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The Hydrolysis of Algal Galactans by Enzymes from a *Cytophaga* Species

BY J. R. TURVEY AND J. CHRISTISON
Department of Chemistry, University College of North Wales, Bangor

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1. Two bacteria were isolated from sea water by the enrichment culture technique, both of which could utilize the galactan sulphate, porphyran, as sole source of carbon. 2. From the cells of one bacterium, classified as a *Cytophaga* sp., hydrolytic enzymes were isolated. 3. Partial purification of the enzymes is described and some of the properties of the principal enzymes have been studied. 4. The action of the enzymes on several galactan sulphates of red algae suggests that an agarase is present in the mixture.

Though some of the structural features of algal galactans can be elucidated by chemical methods, others only become apparent when enzymological methods are also used. This is particularly true when the galactan contains residues of 3,6-anhydrogalactose, the glycosidic linkages of which are preferentially hydrolysed by acidic reagents. For the agarose component of agar, chemical methods have led to the isolation of derivatives of 4-*O*-β-D-galactopyranosyl-3,6-anhydro-L-galactose (agarobiose), thus establishing the nature of one of the polymeric linkages. It was not, however, until Araki & Arai (1956, 1957), using an enzyme from a marine bacterium, isolated from agar the disaccharide 3-*O*-(3,6-anhydro-α-L-galactopyranosyl)-D-galactose (neoagarobiose) and the tetrasaccharide 4^2-β-neoagarobiosylneoagarobiose (neoagarotetraose) that a structural pattern, consisting of 3-linked β-D-galactose units alternating with 4-linked 3,6-anhydro-α-L-galactose units, could be discerned.

Porphyran, the galactan sulphate from the red alga, *Porphyra umbilicalis*, has certain structural features in common with agarose, namely residues of 3,6-anhydro-L-galactose and D-galactose, but differs from agarose in also containing L-galactose-6-sulphate and 6-*O*-methyl-D-galactose (Peat, Turvey & Rees, 1961). In spite of these differences, it is structurally related to agarose in that L-galactose 6-sulphate is known to be the biological precursor of 3,6-anhydro-L-galactose in this alga (Rees, 1961*a*) and in that 6-*O*-methyl-D-galactose seems to replace D-galactose in parts of the molecule. An alternating sequence of either L-galactose 6-sulphate or 3,6-anhydro-L-galactose with either D-galactose or the 6-*O*-methyl ether, proposed for the structure (Turvey & Williams, 1964; Rees & Conway, 1962), has since been established (Anderson & Rees, 1965). The precise location of the various units cannot, at present, be made, but it has been suggested (Turvey, 1961) that the 3,6-anhydrosugar occurs mainly in the periphery of the molecule, the central portion containing most of the L-galactose 6-sulphate. In an effort to obtain further information on the structure of porphyran and related algal galactans, a search was made for bacterial enzymes capable of degrading algal galactans. Such an enzyme system is described in this paper.

MATERIALS AND METHODS

Polysaccharides. Porphyran was obtained from dried and milled *Porphyra umbilicalis* by the method of Peat *et al.* (1961). Alkali-treated porphyran was prepared by the method of Rees (1961*b*) and the galactan of *Corallina officinalis* by the method of Turvey & Simpson (1966). Crude galactans from *Furcellaria fastigiata* and *Laurencia pinnatifida* were obtained by extraction of the air-dried alga with boiling water and precipitation of the polysaccharide with ethanol (2 vol.): κ- and λ-carrageenan were kindly given by Dr D. A. Rees and agarose was kindly given by Dr W. Yaphe. The whole agar used was Japanese agar (British Drug Houses Ltd., Poole, Dorset).

Analytical methods. Protein nitrogen was determined by the method of Warburg & Christian (1942). Relative bacterial numbers were estimated by measuring E_{660} in 1·5cm. circular cells of the culture after given times of incubation, and comparing with the E_{660} value at zero time.

Measurement of enzyme activity. Two methods were used, one based on the decrease in viscosity of a substrate solution, and the other on the estimation of reducing power produced by enzyme action.

