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# Characterisation of Some Enzymic Hydrolysis Products of Xanthan

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## *ABSTRACT*

*Samples of xanthan gum have been hydrolysed with cellulase preparations to produce a number of fragments, which include one, two, and three of the five-sugar repeat units. Enzyme-resistant polymeric material still remains after 72 h digestion. Modified xanthan samples, e.g. acetate-free (AF) pyruvate-free (PF) and acetate-free/pyruvate-free (AF/PF) are hydrolysed more rapidly than the native polymer.*

*Substantial enzymic hydrolysis occurs only at 50°C in the absence of external salt, confirming earlier observations that the xanthan needs to be largely in the disordered conformation.*

*In all cases, the pyruvate and acetate levels in the enzyme-resistant polymeric remainders are higher than those in the oligomeric products.*

*The cellulase preparation AP3 contains* endo-1 → *4-β-*D*-glucanase, and* exo*-β-*D*-glucosidase activities. The* endo*-enzyme(s) act rapidly but to a limited extent on the xanthan backbone, and the* exo*-enzyme(s) act on the resulting polymeric products to release oligosaccharides of d.p. up to 15, plus glucose.*

## INTRODUCTION

Several reports of the enzymic hydrolysis of xanthan gum have appeared (Lesley, 1961; Rinaudo & Milas, 1980; Sutherland, 1981, 1982, 1984; Cadmus *et al.*, 1982; Milas *et al.*, 1986). Of these, the use of fungal cellulase preparations (Rinaudo & Milas, 1980; Sutherland, 1984) required the xanthan molecule to be largely in the disordered conformation for hydrolysis to occur. To achieve this state, both the enzyme preparation and the xanthan substrate were subjected to extensive dialysis, and/or desalting on a Sephadex G-50 column (Pharmacia, Uppsala, Sweden). Incubation at 50°C also ensured that the xanthan was largely in

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the disordered conformation. Sutherland (1984) found that the rate of hydrolysis, measured by viscosity loss, was reduced by the presence of microgels or aggregates in the xanthan sample. Only about 5% of the D-glucose in the polymer was released as the monosaccharide, and some 50% of polymeric material remained even after prolonged enzyme treatment. The other portions of the polysaccharide yielded cellobiose oligosaccharides of d.p. 5–15. Sutherland concluded that the cellulase results probably indicate non-uniformity in the xanthan molecules, there being sites for preferential action by the *endo-β*-D-glucanases present. The most likely sites are regions of the cellulose-like backbone lacking one or more of the trisaccharide sidechains. Some support for this conclusion is found in the ocurrence of cellobiose in the hydrolysis products. Milas *et al.* (1986) compared depolymerization of xanthan brought about by ultrasonic and enzymic treatments. They found that enzymic degradation caused a reduction in pyruvate levels. From these and other results they suggest that (a) the enzyme might act, preferentially, on some irregularities in the molecule (as suggested by Sutherland) and (b) proteins associated with the xanthan could affect enzymic degradation. Overall, Milas *et al.* found that enzymic degradation produced fractions of high polydispersity index, whose behaviour was anomalous, whereas degradation by ultrasonication produced fractions with a low dispersity index, which behaved 'normally'.

Results are reported which provide further information about the enzymic hydrolysis of xanthan, and on the nature of the products. The prime interest was in producing xanthan fragments which could be used in experiments to study the nature of xanthan/galactomannan interactions. Interactions have been studied using gel-permeation chromatography (gpc) (Cheetham *et al.*, 1986; Cheetham & Punruckvong, 1989) gel melting points (Cheetham & Mashimba, 1988) and optical rotation (Cheetham & Mashimba, 1991). The above studies have shown that partially depolymerized xanthan and galactomannan samples may be used as valid models in experiments to probe further the nature of mixed-polysaccharide associations.

## EXPERIMENTAL

### Materials

Cellulose powder (Whatman CF-11) was obtained from W&R Balston, UK, and cellobiose from BDH Chemicals, Poole, UK. Cellopentaose was a gift from Dr G. J. Walker.

