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An Extracellular Agarase from a *Cytophaga* Species

By M. DUCKWORTH and J. R. TURVEY
Department of Chemistry, University College of North Wales, Bangor, Caerns.

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1. An extracellular enzyme has been isolated from cultures of the bacterium growing on agar or porphyran. 2. Partial purification of the enzyme has been achieved by precipitation with ammonium sulphate followed by gel filtration on DEAE-Sephadex A-50. 3. The optimum conditions for the enzyme acting on porphyran are pH 7·2 and a temperature of 40–41°. 4. The enzyme has an endo-action, producing a rapid decrease in viscosity of agar or porphyran solutions with little development of reducing power. 5. From the action of the enzyme on various polysaccharides, it is concluded that the enzyme is specific for the agarose structure.

We have recently described an enzyme system obtained from ultrasonic extracts of a bacterium (N.C.M.B. no. 1327) of the genus *Cytophaga* (Turvey & Christison, 1967*a*). This enzyme system catalysed the hydrolysis of agarose and related polysaccharides and produced a range of mono- and oligo-saccharides from porphyran (Turvey & Christison, 1967*b*). It was, however, believed to be a mixture of several enzymes, including a sulphatase, and its use for obtaining information on structural features of agar-like polysaccharides was therefore limited. Although *Cytophaga* species are known to produce enzymes outside the cell wall, these enzymes are usually bound to the cell wall or to the slime polysaccharide exuded by the cells (Stanier, 1941). Since small, but measurable, enzyme activity can be detected in the supernatant solution obtained after removal of cells from liquid cultures (Turvey & Christison, 1967*a*), it was decided to study this enzyme and this paper describes its isolation, partial purification and some of its properties. A preliminary account of this work has been given (Duckworth & Turvey, 1968).

MATERIALS AND METHODS

The bacterium used (N.C.M.B. no. 1327) has been described previously, as has also the preparation of the polysaccharides (Turvey & Christison, 1967*a*), with the exception of the purified galactan and the alkali-treated galactan of *Laurencia pinnatifida*, which were prepared by the method of Bowker & Turvey (1968).

Culture media. Each 100ml. of liquid medium contained 'aged' sea water (75ml.), peptone (100mg.), $FeSO_4,7H_2O$ (2mg.), yeast extract (G. T. Gurr Ltd., London S.W.6; 100mg.) and either agar (180mg.) or porphyran (300mg.). The pH, after autoclaving, was between 6·8 and 7·2. Solid medium contained the above ingredients but the polysaccharide used was a mixture of agar (1g.) and porphyran (1g.).

Analytical methods. Unless stated otherwise, all units of activity are expressed in viscometric units as defined before (Turvey & Christison, 1967*a*) with the modification that digests were all incubated at 37° and the unit is defined at this temperature. For the reducing-power method of assay, the Nelson–Somogyi colorimetric method was used and the unit of activity is defined at 37°. The measurements of protein N, of relative bacterial numbers and of reducing sugars have also been described (Turvey & Christison, 1967*a*). The estimation of polysaccharide in a solution was made by using the phenol–H_2SO_4 method (Dubois, Gilles, Hamilton, Rebers & Smith, 1956) and the results are expressed in arbitrary units only. An assay for sulphatase activity was made by the method of Rees (1961).

Isolation of enzyme. A sterile 1l. flask, containing liquid medium (250ml.), was inoculated from a solid plate of actively growing bacterium and was then shaken until bacterial growth was exponential (24hr.). Samples (5ml. each) were then transferred aseptically to 20 sterile 1l. flasks, each containing liquid medium (250ml.), which were shaken at 24° for 16hr. The contents of the flasks were then pooled and centrifuged at 0° (low speed) to remove much of the gelled polysaccharide. Bacterial cells were then removed at 0° on a high-speed centrifuge (40000*g*; 10min.). To the cell-free supernatant solution at 4°, solid $(NH_4)_2SO_4$ (461g./l.) was added and the precipitate was left to form for 6hr. The precipitate was recovered at −10° on the centrifuge and was then redissolved at 0° in 0·1M-phosphate buffer (pH 7·0; 50ml.), dialysed at 4° against changes of buffer and either used directly as below or freeze-dried for storage.

