. — Agarose and Sepharose 4B and 6B were gifts from Pharmacia Fine Chemicals, as were the characterized dextran fractions DX2000, DX500, DX150, DX70, DX40, and DX10. Precoated DC-Plastikrolle cellulose was obtained from Merck, and Bio-Gel P-2 from Bio-Rad Laboratories. Agarase from *Pseudomonas atlantica* was a gift from Dr. M. Duckworth, and agarose, carrageenan, porphyran, and segmented agarose[8] from Dr. D. A. Rees. Some of the polysaccharide samples have been described in more detail elsewhere[4]. *Sample 1* was commercial agarose from Marine Colloids Inc., Code nr REX 5468 (sample 1 in Ref. 4). *Sample 2* was agarose from *Gracilaria compressa* containing 3% of pyruvate groups (sample 7 in Ref. 4). *Sample 3* was agarose sulphate extracted from *Gloiopeltis furcata* REX 5804 (sample 8 in Ref. 4). *Sample 4* was alkali-modified REX 5804. *Sample 5* was alkali-modified porphyran with ~50% of D-galactose residues 6-*O*-methylated (sample 3 in Ref. 4). *Sample 6* was agarose extracted from *Gracilaria foliifera*, similar in methoxyl content to sample 5. *Sample 7* was λ-carrageenan extracted from *Chondrus*

crispus, Marine Colloids REX 5400. *Sample 8* was ι-carrageenan extracted from *Agardiella tenera*, Marine Colloids RENJ 5223. *Sample 9* was κ-carrageenan extracted from *Chondrus crispus*, Marine Colloids REX 5401 alkali-modified.

Enzyme. — Agarase from the culture filtrates of an agar-degrading *Pseudomonas*-like bacterium was obtained by affinity chromatography on divinyl sulphone cross-linked Sepharose 4B". The enzyme concentration in the preparation used throughout this investigation was 1.3 mg of agarase/ml, estimated by the absorbance[10] at 280 nm.

Preparation of partially degraded Sepharose 6B for gel-filtration studies. — Two flasks containing 35 g (wet weight) of washed Sepharose 6B beads and 75 ml of 50mM cacodylate buffer (pH 6.8) were slowly shaken at 30°. To one of the flasks was added 100 μl of agarase, and the incubation was then continued for 48 h.

The gels were packed in columns, and 100-μl samples of a solution containing dextrans DX2000, DX500, DX150, DX70, DX40, DX10, and picrylsulphonic acid (TNBS) were applied. The distribution coefficient K_d was calculated as $K_d = (V_e - V_o)/(V_i - V_o)$, where V_i is elution volume of TNBS. The straight line of the plot K_d *vs.* log M_r was calculated by the least-squares method.

Degradation of agarose gel under different conditions. — Sepharose 6B (2 g) was suspended in 25mM cacodylate buffer (8 ml, pH 6.8) and slowly shaken at 30°. Agarase (100 μl) was added and incubated with the gel for 48 h. The gel residues were then washed with water on a glass filter-funnel (Jena Glass D3) and air-dried at 30°. The samples were further dried in an oven at 90°, with a constant air-flow for 3 h, and then placed in a desiccator over P_2O_5.

Degradation of the same amount of Sepharose 6B with agarase was also carried out in a 10-ml Diaflo cell (Amicon) equipped with a PM-10 membrane. A continuous flow of 25mM cacodylate buffer (pH 6.8, ~5 ml/h) was used during the incubation time of 48 h. Similar experiments were also done with mechanical disintegration of the beads, using a magnetic stirrer in the cell. As blank experiments, the beads were disintegrated using a continuous buffer flow.

Molecular weight distribution of polysaccharide chains. — Sepharose 4B beads were washed with water on a glass filter-funnel and suspended in 50mM cacodylate buffer (pH 6.8) at a concentration of 0.25 g of gel/ml of suspension. The agarose samples were either melted and then incubated at 50°, or maintained at 50° before incubation with agarase. The degree of degradation was measured by determination of the reducing sugar, as described in the standard assay[10]. Melted Sepharose 4B was degraded to levels of 0.8 and 2.0 μmol galactose equivalents. Sepharose beads were hydrolyzed to 1.1 and 1.7 μmol galactose equivalents. The third sample of Sepharose beads was incubated at 25° for 120 h with 10 μl of enzyme.