## Enzymes

The cellulase AP3 was supplied by Pfizer Chemical Division, and is derived from a strain of *Aspergillus*. The buff-coloured, water-soluble powder was quoted as having the following activities:

Carboxymethylcellulase − 30 000 μ/g (pH 4·5)
$C_1$-ase (acting on highly oriented cellulose) ≈ 40 000 μ/g (pH 4·0)
Hemicellulase − ≈ 30 000 μ/g (as xylanase − pH 4·5)
β-glucosidase ≈ 14 000 μ/g (as cellobiase − pH 4·0)
The preparation was heat-stable, optimum activity 50–60°C.

Before use, the enzyme was subjected to exhaustive dialysis against distilled water, in an Amicon stirred cell fitted with a 10 000 molecular weight cut off membrane. Dialysis was considered complete when the conductivity of the dialysate reached that of the initial solvent. The enzyme was recovered by freeze-drying, and was stored as a light fawn powder at 4°C. (A small amount of material which precipitated on dialysis was removed by centrifugation prior to freeze-drying. It possessed no cellulase or xanthanase activity).

The activities of the enzyme preparation against cellulose, cellopentaose and cellobiose were determined by incubating, in water, substrate (10 mg/ml) and enzyme (4 μg/ml) for 24 h at 50°C. Soluble products were examined by hplc on two columns, each 7·8 mm × 300 mm. (i) Bio-Rad-HPX 87P and (ii) Bio-Rad-HPX 87H at 0·6 ml/mm flow rate, with refractive index detection. Solvents were water and 0·008 M sulphuric acid respectively, at 85°C.

## Xanthan samples

Glucose and mannose contents of xanthan samples were determined after hydrolysis (2 M TFA; 120°C; 90 min) by hplc on a Bio-Rad-HPX 87(H) ion-exchange column, with 0·008 M sulphuric acid as solvent, at 35°C and a flowrate of 0·6 ml/min. Acetate and pyruvate were determined by hplc (Cheetham & Punruckvong, 1985). Glucuronic acid was determined colorimetrically by the meta-hydroxy diphenyl procedure (Blumenkrantz & Asboe-Hansen, 1973).

Xanthan was derived from two sources:

(a) From crude Keltrol (Kelco Division of E Merck) by stirring (0·3% w/v) in water at room temperature overnight, followed by centrifugation of the dispersion at 200 000 g. The clear supernatant liquid was exhaustively dialysed, and freeze-dried to yield XCW.

(b) From Keltrol XT (Kelco) which dissolved to give a clear solution, which was exhaustively dialysed and freeze-dried. Both freeze-dried samples dissolved readily in water to give clear solutions with the following analytical characteristics: (Subsequently it was found that even undialysed XT was attacked to approximately the same extent as the dialysed material.)

| *Sample* | *Acetate (%)* | *Pyruvate (%)* | *Glucuronic acid (%)* | $\frac{\textit{Glucose}}{\textit{Mannose}}$ | *Nitrogen (%)* |
|---|---|---|---|---|---|
| XCW | 4·1 | 4·3 | 18·6 | 1·18 | 0·65 |
| XT | 6 | 3·5 | 18 | 1·2 | 0·57 |

Acetate-free (AF) and pyruvate-free (PF) samples were prepared by the procedure of Bradshaw *et al.* (1983) and were analysed for pyruvate and residual acetate as described by Cheetham and Punruckvong (1985). Acetate-free/pyruvate-free samples (AF/PF) were made by treatment of AF with trifluoroacetic acid, using the same acid hydrolysis conditions as for the preparation of PF.

## Viscometry

Changes in viscosity during enzyme action were measured on a Contraves Rheomat 115 rotational viscometer (Contraves AG, Zurich) fitted with an MS-1 measuring system. Samples (0·5% w/v) were incubated at 50°C and a shear rate of 10·1 $s^{-1}$.