Viscometric method. The substrate solution contained 1% (w/v) porphyran in 0·033M-phosphate buffer, pH 7·0, containing NaCl (0·3M), and was prepared by dilution of a stock solution of porphyran stored at 0° under toluene. An Ostwald viscometer, of capacity 4ml. and flow-time

58sec. for distilled water at 25°, was used throughout. Substrate solution (5ml.) and enzyme solution in phosphate buffer, pH7·0 (1ml.), were separately equilibrated at 25°, mixed, and a portion (4ml.) was transferred to the viscometer, also at 25°. The flow-time of the digest was determined at once and then at regular intervals. Substitution of phosphate buffer (1ml.) for the enzyme enabled the flow-time for the substrate alone to be determined. The activity of the enzyme was calculated by the method of Tracey (1955). One viscometric unit of activity is defined as the amount of enzyme that would halve the specific viscosity of the digest in 100min. under the above conditions.

Reducing-power method. The digest was identical with that given above and was incubated at 25°. After 1hr., the reaction was stopped by addition of the deproteinizing reagents of Somogyi (1945), the final volume being 10ml. After centrifugation, reducing sugars in the supernatant solution were estimated (as galactose) by the method of Somogyi (1952), but with a heating time for the copper reagent of 20min. at 100°. The reagent was calibrated with solutions of galactose treated in the same way. A blank digest containing the substrate and previously boiled enzyme was also used to measure any initial reducing power. For small amounts of reducing sugar, the reagents of Somogyi (1952) and Nelson (1944) were used in the colorimetric procedure. One reducing-power unit of activity is defined as the amount of enzyme that liberates reducing sugars equivalent to 1mg. of galactose in 1hr. under the stated digest conditions. A comparison of the two units of activity with one particular sample of enzyme showed that 1 viscometric unit was equivalent to 9 reducing-power units. The ratio, however, varied from one enzyme sample to another.

Culture media. Medium I contained $FePO_4$ (0·1%), $NaNO_3$ (0·1%), Difco yeast extract (0·01%) and porphyran (0·1%) in sea water that had been previously stored in the dark for 3 weeks and then filtered (aged sea water). Medium II contained (in each 100ml.) aged sea water (75ml.), peptone (0·1g.), $FeSO_4,7H_2O$ (2mg.), yeast extract (0·1g.) and porphyran (0·3g.). Medium III contained casein hydrolysate (0·1%) in place of the yeast extract and $NaNO_3$ of medium I. Medium IV contained modified Zobell 2216 medium (Oxoid) and porphyran, both at 1% concentration in water. Medium V contained (in each 100ml.) sea water (75ml.), peptone (0·25g.), yeast extract (10mg.), agar (1g.) and porphyran (1g.). The pH of each medium was 6·8–7·2 after autoclaving at 15lb./in.^{2} for 20min.

Isolation of bacteria. Porphyran (1g.) was dissolved in boiling water (10ml.) and the solution cooled to a gel. A small piece of the gel was immersed in fresh sea water (250ml.) and kept for 14 days at 18°. The piece of gel was then transferred to sterile medium I (250ml.) and the mixture was shaken gently. After 7 days at 18° good bacterial growth had occurred. A loopful of the culture was transferred to a second flask of medium I and the culture left for 7 days. Similar transfers were made to fresh medium two further times. A loopful from the fourth flask was diluted with sterile sea water (5ml.) for plating out on medium IV. Isolated colonies of the predominant bacterial species were used to inoculate further cultures in medium I and the whole process was then repeated. The bacteria were stored as slants on medium IV at −20°. For prolonged storage, cells grown on medium V for 4 days at 20° were washed from the surface of the medium with sterile sea water and centrifuged under aseptic conditions. The cells were suspended in mist desiccans (Fry & Greaves, 1951) and stored as described by Heckly (1961).

Isolation of enzymes from culture supernatant solutions. Medium II or III (5l.) was inoculated with a culture of the organism previously grown in the same medium (250ml.) for 2 days at 20°. The culture was aerated at 20° for 48hr. and the cells were then separated on a Sharples ultracentrifuge. To the clear supernatant solution at 4° $(NH_4)_2SO_4$ was added to 70% (w/v) concentration. After 5hr. at 4° the precipitate was recovered on the centrifuge, redissolved in the minimum volume of water at 0° and dialysed against 0·05M-phosphate buffer, pH7·0, for 24hr. The dialysed solution was then freeze-dried.

