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The Specificity of an Agarase from a *Cytophaga* Species

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1. The extracellular agarase from a *Cytophaga* species was shown to have no action on neoagarobiose, neoagarotetraose or their analogues containing 6-*O*-methyl-D-galactose residues. 2. The action of the enzyme on neoagaro-octaose suggests that scission of the central β-D-galactosidic linkage, to form two molecules of tetrasaccharide, is the preferred mode of action; however, both exterior D-galactosidic linkages in the octasaccharide and both in neoagarohexaose are hydrolysed at a somewhat lower rate. 3. Sulphated oligosaccharides produced by prolonged enzyme action on porphyran have a minimum degree of polymerization of about 8–10 units. 4. For such sulphated oligosaccharides to be further hydrolysed by enzyme action, it is suggested that an unmodified neoagarotetraose residue must be present in the oligosaccharide. 6. A new method for determining the degree of polymerization of these large oligosaccharides is described.

In the preceding paper (Duckworth & Turvey, 1969*b*) we described the action of an extracellular agarase from a *Cytophaga* species on agarose and related polysaccharides, such as alkali-treated porphyran and porphyran. These polysaccharides all gave rise to some small oligosaccharides; the neoagarosaccharides from agarose, and neoagarosaccharides, together with the related series of oligosaccharides in which 6-*O*-methyl-D-galactose units replace some of the D-galactose units, from both porphyrans. In addition, native porphyran gave a complex series of highly sulphated oligosaccharides with average D.P.* greater than about 15. It was perhaps significant that, in the smaller oligosaccharides from all three substrates, the tetrasaccharides predominated and disaccharides were in relatively minor amounts. The enzyme, which is specific for hydrolysis of the β-D-galactosidic linkages in agarose, can also catalyse the hydrolysis, but at a lower rate, of the corresponding 6-*O*-methyl-D-galactosidic linkages present in porphyran-type polysaccharides. The enzyme apparently cannot hydrolyse these linkages when they are near an L-galactose 6-sulphate unit, as present in parts of the porphyran molecule.

To understand more clearly the mode of action of the enzyme, its action on a number of oligosaccharides of known structure was studied and the end products of its action on the sulphated oligosaccharides from porphyran were investigated.

* Abbreviation: D.P., degree of polymerization.

MATERIALS AND METHODS

The enzyme and oligosaccharides used in this work are described in previous papers (Duckworth & Turvey, 1969*a*,*b*), as are also most of the chromatographic and analytical methods used. R_F values are all quoted relative to that of D-galactose in t.l.c. on cellulose, with double development in the solvent system butan-1-ol–pyridine–water (2:1:1, by vol.). Electrophoretic mobilities (M_S values) on filter paper in the pyridine–acetic acid buffer, pH 6, are quoted relative to that of D-galactose 6-sulphate. A new method for determining the D.P. of large reducing oligosaccharides was developed for this work.

D.P. of a large oligosaccharide. The method is based on the fact that g.l.c. can be used to separate glycitol acetates from the parent glycose acetates. Reduction of the reducing end unit in an oligosaccharide to the glycitol, followed by hydrolysis and quantitative g.l.c. of the derived acetates, gives a measure of the proportion of reducing end units and hence of the D.P. Details of the gas–liquid chromatograph and of the columns used have been given (Duckworth & Turvey, 1969*b*). Initially, the oligosaccharide must be completely hydrolysed and the constituent sugars identified chromatographically, so that the response of the detector to each sugar and its corresponding glycitol can be calibrated. For oligosaccharides containing 3,6-anhydrogalactose, the content of this sugar was determined in a separate experiment by the usual method.

To the oligosaccharide (2–20mg.) and water (1ml.) in a 10cm.×2·5cm. tube was added a standard solution (0·1–0·9ml.), which contained erythritol (620mg.) in water (100ml.). Then $NaBH_4$ (1–10mg.) was added and the solution left at 18° overnight. The solution was then neutralized and made 0·5M with respect to H_2SO_4, before being heated at 100° for 3hr. The solution was neutralized

($BaCO_3$) and precipitated salts were removed on the centrifuge. The concentrated syrup was dried thoroughly in a vacuum oven over P_2O_5 at 60° for 24hr. The dried mixture was then acetylated and analysed by g.l.c. as described previously (Bowker & Turvey, 1968; Duckworth & Turvey, 1969*a*). Calibration curves for glycitols were constructed by subjecting known weights of the parent monosaccharides to the above procedure. For the free sugars (analysed as a mixture of α- and β-penta-acetates), calibration curves were constructed by the same procedure except that the $NaBH_4$ treatment was omitted. The molar ratio of sugars plus glycitols plus 3,6-anhydrogalactose to glycitols alone was used as a measure of the D.P. Control experiments, in which authentic maltotetraose was subjected to this procedure, gave duplicate values for the D.P. of 3·8 and 3·9.