Chromatography of enzyme. (*a*) On Sephadex G-200. The crude enzyme (305 units; 20·6mg. of protein) was eluted through a column (60cm. × 3cm.) of Sephadex G-200 (fine grade), previously swollen in the eluting solvent, 10mM-tris buffer, pH 7·0, containing 0·1M-NaCl. Eluted fractions (5ml. each) were analysed for enzyme activity and polysaccharide. Two main peaks of activity were detected (Fig. 1) and fractions under each peak were pooled for examination.

(*b*) On DEAE-Sephadex A-50. The gel (medium grade) was converted into the hydroxide form by repeated treatment with M-NaOH and it was then washed exhaustively with water. It was then equilibrated in 0·1M-phosphate

buffer, pH7·0, before being packed in a squat column (20cm.×8cm.). The enzyme (approx. 200 units) in the same buffer was drawn on to the column, which was then eluted with 0·6M-NaCl, fractions being automatically monitored for protein content on the Uvicord spectrometer (L.K.B. Ltd.). Protein-containing fractions were tested for activity, and active fractions were pooled and dialysed at 4° against changes of 0·1M-phosphate buffer. For storage of the enzyme it was necessary to add bovine serum albumin to 0·1% concentration, before the enzyme was dialysed, and the solution was then freeze-dried.

Optimum conditions for the enzyme. For the determination of the optimum temperature, digests containing 1% (w/v) of porphyran in 0·1M-phosphate buffer (pH7·0; 5ml.), which was 0·3M in NaCl, were incubated with enzyme (3 units; 1ml.) at various temperatures. The activity of each digest was determined by the viscometric method. For the optimum pH, the enzyme solution was desalted by passage through a column (60cm.×3cm.) of Sephadex G-25, swollen in water, elution being with water. Only those fractions of enzyme free from phosphate were used. The enzyme (3 units; 1ml.) was added to 1% (w/v) porphyran (5ml.) in 0·3M-NaCl which was also 33mM with respect to phosphate buffers of varying pH. Digests were incubated at 37° and the activity was determined by the viscometric method. An arbitrary measure of activity used in this experiment was the decrease (in sec.) of the flow time of the digest in the viscometer between 3min. and 13min. after mixing enzyme and substrate.

Action of enzyme on polysaccharides. Solutions (20ml. each) of 0·2% polysaccharides in 33mM-phosphate buffer, pH7·0, were prepared by heating at 100° until polysaccharides were dissolved. The solutions were then cooled to 42° and enzyme (12 units) was added to each digest, the temperature then being maintained at 42° for 15min. The digests were then cooled to 37°, and portions (1ml. each) were withdrawn from each digest at intervals for determination of reducing sugars (expressed as galactose) by the Nelson–Somogyi method. When required, portions of digests were also spotted directly on to thin-layer plates of cellulose (Whatman CC 41), which were developed with butan-1-ol–pyridine–water (2:1:1, by vol.) and sprayed with the naphtharesorcinol reagent (Yaphe, 1957).

RESULTS

Enzyme preparation. Extracellular enzyme activity could be detected in cultures of the bacterium at an early stage after inoculation and reached a maximum after 24hr., whereas numbers of bacteria did not reach a maximum for 50hr. However, the cultures were harvested after 16hr., while bacterial growth was still in the exponential phase, to avoid contamination with intracellular enzymes produced by cell lysis. After removal of precipitated polysaccharides and cells, crude enzyme was readily precipitated at 80% saturation with ammonium sulphate, higher concentrations giving greater contamination with polysaccharide. Attempts to separate enzyme from inactive protein and polysaccharide were made with a variety of chromatographic techniques. Gel filtration through Sephadex G-200 gave two peaks of activity (Fig. 1), when elution with 0·1M-sodium chloride–10mM-tris buffer was employed. Peak 1 gave 148 units of enzyme in 18mg. of protein with much polysaccharide also present. Peak 2 contained 150 units of activity in 2·5mg. of protein with much less polysaccharide. Nevertheless, the two enzyme fractions had identical pH and temperature optima, and degraded agarose to give identical patterns of sugars when examined by t.l.c.