## Gel-permeation chromatography

Gel-permeation chromatography was carried out on a system comprising a Rheodyne 7125 injector (Rheodyne Inc., CA, USA) an M6000 (Waters Assoc., Milford, MA, USA) pump and an ERC 7510 refractive index detector (ERMA Optical Works, Tokyo). Analytical gel-permeation chromatography was carried out with two columns in series (a) a Shodex Ion-Pak KS802 (Showa Denko, Tokyo) 50 × 0·8 cm, and (b) a TSK G-3000 PW (Tokyo Soda Co., Tokyo) 30 × 0·75 cm. The eluting solvent was 0·1 M sodium nitrate, and the flowrate 0·2 ml $min^{-1}$. The column was calibrated for low molecular weight oligomers using an enzyme hydrolysate of amylose, $\overline{\mathrm{DP}}17$ (Hayashibara Biochemicals, Tokyo). High molecular weight, enzyme-resistant xanthan was separated from lower molecular weight products by gpc on a column (2·5 × 75 cm) packed with Sephacryl S300 (Pharmacia LKB, Uppsala, Sweden) with water as eluting solvent, or by dialysis in a stirred cell (Amicon, Danvers, MA, USA) with a 30 000 daltons cut-off membrane.

## RESULTS AND DISCUSSION

### Activities present in cellulase AP3 preparations

AP3 cellulase incubated with cellulose for 24 h released glucose, but no cellobiose was detected. Pure cellobiose was mainly converted to glucose by AP3, but some cellobiose still remained after 24 h incubation. Cellopentaose yielded over 90% glucose, and less than 5% cellobiose, after 24 h. If cellobiohydrolase activity was present, it must have been much lower than the $\beta$-glucosidase activity. There was no detectable transferase activity in the experiments with cellobiose and cellopentaose.

### Establishment of hydrolysis conditions

Initial attempts to hydrolyse unmodified xanthan with the cellulase as received, were usuccessful even though the xanthan had been exhaustively dialysed. There was usually an initial fall in viscosity, but low molecular weight products were virtually absent even after incubation up to two days. Exhaustive dialysis of the enzyme preparation was necessary. Sutherland (1984) has shown that the extent of hydrolysis of xanthan depends on both the type of cellulase used, and on the xanthan sample being hydrolysed. The conditions chosen here involved the AP3 cellulase at 50°C, with a xanthan concentration of 0·5% (w/v) in water. The ratio of modified polysaccharide to enzyme typically used was 100:1 (w/v). Hydrolysis of the unmodified xanthan XCW occurred at a much reduced rate, (Fig. 1(a)) and to a lesser extent. In this case a ratio of 20:1 polysaccharide to enzyme was used. Modification of the side chains was carried out, and the resulting effect on the enzymic hydrolysis was examined. Figure 1 shows the effect of cellulase AP3 on the viscosity of various XCW derivatives. Removal of acetate alone produced a derivative AFXCW, which underwent cellulase hydrolysis at the fastest initial rate (Fig. 1(c)). Removal of pyruvate alone caused a slower rate of reaction than for AFXCW as indicated by the reduction in viscosity of PFXCW measured over a 24 h period (Fig. 1(b)). Removal of

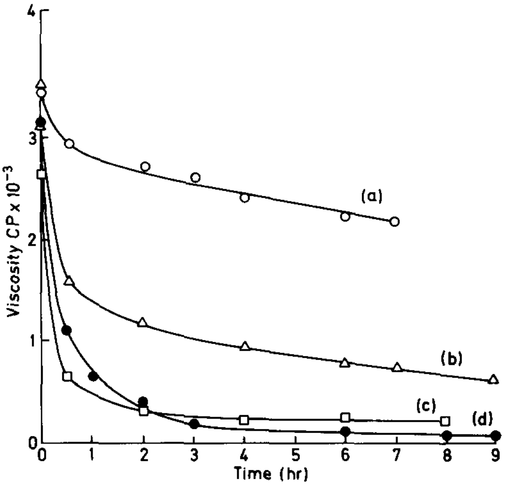

**Fig. 1.** Effect of cellulase AP3 on the viscosity of various XCW derivatives: (a) XCW; (b) pyruvate-free XCW (PFXCW); (c) acetate-free XCW (AFXCW); (d) acetate-free/pyruvate-free XCW (AFPFXCW).