Agarose segments were prepared by Smith degradation of melted agarose, as described by Dea *et al.*[8]. The segments have the same optical-rotation properties as agarose at the sol–gel transitions, but precipitate at the gel setting point without gel formation. Agarose segments (5 mg) were dissolved by melting in 50mM phosphate buffer (pH 6.8, 1 ml) and analyzed as for the other agarose samples.

Gel filtration of melted agarose samples (1 ml) was performed on Sepharose CL-4B packed in a Pharmacia K16 column maintained at 60°. The column was calibrated by gel filtration of dextran fractions DX2000, DX500, DX150, DX70, DX40, DX10, and sucrose.

The straight line for the plot of K_d *vs.* log M_r was calculated by the least-squares method. The carbohydrate content in each sample was measured by the orcinol–H_2SO_4 method[12] with galactose as standard.

Hydrolysis of different algal galactans. — The different polysaccharide samples were dissolved to give 1% solutions in 50mM cacodylate buffer (pH 6.8) and incubated at 50° with agarase for 10 min. The activity was determined as in the standard assay, and the results were expressed as percentages of the activity obtained with agarose as substrate.

Gel filtration of soluble oligosaccharides. — Degradation of melted agarose (80 mg) in 50mM cacodylate buffer (pH 6.8, 6 ml) was continued until no gel residue was found after boiling and cooling. The samples were lyophilized, dissolved in water (1 ml), and centrifuged. The supernatant solution was concentrated in *vacuo* to ~200 µl before being applied to a Bio-Gel P-2 column in water. The column was calibrated with glucose, cellobiose, and cellotriose···cellohexaose dissolved in water (100 µl).

Thin-layer chromatography. — T.l.c. was performed as described by Duckworth and Yaphe[13] on precoated DC-Plastikrolle cellulose. The solvent was 1-butanol–ethanol–water (1:1:1), and sugar components were detected with the naphthoresorcinol reagent[13]. The plot of $R_m = \log[(1/R_F) - 1]$ *vs.* degree of polymerization[14] ($\overline{d.p.}$) was made for the oligosaccharides obtained by enzymic hydrolysis of agarose.

RESULTS

Degradation of agarose gels

Gel-filtration properties of partially degraded agarose gels. — Incubation of 35 g of Sepharose 6B with 100 µl of agarase for 48 h at 30°, with slow shaking, resulted in a 12% swelling of the gel. The dry weight of agarose in the Sepharose 6B gel was 5.8%, and in the agarase-treated gel 3.9%.

The properties of the partially degraded Sepharose gel were studied by molecular-sieve chromatography of characterized dextran fractions. The K_d value of the dextran fraction was plotted *versus* log M_r (Fig. 1). The plot for agarase-degraded Sepharose 6B is not parallel with that for Sepharose 6B. With dextran $M_r = 1 \times 10^4$, the gel separates approximately as Sepharose 4B, but for dextran of high M_r, the separation corresponds to Sepharose 2B.

Effects of different incubation conditions. — After incubation of 2 g of Sepharose 6B with 100 µl of agarase for 48 h, the dry weight of the gel material was measured. As shown in Table I, 65 ±3% of Sepharose 6B was solubilized in batch experiments. Using the same amount of substrate and enzyme in a 10-ml Diaflo cell equipped

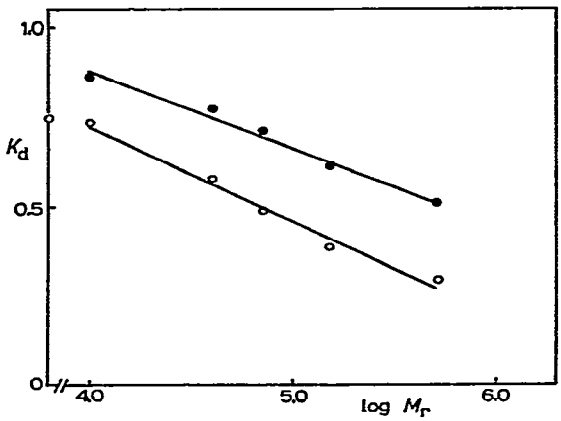

Fig. 1. The dextran molecular weight–selectivity curve for Sepharose 6B (○—○) and agarase-treated Sepharose 6B (●—●). The gels were packed in columns, 1.0 × 48.7 cm for Sepharose 6B, and 1.0 × 42.4 cm for the agarase-treated gel. The flow rate of 50mM phosphate buffer (pH 6.8) was 6 ml/h, and 100-μl samples of dextran DX2000, DX500, DX150, DX70, DX40, DX10, and picryl-sulphonic acid (TNBS) were applied.