Chromatography on DEAE-Sephadex A-50 gave almost complete removal of contaminating polysaccharide but the use of long columns was precluded by a large loss (60–70%) in enzyme activity. Polysaccharide could, however, be satisfactorily removed on a short column, being retained by the first few millimetres of gel, and enzyme was eluted in one peak with only 30% loss in activity. A typical purification scheme from 250ml. of culture medium is shown in Table 1. At this stage it was noted that all enzyme activity was lost if the column eluate was dialysed against distilled water and freeze-dried. Dialysis against phosphate buffer resulted in some retention of activity but freeze-drying still gave 30–40% loss of activity. Addition of bovine serum albumin was necessary to stabilize the enzyme during freeze-drying.

Optimum conditions. The optimum substrate

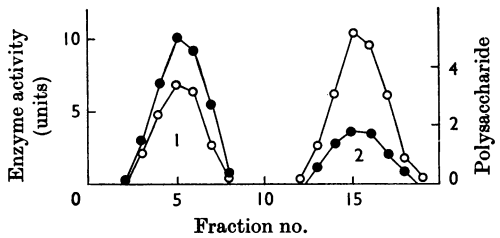


Fig. 1. Gel filtration of enzyme on Sephadex G-200 in 0·1M-sodium chloride–10mM-tris buffer, pH7. ○, Enzyme; ●, polysaccharide (colorimeter units).

Table 1. *Stages in the purification of the enzyme*

Stage	Activity (units)	Specific activity (units/mg. of protein)	Polysaccharide*
Culture supernatant solution	350	10	~2,000
Ammonium sulphate precipitate	175	58	280
DEAE-Sephadex A-50 and dialysis	93	246	15

* Arbitrary units (see Materials and Methods section).

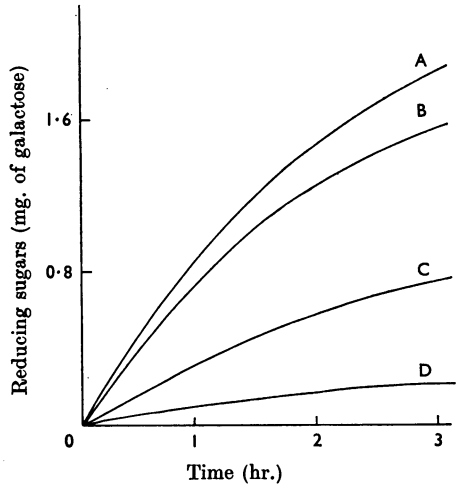


Fig. 2. Action of enzyme on various polysaccharides. A, Agarose; B, alkali-treated porphyran; C, porphyran; D, alkali-treated galactan of *L. pinnatifida*. Reducing power is expressed as apparent galactose (mg.) in total digest. Digests contained 0·2% polysaccharide in 33mM-phosphate buffer (pH 7·0; 20ml.) and enzyme (12 units), and were incubated for 15min. at 42° and then at 37°.

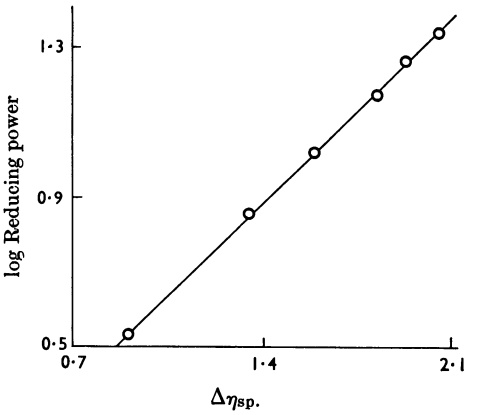


Fig. 3. Plot of change in specific viscosity against logarithm of increase in reducing power. The digest contained porphyran and enzyme as in Fig. 2 but the reaction was followed by both viscometry and determination of reducing power.