Isolation of enzymes from bacterial cells. The cells, obtained as described, were contaminated with a gel-like material consisting partly of polysaccharides. The mixture was suspended in 0·1M-phosphate buffer, pH7·0, to give a thin slurry. Portions (2–5ml.) of the slurry were treated in an ice bath with ultrasonic vibration for 10min. After centrifugation, the clear supernatant solution was retained and the cell debris was resuspended in the buffer for further ultrasonic treatment. As many as four such treatments were necessary to liberate all the enzyme from the more viscous cell suspensions. The combined enzyme solutions were freeze-dried. To avoid contamination of the cells with precipitated gel, later preparations were made from bacterial cells grown on medium V in large flasks at 20° for 10 days. The cells were washed from the surface of the medium with 0·1M-acetate buffer, pH7·0, and were subjected to two ultrasonic treatments. To the combined cell-free solutions at 4° $(NH_4)_2SO_4$ was added to 70% (w/v) concentration and the precipitated enzyme was recovered on the centrifuge after 5hr. at 0°. The precipitate was redissolved in water at 0°, dialysed against changes of the acetate buffer for 24hr. and was used directly for further studies.

Partial purification of the enzyme. The freeze-dried enzyme was dissolved in water at 0° and acetone (1 or 2vol.) was then added slowly and with stirring while the mixture was cooled to −5°. After 2hr. at −5°, precipitated enzyme was recovered on the centrifuge, redissolved in water at 0°, dialysed against 0·02M-phosphate buffer, pH7·0, for 24hr. and freeze-dried for storage. For fractionation with $(NH_4)_2SO_4$, enzyme precipitated by acetone (1vol.) was used. The enzyme was dialysed against water for 48hr. at 0° and solid $(NH_4)_2SO_4$ was then added to the desired concentration. After 3hr. the precipitate was recovered on the centrifuge, dissolved in water at 0° and dialysed for 24hr. Activity was measured by the viscometric method.

Gel filtration. The enzyme (850 viscometric units) in 0·1M-acetate buffer, pH7·0 (80ml.), was allowed to percolate through a column (50cm. × 5cm.) of Sephadex G-25 previously equilibrated in the same buffer. Elution was with the same buffer and the collected fractions were tested for protein, for nucleic acids and for enzyme activity. Active fractions were combined, dialysed against 0·1M-phosphate buffer, pH7·0, and were then freeze-dried. A portion of this enzyme preparation was dissolved in 0·05M-tris–HCl buffer, pH7·3 (5ml.), containing NaCl (0·2M). It was passed through a column (50cm. × 2·5cm.) of Sephadex

G-200 in the same buffer–salt solution and eluted with the same solution. Collected fractions were tested for protein and enzyme activity.

Optimum conditions. For the determination of the optimum temperature, the digest conditions were those described for activity determination by the reducing-power method except that varied incubation temperatures were used. For optimum pH, both viscometric and reducing-power assay procedures were used with the pH 7·0 buffer replaced by phosphate buffer of the appropriate pH and the digests were incubated at 35°. For studying the effect of various buffers on enzyme activity, the viscometric assay procedure was used with the phosphate buffer replaced by other buffers, all at 0·033 M and pH 7·0.

Substrate specificity. Where a solution of the substrate did not readily gel on cooling to 35°, the digest contained polysaccharide (50 mg.) in water (5 ml.) and enzyme (0·3 reducing-power unit) in 0·2 M-phosphate buffer, pH 7·0 (1 ml.). Incubation was at 35° and, after 2 hr., the liberated reducing sugars were estimated (as galactose). For the polysaccharides, agarose and alkali-treated porphyran, aqueous 0·2% (w/v) suspensions were dissolved by heating to 100° and were then cooled to 42°. To each solution (10 ml.), maintained at 42°, was added 0·2 M-phosphate buffer, pH 7·0 (9 ml.), and enzyme solution (0·5 ml.), both preheated to 42°. After 15 min., a further portion (0·5 ml.) of enzyme solution was added and the digest was maintained at 42° for a further 15 min. When cooled to 35°, the substrates no longer gelled and the solutions were used directly in the procedure described for non-gelling polysaccharides.