TABLE I

DRY-WEIGHT DETERMINATIONS OF GEL RESIDUES AFTER INCUBATION[a] OF AGARASE AND SEPHAROSE 6B

Conditions	*Solubilized gel* (%)
Batch	0
Batch, agarase	65 ± 3
Diaflo cell, buffer flow, agarase	72 ± 4
Diaflo cell, buffer flow, stirred, agarase	95 ± 4
Diaflo cell, buffer flow, stirred	5 ± 2

[a]For 48 h at 30°.

with an Amicon PM-10 membrane and with continuous buffer flow, 72 ±4% of the gel was degraded. When the gel was disintegrated in the cell with magnetic stirring during the incubation, 95 ±4% was solubilized. In control experiments in which no enzyme was added, the gel was completely homogenized by the magnetic stirrer, but only 5 ±2% was eluted from the cell. In all cases with no mechanical treatment of the gel, the beaded structure remained intact.

Analysis of polysaccharide chain-lengths. — The molecular weight distribution of the polysaccharides in the agarose samples was analyzed by gel filtration of melted samples at 60° on Sepharose CL-4B. The enzyme substrate, Sepharose 4B, showed a wide distribution curve (Fig. 2). Most of the material was eluted at volumes corresponding to M_r 1×10^5–1×10^6, but larger particles were also found. Up to 6% of the carbohydrate content had $M_r < 1 \times 10^4$.

Fig. 2 also shows a gel-filtration experiment with agarose segments. Most of the material was eluted at a volume corresponding to 1×10^4 in M_r.

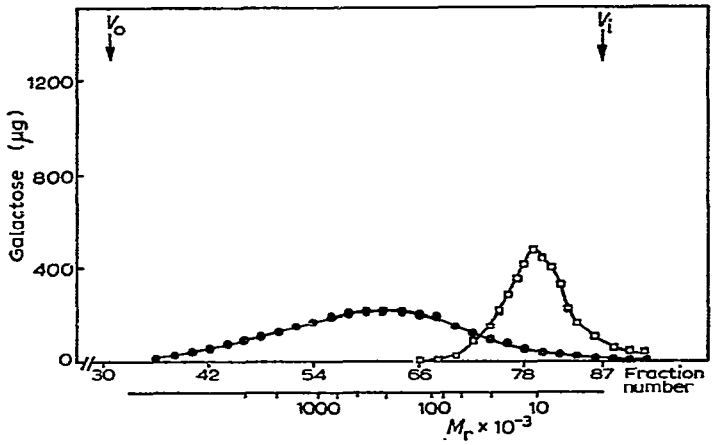

Fig. 2. Gel filtration of melted agarose samples (1 ml) on Sepharose CL-4B at 60° in 50mM phosphate buffer (pH 6.8); column, 1.6 × 88 cm; flow rate, 9.6 ml/h. Fractions (1.9 ml) were collected, and analyzed for carbohydrate by the orcinol–H_2SO_4 method with galactose as standard. The column was calibrated with DX2000, DX500, DX150, DX70, DX40, DX10, and sucrose: ●—●, Sepharose 4B; □—□, agarose segments.