both pyruvate and acetate yielded results similar to those for acetate-free samples (Fig. 1(d)). On the other hand undialysed, unmodified xanthan XT underwent hydrolysis at a rate, and to an extent, similar to that of PFXCW. Xanthan XT is a preparation which dissolves readily to give a clear solution. Cell debris and probably some protein have been removed by the manufacturer. The behaviour of XT indicates that microgels or aggregates are not present to a significant extent. Sutherland (1984) noted that the rate of xanthan hydrolysis was reduced by such aggregates.

## Gel-permeation chromatography of the hydrolysis products

### *Xanthan XCW and its derivatives*

Figure 2 shows the elution profiles of various xanthan hydrolysates, with the analytical columns KS802 and G-3000 PW in series, and 0·1 M $NaNO_3$ as solvent. In each case the incubation time was 24 h, using cellulase AP3. Figure 2(a) shows the limited degree of hydrolysis (≈20%) undergone by unmodified XCW. The high molecular weight material which is resistant to hydrolysis elutes near the void volume of this system (Peak (i)) and constituted in excess of 80% of the products. Peaks (iii) & (iv) consists of two and one of the five-sugar xanthan repeat

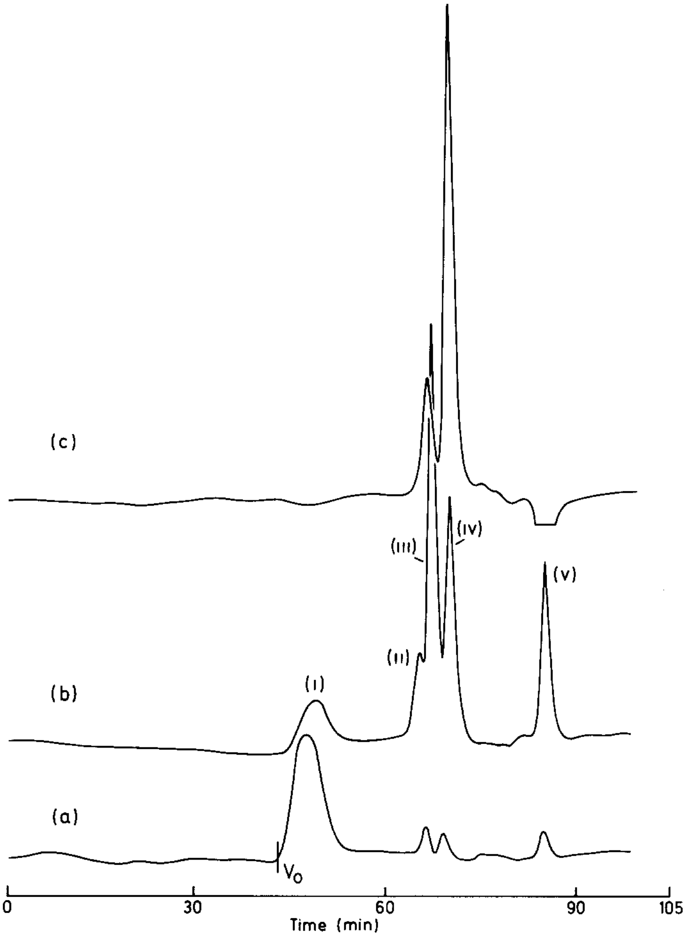

**Fig. 2.** Gel permeation chromatography elution profiles of various cellulase AP3 hydrolysates of xanthan: (a) unmodified XCW: (b) acetate-free XCW (AFXCW); (c) low molecular weight fraction isolated from the action of cellulase AP3 on AF/PF XCW. (i) Residual, cellulase resistant polymeric material; (ii) DP 15 oligomer; (iii) DP 10 oligomer; (iv) DP 5 oligomer; (v) unidentified.

