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An HPLC Method for the Determination of Acetyl and Pyruvyl Groups in Polysaccharides

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SUMMARY

A method for the quantitative analysis of pyruvate and acetate groups in polysaccharides has been developed, using HPLC on a cation exchange resin in the protonated form and u.v. detection at 210 nm. Procedures for release of acetate and pyruvate from polysaccharides are described.

In the case of xanthan gum, most of the pyruvate is removed if the sample is autoclaved in water during preparation, and about 25% is lost if autoclaved in 0·1 M sodium chloride solution.

1. INTRODUCTION

A number of polysaccharides containing either O-acetyl or O-pyruvyl (1 carboxyethylidene) groups, or both, have been isolated. Many microbial exopolysaccharides fall into this category (Sutherland, 1967).

The location and quantitative analysis of these groups are important steps in the full structural elucidation of the polysaccharide. Acetyl groups may function in increasing the solubility of the polymer (e.g. in Konjac glucomannan), and pyruvyl groups affect the polyanion properties of the molecule.

Variations in the pyruvate content of xanthan from different strains prepared by different culture procedures have been reported (Cadmus *et al.*, 1976; Davidson, 1978; Evans *et al.*, 1979). Some of the variations in reported values no doubt reflect real differences in pyruvate content, but in addition the lability of the pyruvate ketal (Rinaudo *et al.*, 1983)

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may lead to losses during isolation. Sutherland (1981*a*) has reported an affinity procedure for the small-scale separation of xanthan preparations into fractions of differing pyruvate content. Larger samples may be handled by fractional precipitation with ethanol (Sandford *et al.*, 1978).

Overall, these studies have shown that pyruvate and acetate contents of xanthan may vary considerably depending on the source. They have also indicated that in certain preparations some molecules may have their sidechains 50% substituted with pyruvate, while others are essentially pyruvate-free (Sutherland, 1981*b*).

Acetyl groups on polysaccharides may be estimated by a variety of methods: the hydroxamic acid procedure (Hestrin, 1949; McComb & McCready, 1957; Sutherland & Wilkinson, 1968); titration with acid after release of acetate under alkaline conditions (Jeanes *et al.*, 1961; Rinaudo *et al.*, 1983); gas chromatography (Turner & Cherniak, 1981); and n.m.r. spectroscopy (Smith *et al.*, 1981; Rinaudo *et al.*, 1983).

Pyruvyl groups may be assayed by hydrolysis with acid and (i) formation of the 2,4-dinitrophenylhydrazone (Koepsell & Sharpe, 1952; Sloneker & Orentas, 1962) or (ii) enzymically, using lactate dehydrogenase (Hadjivassiliou & Rieder, 1968; Duckworth & Yaphe, 1970; Jeanes *et al.*, 1976).

The above methods are either indirect and subject to possible interference (colorimetric), require derivatization (GLC) or are expensive in enzymes or equipment (n.m.r.).

Studies in this laboratory are concerned with the properties and interactions of a number of modified xanthan molecules, and a simple and rapid method for the analysis of pyruvate and acetate was sought.

2. EXPERIMENTAL

2.1 Materials

Pyruvic acid of Sigma type 1 was used. Acetic acid (AJAX) was purified by distillation. Xanthan samples were derived from Keltrol (Kelco Division of Merck & Co. Inc., USA). Konjac glucomannan (from *Amorphophallus* Konjac) was kindly provided by Dr B. V. McCleary. Standard curves for each acid were prepared. Sonication of samples was carried with a Sonicor ultrasonic probe (Sonicor Ins. Corp., USA).

2.2 HPLC analyses

HPLC was carried out on a system consisting of a Rheodyne 7125 injector, M 6000 pump (Waters), Varichrome u.v.-visible detector (Varian) set at 210 nm, and a Bio-Rad HPX-87H column (300 × 7·8 mm) fitted with an ion exclusion precolumn cartridge (Bio-Rad.).

Initial work was recorded on a National VP-6513A chart recorder. Quantitative analyses were carried out on a 3390-A Integrator (Hewlett-Packard). The eluent was 8 mM sulphuric acid, at a flowrate of 0·6 ml min^{-1}. Column temperature was maintained at 35°C by an Eldex column heater.

2.3 Sample preparation

Polysaccharide was dissolved in water to a concentration of $\simeq 5$ mg ml^{-1}, by stirring overnight at room temperature and then stirring at 90° for 1 h.

The exact concentration was determined by the phenol/sulphuric acid procedure (Dubois *et al.*, 1956). In the case of the highly viscous xanthan solutions, sonication was carried out ($\simeq 2$ min) using a Sonicor ultrasonic probe fitted with a needle tip (4 mm diameter). This facilitated subsequent handling.