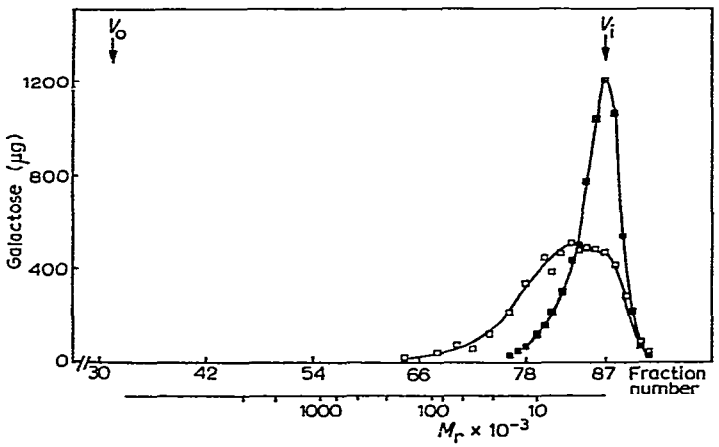

Fig. 3. Gel filtration (as in Fig. 2) of Sepharose 4B degraded by agarase as a *solution* at 50°: □—□, Sepharose 4B degraded to 0.8 μmol galactose equivalent; ■—■, Sepharose 4B degraded to 2.0 μmol galactose equivalents.

Enzymic degradation of agarose *solutions* was performed at 50°, and the molecular weight distributions of these samples are shown in Fig. 3. The samples were degraded to 0.8 and 2.0 μmol galactose equivalents, respectively. In the last sample, almost no polysaccharide chains with M_r greater than 1×10^4 were found. The first sample shows a wider distribution of material from 1×10^5 in M_r to small oligosaccharides, but most material had $M_r < 1 \times 10^4$.

Enzymic degradation of agarose *gels* was made at 50° to 1.1 and 1.7 μmol galactose equivalents, respectively, and the elution patterns can be seen in Fig. 4.

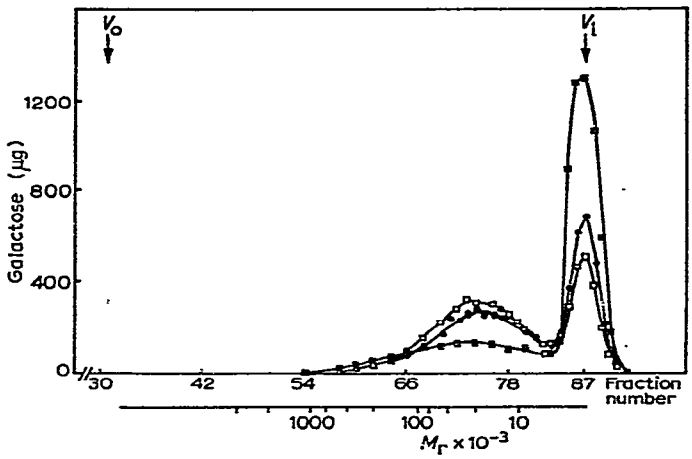

Fig. 4. Gel filtration (as in Fig. 2) of Sepharose 4B degraded as a *gel* at 50°: □—□, Sepharose 4B degraded to 1.1 µmol galactose equivalents; ●—●, Sepharose 4B degraded to 1.7 µmol galactose equivalents; ■—■, Sepharose 4B degraded at 25° for 120 h.

TABLE II

AGARASE ACTIVITY MEASURED WITH DIFFERENT POLYSACCHARIDES AS SUBSTRATES UNDER STANDARD ASSAY CONDITIONS

Polysaccharide sample	*Agarase activity* (%)
1. Commercial agarose (Marine Colloids REX 5468)	100
2. Agarose from *Gracilaria compressa* (3% of pyruvate groups)	88
3. Agarose sulphate from *Gloiopeltis furcata* (Marine Colloids REX 5804)	12
4. Alkali-modified REX 5804	9
5. Porphyran, alkali modified (50% of D-galactose residues 6-*O*-methylated)	40
6. Agarose from *Gracilaria foliifera* (50% of D-galactose residues 6-*O*-methylated)	59
7. Lambda carrageenan from *Chondrus crispus* (Marine Colloids REX 5400)	0
8. Iota carrageenan from *Agardiella tenera* (Marine Colloids RENJ 5223)	1
9. Kappa carrageenan from *Chondrus crispus* (Marine Colloids REX 5401), alkali-modified	5

The third curve represents the same type of degradation, except that the temperature was 25° and the incubation time 120 h. The elution pattern is clearly distinguishable from that obtained with melted agarose as substrate. Two peaks were found and most of the material in one of them was eluted at a position corresponding to M_r 2.5×10^4. The other peak was eluted at the total volume of the column and comprised small oligosaccharides. The three curves show that, even after prolonged incubation, some material of M_r up to 5×10^5 was left, and that the enzyme degraded only material with M_r 10^4–10^5 into soluble oligosaccharides.