units, respectively. Peak (V) is as yet unidentified, but is not cellobiose or cellotriose. Glucose was detected in the hydrolysate by hplc, and enzymically by glucose oxidase/peroxidase. Figure 2(b) shows the gpc profile for an acetate-free samples (AFXCW) treated with AP3 cellulase. Hydrolysis has proceeded much further than that of the native XCW. Peak (ii) probably consists of three xanthan repeat units. Peak (iii) material (decasaccharide) is the dominant species. Figure 2(c) shows the profile for the low molecular weight fraction isolated from the action of cellulase AP3 on AF/PFXCW. It was separated from residual high molecular weight (Peak (i)) material by gel-permeation chromatography on a column of sephacryl S300. Analysis of the high molecular weight (Peak (i)) and low molecular weight (Peaks (ii)–(iv)) fractions from AP3 action on PFXCW (Table 1) shows that the high molecular weight (AP3 resistant) material had higher (Pyr 2·1; OAc 4·1) pyruvate and acetate levels than that of the low molecular weight material (Pyr 0·93; OAc 3·1). This confirms that xanthan molecules highest in pyruvate and acetate are the most resistant to enzymic hydrolysis by cellulases. Peak (IV) material predominates in Fig. 2(c), indicating the slightly greater degree of hydrolysis in 24 h of AF/PF xanthan compared with AF xanthan (Fig. 2(b)).

### *Xanthan XT*

Figure 3 shows gpc profiles of the products formed by the action of cellulase AP3 on unmodified xanthan XT (1:20 w/w). Important features are:

(a) There is still polymeric material present after 36 h, after which time the gpc profiles remain essentially unchanged.
(b) Several polymeric products are formed (material eluting 40–50 min) at the same time large amounts of oligomeric material is produced (material eluting 60–80 min).
(c) There is virtually no material of intermediate size formed.
(d) The DP of the oligomeric material first formed is ≈ 10, which corresponds to two repeat units of xanthan.
(e) With increased incubation time, some of the DP 10 material is further hydrolysed by one of the enzyme activities to DP 5 material, but some DP 10 material remains even on extended hydrolysis (72 h).

After 72 h digestion, the low molecular weight fractions were separated from the residual polymeric material by dialysis in a stirred cell. Both low and high molecular weight fractions were analysed for pyruvate, acetate (Table 1), glucose, mannose and glucuronic acid levels. The glucose/mannose/glucuronic acid ratios were close to the 2:2:1 found in xanthan. The polymeric material (as for XCW) had higher (Pyr 2·8; OAc 4·3) pyruvate and acetate levels than the low molecular weight material (1·92 Pyr; OAc 3·8). Further digestion of the isolated polymeric material with fresh cellulase showed very little additional attack by the

**TABLE 1**
Composition (% by Weight) of Some Xanthan Fragments

| *Sample* | *Pyr* | *OAc* | *Nitrogen* |
|---|---|---|---|
| XCW | 4·3 | 4·1 | 0–0·5 |
| XCWE[a] | 3·57 | 3·01 | |
| PFXCWE | 1·83 | | |
| PFXCWE (large)[b] | 2·1 | 4·1 | |
| PFXCWE (small)[b] | 0·93 | 3·1 | |
| AFPFXCW | trace | trace | |
| AFPF XCWE (large) | trace | 0·71 | |
| AFPF XCWE (small) | trace | trace | |
| XT | 3·5 | 6 | 0·57 |
| XTE (large) | 2·8 | 4·3 | |
| XTE (small) | 1·92 | 3·8 | |

[a] The suffix 'E' indicates that the sample has been incubated with the enzyme AP3.
[b] Large and small refer, respectively, to the high molecular weight and low molecular weight fragments obtained by AP3 enzyme treatment of the particular xanthan sample.