Enzymolysis of neutral oligosaccharides. The oligosaccharide (1–5mg.) in water (1ml.) was incubated at 37° with freeze-dried enzyme (15units) under a layer of toluene. After 48hr., the digest was concentrated to a small volume and examined by t.l.c. If hydrolysis had occurred, the remaining solution was fractionated by preparative paper chromatography or t.l.c. and the components were recovered for quantitative analysis. Where it was required to follow the course of hydrolysis, the amount of enzyme was decreased to 0·5unit, and small samples of the digest were removed at intervals for analysis by t.l.c.

For oligosaccharide alcohols, the oligosaccharide was reduced with $NaBH_4$, neutralized and desalted (Duckworth & Turvey, 1969*b*), before being subjected to enzymolysis as described above.

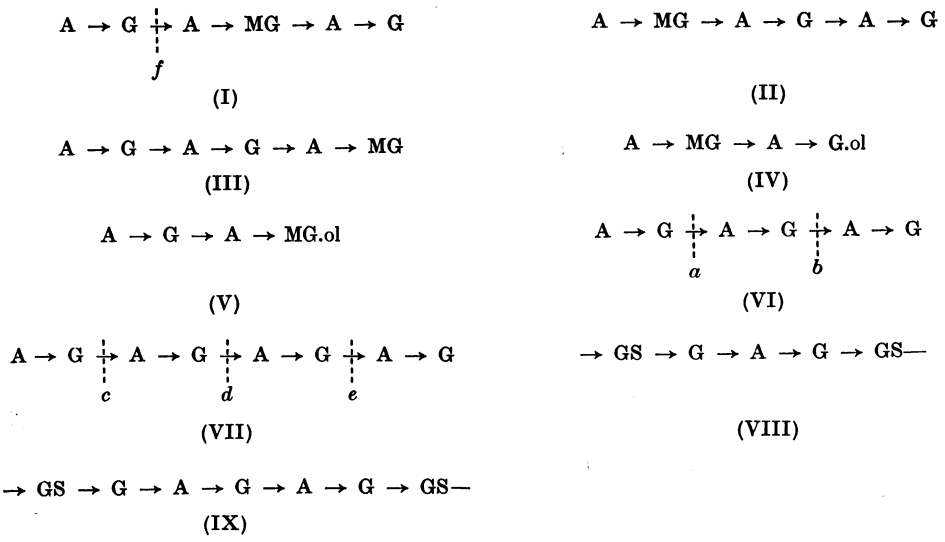
Enzymolysis of sulphated oligosaccharides from porphyran. Combined sulphated oligosaccharides (S_1–S_8; 40mg.) from porphyran (Duckworth & Turvey, 1969*b*) in water (15ml.) were incubated at 37° with enzyme (150units) under a layer of toluene. After 100hr., the digest was added to ethanol (3vol.) and the precipitate was discarded. The supernatant solution was concentrated, desalted, and then fractionated on a column (60cm.×2cm.) of DEAE-Sephadex A-25 (Cl^- form), neutral oligosaccharides being eluted with water (50ml.). The sulphated oligosaccharides were then eluted by a gradient concentration of 0→2M-NaCl over 50ml., six separate peaks being detected. The six fractions (S_{10}–S_{15}) were separately bulked, concentrated, desalted and freeze-dried. A small portion of each fraction was incubated with enzyme (10units) in water (1ml.) and the reaction followed by determination of reducing sugar.

RESULTS

The enzyme had no detectable action on neoagarobiose [3-*O*-(3,6-anhydro-α-L-galactopyranosyl)-D-galactose], on 6^1-*O*-methylneoagarobiose, on neoagarotetraose (4^2-*O*-β-neoagarobiosyl-neoagarobiose) or on the mixture of 6^1-*O*-methyl- and 6^3-*O*-methyl-neoagarotetraose obtained by enzyme action on porphyran. Neoagarohexaose (R_{Gal} 0·46) was rapidly hydrolysed to give neoagarobiose (R_{Gal} 1·30) and neoagarotetraose (R_{Gal} 0·80) in the molar ratio 1·0:1. After borohydride reduction, the hexasaccharide alcohol was also rapidly hydrolysed to give a band of sugars with R_{Gal} 1·30 and another band with R_{Gal} 0·80. The bands were separated by t.l.c., separately eluted and then hydrolysed with acid. The products were analysed, by g.l.c., as the acetates. For the disaccharide band, the molar ratio of galactitol to galactose in the hydrolysate was 1:1·1 and for the tetrasaccharide band the ratio was 1:3·1. If each band consisted of an equimolar mixture of reducing sugar and sugar alcohol, the ratios would be 1:1 and 1:3 for the disaccharide and tetrasaccharide bands respectively.