Degradation of melted agarose

Hydrolysis of different algal galactans. — The activity of the enzyme was measured under standard assay conditions, with melted agarose, carrageenan, and porphyran as the substrates. The results are shown in Table II, where the activity is expressed as a percentage of the activity towards agarose.

Preparation of oligosaccharides of agarose. — Oligosaccharides prepared by enzymic hydrolysis of melted agarose were separated on Bio-Gel P-2 in water; Fig. 5 shows the chromatogram. The column was calibrated with glucose and the cellodextrins G_2 to G_6. The calibration plot for G_1 to G_6, K_{av} *versus* log M_r, gives a slightly curved line (Fig. 6). The main material was eluted at the volumes corresponding to a tetrasaccharide and a hexasaccharide. Extrapolation of the plot of K_{av} to values corresponding to the octasaccharide and decasaccharide indicates that these are the molecular sizes of the two smaller peaks.

Analysis of oligosaccharides from agarose. — Material from the different peaks after gel filtration on Bio-Gel P-2 was analyzed by t.l.c. Small amounts of impurities were detected, comprising the oligosaccharides nearest in d.p. The hexasaccharide peak and larger oligosaccharides all contained small proportions of a component that was faster moving than the disaccharide and had R_{Gal} 1.35. The same spot was also found in samples of agarase-degraded, alkali-treated porphyran which had

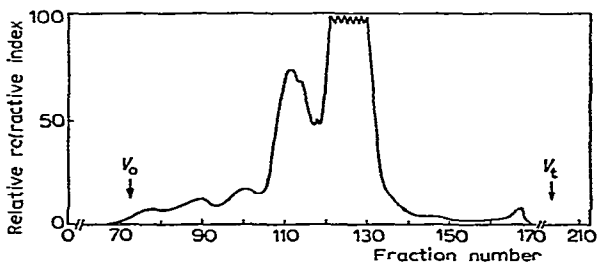

Fig. 5. Gel filtration of soluble oligosaccharides obtained by enzymic hydrolysis of agarose (80 mg) on Bio-Gel P-2 in water; column, 1.0 × 131.5 cm, with V_t 105 ml, and flow rate 3.9 ml/h. Fractions (0.5 ml) were collected after the effluent had been monitored by a refractometer.

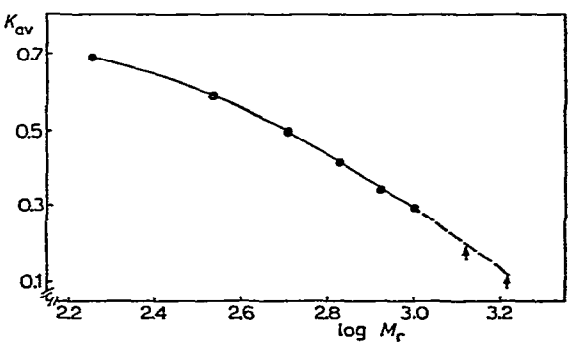

Fig. 6. The cellodextrin molecular weight–selectivity curve for Bio-Gel P-2. The arrows indicate the log M_r for an octasaccharide and a decasaccharide.

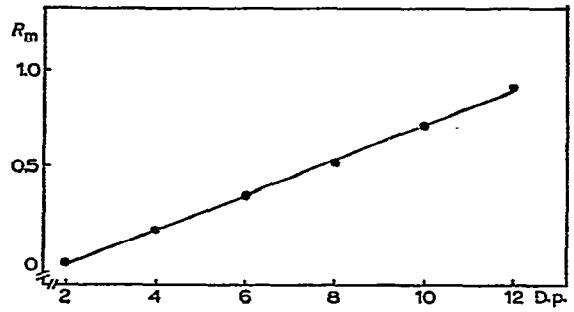

Fig. 7. The plot of $R_m = \log [(1/R_F) - 1]$ *versus* $\overline{\text{d.p.}}$ for the oligosaccharides obtained by enzymic hydrolysis of agarose.

50% of the D-galactose residues 6-*O*-methylated. The product is thus probably 6-*O*-methylneoagarobiose, which is known to move faster than neoagarobiose in this t.l.c. system. The octasaccharide and decasaccharide contained small proportions of the different saccharides from $\overline{\text{d.p.}}$ 2–10.