RESULTS

Isolation of enzyme. The enrichment culture technique used in this study gave pure cultures of two bacteria. Of these, one bacterium gave more active enzyme preparations than the other and so was used for the isolation of enzymes on a large scale. This bacterium has been classified as a *Cytophaga* sp. by Dr J. M. Shewan and is held in the culture collection at the Torry Research Station as no. N.C.M.B. 1327. A study of the amount of enzyme produced by this organism in a 5 l. liquid culture grown for 48 hr. showed that the culture supernatant solution contained 170 viscometric units, whereas ultrasonic extracts of the bacterial cells furnished 680 units. At 20°, the enzyme concentration in the culture supernatant solution reached a maximum after about 24 hr. incubation. After longer incubation periods, the enzyme yield was smaller until cell lysis caused a further increase (after about 80 hr.). In the same culture, cell growth (measured turbidimetrically) did not reach a maximum until 55 hr. incubation. For optimum yield of enzymes from the cells incubation was therefore prolonged to 48 hr. Even better yields of enzymes were obtained from cells grown on solid media. After 10 days, the cells grown on 1·2 l. of solid medium furnished 5187 units of enzyme (viscometric), with only 450 units dispersed in the medium. The choice of solid medium was also dictated by the relative absence of a contaminating gel-like material, which interfered with the ultrasonic treatment of cells grown in liquid medium.

Table 1. *Fractionation of enzymes with ammonium sulphate*

To the enzyme solution at 0° solid $(NH_4)_2SO_4$ was added to the indicated saturation. The precipitated enzyme was recovered, dialysed for 48 hr., and its activity measured by the viscometric method.

Concn. of $(NH_4)_2SO_4$ (% saturation)	Activity (viscometric units)	Sp. activity (units/mg. of protein)
—*	1336	2·29
0–30	57	0·67
30–50	208	0·87
50–60	38	2·18
60–85	596	16·5

* Initial enzyme solution.

Partial purification of enzyme. The enzyme obtained from bacterial cells contained other cell constituents and a convenient initial step in the isolation of enzyme was acetone precipitation. Addition of acetone (2 vol.) gave enzyme in 73% yield with specific activity 0·3–0·5 viscometric unit/mg. of protein. Purer enzyme, in decreased yield, however, was obtained with 1 vol. of acetone, specific activities as high as 2·29 units/mg. of protein being observed. Ammonium sulphate fractionation of such an enzyme gave the results shown in Table 1.

Gel filtration of enzyme. Percolation of enzyme through Sephadex G-25 resulted in the elimination of some low-molecular-weight material, and all the enzyme activity (93% recovery) appeared in the material excluded by the gel. Percolation of a portion of this enzyme preparation through Sephadex G-200 gave the elution pattern shown in Fig. 1. There are two peaks of activity in this pattern, the first peak representing about 20% of the total activity measured viscometrically. In a second experiment, in which enzyme was eluted through Sephadex G-25 and the excluded fraction then passed through Sephadex G-200, the activity was estimated by the reducing-power method. Two peaks of activity were again detected in similar fractions to those above, but the first peak contained about 70% of the total activity.

Optimum conditions. With porphyran as substrate, the optimum temperature was 34°. When the pH was varied, several optima were revealed when the reducing-power procedure for enzyme activity was used (Fig. 2). One clearly defined optimum occurred at pH 5·2 and two further optima