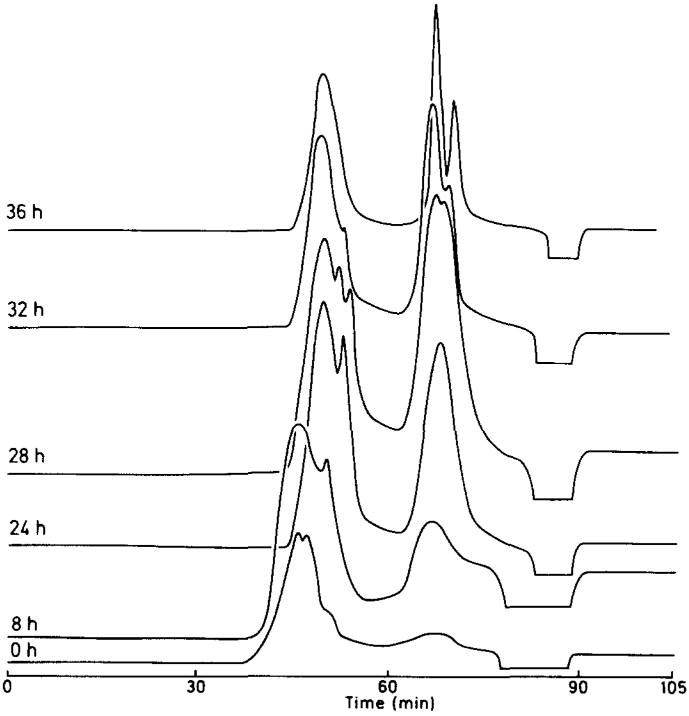

**Fig. 3.** Gel-permeation elution profiles of products formed by the action of cellulase AP3 on unmodified XT xanthan, after incubation times of 0, 8, 24, 28, 32 and 36 h.

enzyme in 12 h incubation, approximately 5% of additional low molecular weight material being produced, as shown by gpc. For unmodified XT, the residual polymeric material constituted 60–70% of the products.

High resolution proton nmr spectra were obtained from the low molecular weight fraction (Peaks (iii) & (iv), Fig. 2(c)). As this consisted of a mixture of five and ten monosaccharide units, nmr peak assignments will be reported after their preparative separation. The cellulase-resistant fraction yielded poor spectra even at 85°C. Cell-permeation chromatography indicated a molecular weight of approx $10^5$.

To summarise the data on cellulase activity:

1. Exhaustively dialysed enzyme is needed for substantial hydrolysis to occur.
2. Unmodified XCW undergoes limited hydrolysis with cellulase, whereas unmodified XT is attacked faster and to a greater extent.
3. Side-chain modifications, especially acetate removal, increase the susceptibility of xanthan to cellulase hydrolysis.
4. The gpc profiles of small molecular weight fragments arising from cellulase digestion of unmodified xanthan samples are very similar to those obtained from modified samples. Structurally they differ only in pyruvate and acetate levels, depending on the level of pyruvate and acetate in the starting materials.
5. Polymeric material resistant to hydrolysis by cellulase occurs in all cases.
6. The cellulase-resistant polymeric material invariably has a higher pyruvate (and usually a higher acetate) content than the corresponding small molecular weight fragments (Table 1).
7. During enzyme hydrolysis, turbidity develops, which at the end of the hydrolysis can be centrifuged to a precipitate. The precipitate is high in nitrogen and it was taken to be enzyme plus some bound xanthan. Cellulase heated alone under the same conditions does not yield a precipitate.

The reason why unmodified Keltrol XT should be attacked by cellulase AP3 to a greater extent than XCW is not known, though XT has a lower (3·5%) pyruvate level than XCW (4·3%). The crude Keltrol from which XCW is derived has protein and other cell wall debris present, and this is partially removed during XCW preparation. Our experience with xanthan has shown, however, that the solution properties of xanthan depend on the sample used, and on its thermal and storage treatment. The same seems to apply to attack by cellulase, for which xanthan is not the 'natural' substrate.