A plot of $R_m = \log [(1/R_F) - 1]$ *versus* $\overline{\text{d.p.}}$ has been found to be a straight line for homologous series of oligosaccharides[14]. Such a plot for the different oligosaccharides can be seen in Fig. 7, and a straight line was obtained. The R_{Gal} values for oligosaccharides with $\overline{\text{d.p.}}$ 8, 10, and 12 agreed well with the values for the neoagarosaccharide series[13].

DISCUSSION

Agarase prepared by affinity chromatography of culture filtrates from our agar-degrading, *Pseudomonas*-like bacterium was earlier shown to have strong gel-degrading properties[11]. This agarase preparation is easy to obtain and, although it is not pure, the contaminants are of high molecular weight[10]. Two different agarose-degrading enzymes have been found on gel filtration of this agarase preparation but, as they may cooperate in the degradation of agarose gels, they were not separated for this study.

The degradation of agarose gels can be analyzed in two ways. First, an analysis of the bulk properties of the gel by molecular-sieve chromatography and also by measuring the amount of solubilized gel material under different hydrolyzing conditions. Second, by analyses of the polysaccharide chains that give the gel its properties, and examining how they change after hydrolysis. The results may then be discussed in terms of models for the agarose gel structure[4,8].

The incubation of a relatively large volume of Sepharose 6B with enzyme resulted in swelling of the gel, and the gel became more transparent. Syneresis[4] in agarose is a result of tensions in the gel formed when one polysaccharide chain is incorporated in many different double helices forming a gel network. When an enzyme splits glycosidic bonds in the gel, the tensions are lowered and water is taken up by the gel. The light-scattering properties of agarose gels depend on the size of the

aggregates of double helices[15]. Clearing of the gel by partial enzymic degradation shows that the size of these aggregates has decreased.

The molecular-sieving properties of the partially degraded agarose gel were studied by gel filtration of well-defined dextran fractions. A plot of K_d *versus* log M_r was not parallel with the corresponding plot for Sepharose 6B. Although the dry weight of the partially degraded gel was 3.9%, molecules with $M_r \sim 10^4$ and very large molecules were eluted approximately as from an agarose gel of 4% and 2%, respectively. Polysaccharide chains surrounding big voids in the gel seem to be more rapidly degraded than polysaccharide chains surrounding small voids. The dominating agarase in the preparation, agarase I[10], has M_r 212,000, and such large proteins cannot penetrate the whole gel volume in Sepharose 6B. The concentration of the enzyme thus becomes higher in larger voids in the gel than in the denser parts.

The influence of different substituents in agarose on the activity of an agarase from a *Cytophaga* sp. has been studied by Duckworth and Turvey[16]. They found that 6-*O*-methyl-β-D-galactose linkages in agarose were hydrolyzed, but that sulphated oligosaccharides must have one unmodified neoagarotetraose residue in order to be hydrolyzed. Similar results were obtained with our agarase: sulphated agarose was hydrolyzed to a very low extent, but alkali-treated porphyran and agarose rich in 6-*O*-methyl-D-galactose residues were hydrolyzed at a reduced rate (Table II).

Incubation of relatively large amounts of enzyme with Sepharose 6B was performed in test tubes or a Diaflo cell with continuous buffer flow. It was possible by this method to determine whether product inhibition or mechanical disintegration of the beads by a magnetic stirrer was important for the total hydrolysis of agarose gel. The results show that product inhibition is low and that only 7% more gel was degraded in the Diaflo cell than in batch experiments. More important is the mechanical treatment of the agarose beads, as 95% of the gel material was solubilized in combination with enzyme, and 5% by mechanical disruption only. Agarose sulphate was degraded to a very low extent (Table II), indicating that the sulphate groups in the gel inhibit degradation of the polysaccharide chains and also protect other degradable chains from the enzymes. When the gel was broken by mechanical treatment, these hidden polysaccharide chains were exposed to enzyme and hydrolyzed.

Analysis of the molecular weight distributions of the polysaccharide chains in different agarose samples was performed by gel filtration of melted agarose at 60° on Sepharose CL-4B. At 60°, the polysaccharide chains in agarose have the same conformation as they have after melting of the gel at higher temperatures[4]. Thus, the polysaccharide chains are supposed to be in the random coil form at that temperature, and calibration of the column with defined dextran fractions should therefore give a fairly good estimation of their molecular weights.