Neoagaro-octaose was also hydrolysed rapidly to give products identified chromatographically as neoagarobiose and neoagarotetraose. Quantitative determination showed that the molar ratio of tetrasaccharide to disaccharide was 1·4:1. When the amount of enzyme used to hydrolyse the octasaccharide was decreased and the products were analysed by t.l.c. at various times, neoagarohexaose, neoagarotetraose and neoagarobiose were all detected in the early stages of hydrolysis, but the hexasaccharide disappeared in the later stages of reaction. The tetrasaccharide, however, predominated at all stages of the reaction.

The action of the enzyme on alkali-treated porphyran and native porphyran had yielded, among other components, an *O*-methyl hexasaccharide fraction, which was shown to be a mixture in which the principal components were 6^3-*O*-methylneoagarohexaose (I) and 6^5-*O*-methylneoagarohexaose (II), together with a minor component 6^1-*O*-methylneoagarohexaose (III) (Duckworth & Turvey, 1969*b*) (Scheme 1). Enzyme action on this fraction (5mg.) was slow and hydrolysis was incomplete, even after two successive treatments with enzyme for 48hr. each. Examination by t.l.c. showed that the major products of hydrolysis were neoagarobiose (R_{Gal} 1·30) and a 6-*O*-methylneoagarotetraose (R_{Gal} 1·05), but that 6^1-*O*-methylneoagarobiose (R_{Gal} 1·7) and neoagarotetraose (R_{Gal} 0·80) were minor products. Reduction of the original hexasaccharide mixture (2mg.) with sodium borohydride to the alcohols, followed by enzyme action (three successive treatments each for 48hr.), gave a similar pattern of products on t.l.c. From this mixture, the bands with R_{Gal} 1·05 and R_{Gal} 1·30 were isolated. Each band was hydrolysed, acetylated and examined by g.l.c. for its content of glycitols and reducing sugars. The band with R_{Gal} 1·05 gave galactitol acetate and 6-*O*-methyl-D-galactose acetates as major products, together with traces of 6-*O*-methyl-D-galactitol acetate and galactose acetates. This band must therefore have consisted of 6^3-*O*-methylneoagarotetraitol (IV) as the major component, with 6^1-*O*-methylneoagarotetraitol (V) as a minor component and, perhaps, traces of the corresponding reducing tetrasaccharides. The band with R_{Gal} 1·30 gave predominantly galactose acetates, with only



Scheme 1. Schematic representation of oligosaccharide structures. Key: A, 3,6-anhydro-α-L-galactopyranosyl unit; G, β-D-galactopyranosyl unit; MG, 6-*O*-methyl β-D-galactopyranosyl unit; GS, α-L-galactopyranosyl 6-sulphate unit; G.ol, galactitol; MG.ol, 6-*O*-methyl-D-galactitol.

Table 1. *Sulphated oligosaccharides from porphyran*

Combined sulphated oligosaccharides (40mg.) obtained by enzyme action on porphyran were incubated with enzyme (150units) until enzyme action ceased. The products were then separated on DEAE-Sephadex into a neutral fraction, and then by gradient elution with 0–2M-NaCl into six bands of sulphated oligosaccharides.

Oligosaccharide band	Weight (mg.)	3,6-Anhydro-galactose content (%)	Sulphate content as SO_4^{2-} (%)	*M*s	D.P.
S_{10}	3·0	22·0	12·0	0·54	12
S_{11}	4·1	19·5	13·8	0·63	10
S_{12}	4·2	14·2	17·0	0·74	14
S_{13}	5·4	14·0	21·0	0·74	12
S_{14}	8·6	11·1	19·0	0·80	16
S_{15}	5·4	10·5	15·0	0·89	14

traces of galactitol acetate, and must therefore have contained predominantly neoagarobiose.