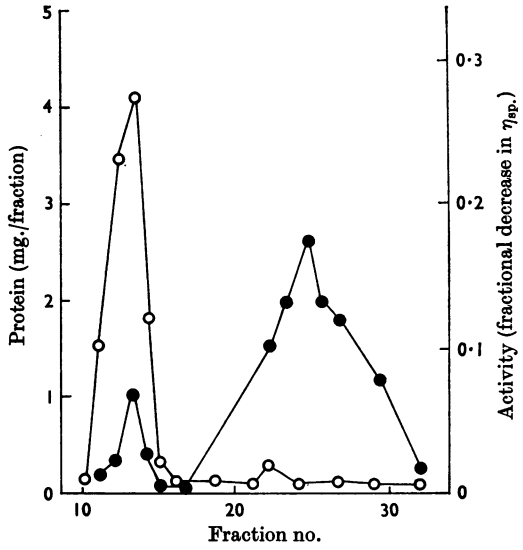

Fig. 1. Gel filtration of *Cytophaga* enzymes in 0·1 M-acetate buffer, pH 7·0, through Sephadex G-200. ○, Protein; ●, enzyme activity assayed viscometrically and shown as the fractional decrease in specific viscosity of digests after incubation for 20 min.

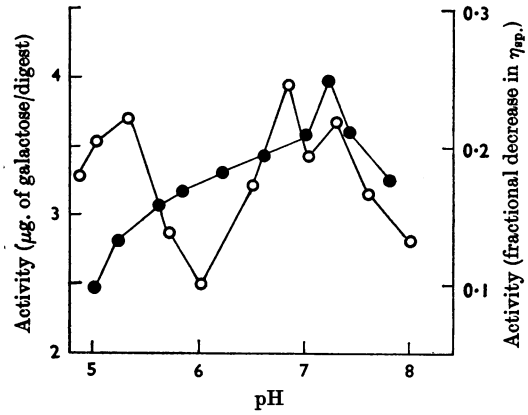

Fig. 2. Optimum pH for the *Cytophaga* enzyme. ○, Determined by reducing-power method; ●, determined by viscometric method and expressed as the fractional decrease in $\eta_{sp.}$ in 10 min.

appeared at pH 6·8 and 7·4. When the viscometric method for enzyme activity was used, a broad optimum with a peak at pH 7·2 was obtained. Table 2 shows the effect of different buffers on enzyme activity, and the action of the enzyme on various substrates is recorded in Table 3.

Table 2. *Effect of various buffers on enzyme activity*

Substrate solution (5 ml.), containing porphyran (50 mg.) in the appropriate buffer (all at pH 7·0 and 0·033 M, and 0·3 M with respect to NaCl), was incubated at 25° with enzyme solution (1 ml.). Enzyme activity was determined by the viscometric method.

Buffer	Activity (viscometric units)
Phosphate	3·50
Borate–HCl	3·84
Acetate–acetic acid	2·80
Tris–maleate	2·94
Citrate–phosphate	3·48

Table 3. *Action of the* Cytophaga *enzyme on various substrates*

For incubation conditions *A*, polysaccharide solution (0·1%, 5 ml.) was incubated at 35° with enzyme (0·3 reducing-power unit) in 0·2 M-phosphate buffer, pH 7·0 (1 ml.), for 2 hr. For incubations *B*, the polysaccharide (0·2%, 10 ml.) was treated with 0·2 M-phosphate buffer, pH 7·0 (9 ml.), and enzyme (0·15 unit, 1 ml.) at 42° until gelling no longer occurred on cooling to 35°. This partly hydrolysed polysaccharide solution was then further incubated under conditions *A*.

Substrate	Reducing power (mg. of galactose/ 100 ml.)
Under conditions *A*	
Porphyran	8·0
κ-Carrageenan	0
λ-Carrageenan	0·6
Corallina galactan	0·1
Furcellaria galactan	0·9
Laurencia galactan	1·25
Under conditions *B*	
Porphyran	7·4
Agarose	11·2
Alkali-treated porphyran	4·9