When the agarose in Sepharose 4B was analyzed, a very wide distribution of molecular weights was found for the polysaccharide chains (Fig. 2). Up to 6% of the total material was eluted at positions corresponding to $M_r < 1 \times 10^4$. This fraction of low molecular weight may be a result of the treatment of agarose during the production of Sepharose. Hickson and Polson[17] studied some physical characteristics

of agarose and found it to be fairly monodisperse, which is not found on gel-filtration analysis.

In order to understand how the gel is degraded, it is important to know which parts of the structure are first hydrolyzed by agarase. Degradation of Sepharose 4B as *beads* was compared to degradation of the melted beads (Figs. 3 and 4). Analysis of such degraded gel samples always showed two peaks independent of the degree of degradation. One corresponds to oligosaccharides, was eluted at the total volume of the column, and increased in area with the degree of degradation. The other peak is rather broad and the material is very slowly degraded into soluble oligosaccharides on long incubation. The main material in this peak was eluted at a position corresponding to M_r 25,000. These results clearly show a restriction of the enzymic activity by the gel structure. Some polysaccharide chains having $M_r > 10^5$ are not degraded to smaller polysaccharide chains, and these few non-degradable chains are probably of great importance for maintaining agarose as a gel when most of the polysaccharide material is solubilized.

In order to find out if the first point of attack is at the end of each double helix, agarose segments were analyzed by gel filtration at 60° on Sepharose CL-4B. These segments were prepared by Smith degradation of the polysaccharide chains in agarose, which are thought to be hydrolyzed at the position of the helix termination[8]. The main material was eluted at a position corresponding to M_r 1×10^4, which shows that the formation of the broad peak is not dependent on breakage between each double helix.

The finding that most segments have M_r 1×10^4 means that ~6% of the polysaccharide material in the native agarose is probably bound in only one double helix. These polysaccharides may represent part of the material that was eluted from the PM-10 membrane in the Diaflo cell when the gel beads were mechanically disrupted during buffer flow.

In conclusion, degradation of agarose gels is proposed as a two-step reaction. First, a rapid hydrolysis of very large polysaccharide chains to molecules with M_r 10^4–10^5, and then a slower solubilization of this material. Although up to 72% of the agarose gel can be solubilized by agarase (Table II), the gel structure is maintained. It contains long polysaccharide chains with M_r 10^5–10^6 which are not degradable by enzymes. Mechanical disintegration of the gel during enzymic hydrolysis liquefies the gel almost completely; the long polysaccharide chains are probably broken by this treatment, during which agarase can attack chains that earlier were hidden. The polysaccharide chains connecting different aggregates are thought to contain a higher-than-average proportion of "kinks"[4] (D. A. Rees, personal communication). These anomalies in the primary structure may prevent agarase from hydrolyzing the polysaccharide chains, thus leaving long chains that can maintain the gel structure.

The soluble oligosaccharides obtained by hydrolysis of melted agarose were fractionated on Bio-Gel P-2, in order to study the distribution of oligosaccharides. The column was calibrated with cellodextrins having $\overline{\text{d.p.}}$ 1–6, and the calibration curve was extrapolated to $\overline{\text{d.p.}}$ 10. The two main peaks were eluted at the position

of a tetrasaccharide and of a hexasaccharide, and the two other peaks correspond to octasaccharide and decasaccharide.

The soluble products formed by enzymic degradation of agarose were shown by t.l.c. to be a homologous series of oligosaccharides, since the plot of R_m *versus* $\overline{\text{d.p.}}$ was linear. The R_{Gal} values for the products corresponding to $\overline{\text{d.p.}}$ 8, 10, and 12 were compared to the value for the neoagarosaccharide series given by Duckworth[13]. The small difference probably depends on the butanol concentration in the t.l.c. system, which is very critical.

In order to confirm that agarase actually hydrolyzes the β-linkage in agarose, the products were compared with those formed by agarase from *Pseudomonas atlantica*, which is known to be a β-agarase[18]. When this enzyme hydrolyzed agarose, as well as the octasaccharide from the gel-filtration step, neoagarotetraose was formed and its mobility in t.l.c. was identical to that of the tetrasaccharide from gel filtration.

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