## CONCLUSIONS

The cellulases apparently bind to, and attack, regions of xanthan which are in the disordered conformation. This is not surprising, as in the disordered conformation, the cellulase-like backbone is largely in the two-fold 'ribbon' conformation similar to that in most cellulose samples which are susceptible to cellulase hydrolysis. Removal of acetate favours faster attach by cellulase. The acetate group is attached to $C_6$ of the inner mannose residue, and has a stabilising influence on the ordered xanthan conformation (Holzwarth & Ogletree, 1979). Its removal favours the disordered conformation, and in addition probably allows the cellulase to bind more firmly by allowing a closer approach to the backbone of the xanthan molecule.

Pyruvate removal, on the other hand, tends to stabilize the ordered conformation (Holzwarth & Ogletree, 1979) but probably allows the enzyme a closer approach and increased binding to the backbone. Thus pyruvate-free xanthan is attacked at a greater rate than unmodified xanthan, but at not as fast a rate as acetate-free samples.

The products of prolonged cellulase digestion of xanthan and its sidechain modified derivatives in all cases are:

(a) A cellulase-resistant, polymeric ($Mw \approx 10^5$) material. This is not attacked further after isolation followed by addition of fresh enzyme.
(b) Deca- and pentasaccharides, consisting of two- and one- of the five-sugar xanthan repeat units. These constitute 60–70% of the products from sidechain-modified XCW and XT.

The reasons for lack of cellulase attack on the polymeric remainder could include:

(i) Some xanthan chains contain regions which have the trisaccharide sidechains fully substituted by pyruvate and acetate, and which resist conversion to the disordered conformation. They would also prevent close approach of the enzyme to the backbone, and thus prevent it binding. This reason had been proposed by Sutherland (1984). Analytical values for pyruvate and acetate support this (Table 1).
(ii) There are possibly some other structural inhomogeneities in a number of xanthan molecules. These could include attachment of protein, a different sequence of backbone monosaccharides, or some sidechain variations or omissions.

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## REFERENCES

Blumenkrantz, N. & Asboe-Hansen, G. (1973). *Anal. Biochem.*, **54**, 484.

Bradshaw, I. J., Nisbet, B. A., Kerr, M. H. & Sutherland, I. W. (1983). *Carbohydr. Polym.*, **3**, 2.

Cadmus, M. C., Jackson, L. K., Burton, K. A., Plattner, R. D. & Slodki, M. E. (1982). *Appl. Env. Microbiol.*, **44**, 5.

Cheetham, N. W. H. & Punruckvong, A. (1985). *Carbohydr. Polym.*, **5**, 399.

Cheetham, N. W. H. & Mashimba, E. N. M. (1988). *Carbohydr. Polym.*, **9**, 195.

Cheetham, N. W. H. & Mashimba, E. N. M. (1991). *Carbohydr. Polym.*, **14**, 17.

Cheetham, N. W. H., McClearly, B. V., Teng, G., Lum, F. & Maryanto (1986). *Carbohydr. Polym.*, **6**, 257.

Cheetham, N. W. H. & Punruckvong, A. (1989). *Carbohydr. Polym.*, **10**, 129.

Holzwarth, G. & Olgetree, J. (1979). *Carbohydr. Res.*, **76**, 277.

Lesley, S. M. (1961). *Can. J. Microbiol.*, **7**, 815.

Milas, M., Rinaudo, M. & Tinland, B. (1986). *Carbohydr. Polym.*, **6**, 95.

Rinaudo, M. & Milas, M. (1980). *Int. J. Macromol.*, **2**, 45.

Sutherland, I. W. (1981). *Carbohydr. Polym.*, **1**, 107.

Sutherland, I. W. (1982). *J. Appl. Bacteriol.*, **53**, 385.

Sutherland, I. W. (1984). *Carbohydr. Res.*, **131**, 93.