DISCUSSION

Although there are many reports of agar-digesting bacteria in the literature, in only a few cases has any attempt been made to examine the enzymes involved in the breakdown of agar and to examine the reaction in detail (Ishimatsu, Kibesaki & Maitani, 1954; Araki & Arai, 1956; Yaphe, 1957; Swartz & Gordon, 1959). Carrageenan is the only other algal galactan whose breakdown by bacteria has been investigated. A search was therefore made for bacteria capable of degrading porphyran and other algal galactans. With porphyran as sole source of carbon, two bacteria were isolated from sea water and both furnished enzyme preparations that hydrolysed porphyran. Of these two bacteria,

one gave enzyme preparations more active towards porphyran than the other and attention was concentrated on the more active organism. It has been classified as a *Cytophaga* sp. (Order Myxobacteriales). The genus *Cytophaga* is characterized by formation of a bacterial slime and by the fact that many species can liquefy agar (Stanier, 1941). We found that this slime formation hindered the isolation of enzymes from the cells, since the cells were protected from the ultrasonic vibrations and repeated treatment of the cells was necessary to isolate all the enzyme. Although activity against porphyran was detected in the cell-free supernatant solutions, the amount of activity was only one-quarter of that obtainable from the cells. Accordingly, ultrasonic extracts of cells were used as a source of crude enzyme.

The crude enzyme could be freed from much of the low-molecular-weight cell constituents by acetone precipitation, but when ammonium sulphate fractionation was attempted it became clear that more than one enzyme with activity against porphyran was present, two peaks of activity being observed (Table 1). Gel filtration on Sephadex G-200 again shows the existence of two peaks of activity (Fig. 1), and suggested that these two peaks corresponded to different enzymes since the ratio of the activity determined by the viscometric method to that determined by the reducing-power method varied markedly. The enzyme eluted first from the gel had the properties of an exo-enzyme, since it gave only a small decrease in viscosity when acting on porphyran but produced a relatively large increase in reducing power. The second eluate showed the reverse and is probably an endo-enzyme. A study of the pH optima confirmed that at least two hydrolytic enzymes are present. One, with a pH optimum at 5·2, was detected only by the reducing-power method (Fig. 2), whereas others, with pH optima at about 7, gave a large decrease in viscosity as well as production of reducing sugars.

In spite of this heterogeneity in the enzyme system, it was decided to continue with the mixture of enzymes in studying the action on a range of substrates, rather than to select any one enzyme with the possibility of its action being restricted to a smaller number of substrates. Table 3 shows that, of the galactan sulphates incubated under conditions *A*, only porphyran was degraded to any extent. The carrageenan fractions are not attacked, in agreement with the findings of Yaphe (1957) that the agarase of the marine bacterium *Pseudomonas atlantica* could not attack carrageenan. The slight action on the extract of *Furcellaria fastigiata*, the galactan of which resembles the carrageenans in structure (Painter, 1960), is probably due to the fact that a crude extract of the alga was used. The galactan sulphate of *Corallina officinalis* contains residues of D- and L-galactose, some of which are sulphated, but is devoid of 3,6-anhydrogalactose (Turvey & Simpson, 1966). It is significant that this galactan sulphate is not attacked by the *Cytophaga* enzyme, suggesting that the site of action of the enzyme on porphyran is in the region of the 3,6-anhydro-L-galactose residues. The galactan of *Laurencia pinnatifida* contains the same sugar residues as porphyran, linked in the same way, but has some additional residues that must inhibit enzyme action since only a low activity on this substrate is apparent.

In incubations *B* (Table 3) agarose is a better substrate than porphyran, suggesting that the principal enzyme in the mixture from *Cytaphaga* is an agarase. If this enzyme is specific for 3,6-anhydro-L-galactose residues linked to D-galactose (agarose type), no action would be expected on the carrageenan-type polysaccharides (containing the D-isomer, and not the L-isomer, of 3,6-anhydrogalactose). Further, no action might be expected on those regions of porphyran that contain the residue L-galactose 6-sulphate, in place of the anhydrosugar. Nevertheless, alkali-treated porphyran, which more closely resembles agar than does native porphyran (inasmuch as the sulphated units have been converted into units of 3,6-anhydro-L-galactose; Rees, 1961*b*), failed to give the expected greater susceptibility to the enzyme action in comparison with native porphyran. The reason for this is not yet clear. It is possible that the *Cytophaga* enzyme that degrades porphyran is not identical with agarase, but evidence presented in the following paper (Turvey & Christison, 1967) suggests that this is not the case.

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