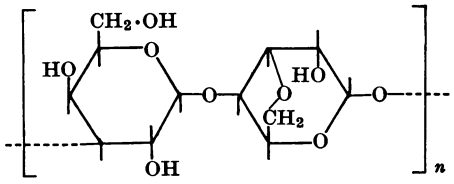
concentration for the enzyme could not be determined since the substrate solutions gelled before this concentration could be reached. The enzyme showed a sharp pH optimum at pH 7·2 but was reasonably active over the range pH 5·5–8. This should be compared with the multiple optima obtained with enzymes from ultrasonic extracts of cells (Turvey & Christison, 1967*a*), one at pH 5·2 and others in the region of pH 7. The temperature optimum was 40–41°, compared with 34° for the ultrasonic extracts. When the optimum conditions were used to compare enzyme activity measured by the reducing-power method with that measured by the viscometric method, it was found that the extracellular enzyme produced far less reducing power than the ultrasonic extracts when equal (viscometric) activity was used. For ultrasonic extracts of cells, 1 viscometric unit of activity was equivalent to 9 reducing-power units, whereas for the purified extracellular enzyme the equivalent figure was 0·04 reducing-power unit. Sulphatase activity could not be detected in the extracellular enzyme.

Hydrolysis curves for a number of polysaccharides are shown in Fig. 2. When the native galactan sulphate of *L. pinnatifida* was used as substrate, production of reducing power was too small to be recorded in Fig. 2 and was clearly seen only after 10hr. incubation.

DISCUSSION

That the extracellular enzyme produced by the *Cytophaga* species is an endoenzyme is shown by the very small production of reducing sugars for a significant fall in the viscosity of the substrate. The enzyme system from ultrasonic extracts of cells (Turvey & Christison, 1967*a*) produced over 200 times the reducing power for a similar fall in viscosity, indicating that it contained exoenzymes or oligosaccharases, in addition to endoenzyme. As a final test, when the change in specific viscosity was plotted against the logarithm of the reducing power produced with porphyran as substrate, a straight line was produced (Fig. 3). A similar relationship has been reported for a cellulase preparation from *Myrothecium verrucaria* (Storvick & King, 1960).

Purification of the enzyme presented two related problems, only one of which was solved satisfactorily. The enzyme is always found associated with polysaccharide in the cultures. Indeed Stanier (1941) suggests that this enzyme is usually bound to the bacterial slime or to the cell wall. Passage of the enzyme through Sephadex G-200 gave two apparent peaks of activity, one of which was associated with more polysaccharide than the other (Fig. 1). Since the properties of the two enzyme fractions seemed to be identical, the two fractions probably represent different polysaccharide complexes with the same enzyme. Although the procedure adopted in this study resulted in almost complete removal of polysaccharide from the enzyme, it produced a second problem, namely, a decrease in the stability of the enzyme. Only by adding a protective colloid, such as serum albumin,



(I)

was activity retained during freeze-drying and storage. For this reason, also, further purification of the enzyme was found impractical since complete loss of enzymic activity usually resulted.

The rate of action of the enzyme on the polysaccharide substrates is of interest since it clearly demonstrates that the enzyme is an agarase, similar to those reported by other workers (Araki & Arai, 1956, 1957; Yaphe, 1957; Swartz & Gordon, 1959). From Fig. 2 it is seen that the fastest action is on agarose, with that on alkali-treated porphyran, native porphyran, alkali-treated galactan from *L. pinnatifida* and the native galactan from *L. pinnatifida*, decreasing in that order. This order parallels the degree to which these polysaccharides depart from the idealized agarose structure. Agarose is believed to be a strictly alternating polymer of 3-linked β-D-galactose units with 4-linked 3,6-anhydro-α-L-galactose units (I). In alkali-treated porphyran, the only major departure from structure (I) is that about 50% of the D-galactose units are 6-*O*-methylated (Anderson & Rees, 1965). This feature obviously inhibits enzyme action to a small extent. In native porphyran, there is a further departure from the agarose structure in that some of the 3,6-anhydro-L-galactose is replaced by its biological precursor, L-galactose 6-sulphate, in addition to the 6-*O*-methyl-D-galactose (Turvey & Williams, 1964; Anderson & Rees, 1965). This sulphate obviously inhibits the enzyme to a marked extent. In the alkali-treated galactan from *L. pinnatifida*, the structural features present in alkali-treated porphyran are all present but, in addition, some of the D-galactose units are 2-sulphated and approximately one-third of the 3,6-anhydro-L-galactose units are 2-*O*-methylated (Bowker & Turvey, 1968). Enzyme action on this substrate is very small, and that on the native galactan of *L. pinnatifida* is virtually negligible.

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