The sulphated oligosaccharides (S_1–S_8) obtained previously by enzyme action on porphyran (Duckworth & Turvey, 1969*b*) were all further degraded by enzyme action. They were therefore combined and incubated with enzyme until the reducing power reached a constant value. The products were separated into a neutral fraction (9·8% by wt.) and a sulphated fraction, which was further subfractionated on a column of DEAE-Sephadex A-25 to yield six bands of sulphated oligosaccharides, S_{10}–S_{15} (Table 1) in order of elution from the column by increasing salt concentration. The neutral oligosaccharides, when examined by t.l.c., showed a pattern identical with that obtained by enzyme action on alkali-treated porphyran. When each of the fractions S_{10}–S_{15} was subjected to further action by the enzyme, no appreciable hydrolysis of any could be detected by determinations of reducing power. Each fraction was then analysed (see Table 1), the new g.l.c. method being used to estimate the D.P. of these oligosaccharides. Although it was not possible to check the absolute accuracy of this method, trial experiments with maltotetraose had established that it gave reasonable results (D.P. found 3·8, 3·9). Electrophoretic examination of the fractions S_{10}–S_{15} in neutral buffer showed that each migrated as a single zone (Table 1), but t.l.c. (triple development) revealed that each consisted of two or three closely related oligosaccharides.

DISCUSSION

It has already been suggested that the extracellular enzyme from the *Cytophaga* is specific for the β-D-galactosidic linkages in agarose, but that it can hydrolyse, at a lower rate, a 6-*O*-methyl-β-D-galactosidic linkage such as is present in alkali-treated (porphyran (Duckworth & Turvey, 1969*b*). The fact that the enzyme cannot hydrolyse neoagarobiose shows that there is no 3,6-anhydro-α-L-galactosidase activity. The lack of action on neoagarotetraose indicates that at least a three-unit oligosaccharide must be held in the active site of the enzyme before a β-D-galactosidic linkage outside these three units can be hydrolysed. The results obtained with neoagarohexaose and the corresponding hexaitol as substrates, however, suggest that scission of bonds can occur at either position *a* or *b* in structure (VI). It should be emphasized that this conclusion depends implicitly on the assumption that reduction of the hexasaccharide to the corresponding alcohol does not affect the specificity of the enzyme, an assumption that may not be true. It is difficult to envisage an active site for which the tetrasaccharide is not sufficiently large to give hydrolysis, but which can lead to hydrolysis in the two positions indicated for the hexasaccharide.

With neoagaro-octaose (VII) scission of the bond at *d* should give rise to 2 mol.prop. of neoagarotetraose, whereas scission at either *c* or *e* should lead to 1 mol.prop. of neoagarobiose and 1 mol.prop. of neoagarohexaose, the latter subsequently giving a further 1 mol.prop. of neoagarobiose with 1 mol.prop. of the tetraose. Although the hexasaccharide could be detected as one intermediate, suggesting that some scission did occur at *c* or *e*, the results suggest that this is a slower reaction than scission at *d*. By analogy with the hydrolysis of neoagarohexaose, we assume that scission at *c* and *e* occurs at equal rates and, from the molar ratio of disaccharide to tetrasaccharide found in the final products (1:1·4), this suggests that scission at *d* occurs at over twice the rate at either *c* or *e*. This implies that interior β-D-galactosidic linkages in agarose are hydrolysed faster than those nearest the chain ends. The predominance of tetrasaccharides in the hydrolysates from agarose and porphyran is thus explained and also the endoenzyme character of this particular enzyme.

The results of enzyme action on the mixture of 6-*O*-methylneoagarohexaoses (I–III) are difficult to interpret, but they suggest strongly that one predominant activity is scission of the bond *f* in structure (I), other points of scission being hydrolysed less rapidly.

The original mixture of sulphated oligosaccharides obtained from enzymic hydrolysates of porphyran (Duckworth & Turvey, 1969*b*) was shown not to consist of fundamental products, in that it could be fragmented further by prolonged enzyme action. When enzyme action had virtually ceased, the products contained six bands of sulphated oligosaccharides (Table 1), but each band was not a single molecular species. Each band did, however, migrate on electrophoresis as a single species, suggesting that the components in each band had similar ratios of charge to molecular size. Despite the fact that these oligosaccharides were not pure, some interesting conclusions can be drawn from their analytical data. If we consider component S_{11} as an example, the analyses suggest that in a group of oligosaccharides of average D.P. 10 units there are on average three sulphate groups to two 3,6-anhydrogalactose units. Similarly for component S_{10}, in an average 12-unit dextrin, there are three sulphate groups and three anhydro sugar units. It should be noted that there were no oligosaccharides isolated in which only one sulphate group occurred, and that all the oligosaccharides isolated had, on average, two or more 3,6-anhydrogalactose units/molecule. This suggests that each oligosaccharide had one anhydro sugar unit at the non-reducing end and another as the unit penultimate to the reducing end. Where there are more than two anhydro sugar units/molecule, one of these must be in a position away from either end of the molecule as in the partial structure (VIII), and yet the oligosaccharide is resistant to further enzyme action. It would appear that at least two anhydro sugar units in a structure such as (IX) are necessary for the enzyme to be able to hydrolyse the molecule.

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