2.3.1 Pyruvate

To polysaccharide solution (1 ml) was added phosphoric acid (0·1 M, 1 ml). The sample was sealed, placed in a metal heating block, maintained at 90°C for 90 min, made up to exactly 3 ml with water, filtered and injected (10–20 μl). Elution time was 10 min.

2.3.2 Acetate

To polysaccharide solution (1 ml) was added potassium hydroxide solution (0·2 M, 1 ml). The sample was flushed with nitrogen, sealed and held at 45°C for 6 h. The solution was made acidic with phosphoric acid, and diluted to exactly 3 ml with water, filtered as for pyruvate and injected. Elution time was 15·8 min.

2.3.3 Estimation by colorimetric methods

For comparison purposes, pyruvate and acetate were also estimated by the 2,4-dinitrophenylhydrazone (Sloneker & Orentas, 1962) and the hydroxamic acid (Hestrin, 1949) procedures, respectively.

3. RESULTS AND DISCUSSION

3.1 Comparative results

Figure 1 shows the chromatograms for (a) a standard mixture of acetic and pyruvic acids, and (b) pyruvic acid released from xanthan gum. Table 1 shows the results for a number of samples: pyruvate and acetate estimations by the present HPLC method and, for comparison, by colorimetric procedures. The results for pyruvate estimated by colorimetry are higher than those found by HPLC, with the exception of two xanthan samples which had been depolymerized by sonication. It has been noted previously (Cheetham & Norma, unpublished results) that xanthan samples sonicated for 15 min or longer and isolated by filtration and dialysis were found to have very low nitrogen content (0–0·2%). Crude xanthan had 2·0% nitrogen, and even when purified by fractional precipitation had 0·8% nitrogen present. The nitrogen con-

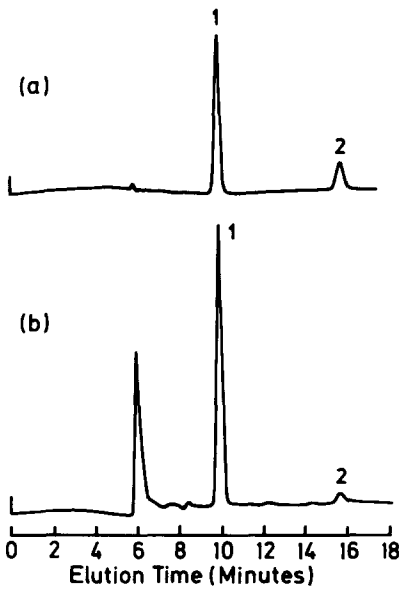

Fig. 1. Elution profiles of (a) a standard mixture of pyruvic (1) and acetic (2) acids, and (b) pyruvic acid released from a sample of xanthan by treatment with phosphoric acid. Column, Bio-Rad HPX-87H, 300 x 7·8 mm; flowrate, 0·6 ml min^{-1}; solvent, 8 mM sulphuric acid; temperature, 35°C.

TABLE 1
Pyruvate and Acetate Contents, % w/w, of Polysaccharides, Estimated by HPLC and Colorimetric Methods

Sample	*Method of dissolving*	*Pyruvate*		*Acetate*	
		HPLC	*Colorimetric*	*HPLC*	*Colorimetric*
Xanthan[a]	Heat/stir	4·39	5·31	6·17	5·4
Xanthan[a]	Autoclaved[c]	3·23	4·31	4·26	3·68
Xanthan[b]	Heat/stir	4·59	4·39	4·47	4·17
Xanthan[b]	Autoclaved[c]	2·54	2·2	3·9	3·36
Xanthan[d]	Heat/stir	3·87	4·19	5·23	4·58
Konjac[e]	Heat/stir	–	–	4·09	3·94

[a] Native.
[b] Sonicated, 10 h.
[c] 120°/20 min, in 0·1 M NaCl.
[d] Oxidative-reductive depolymerization with ascorbic acid.
[e] Konjac glucomannan has been reported to have 3·7% acetyl groups (Dea *et al.*, 1977).

tent was taken to be due to protein and cell debris in the original xanthan preparations. Apparently, prolonged sonication depolymerizes the protein and cell material to the extent that it is lost during dialysis. Xanthan itself is difficult to depolymerize.

We attribute the higher pyruvate readings in unsonicated samples to colour developed between the 2,4 dinitrophenylhydrazine reagent and protein and/or cell debris. The pyruvate values determined by the HPLC method are close to the 4·6% which corresponds to 1 mole of pyruvate to two repeat units. An exception is the sample dissolved by autoclaving, as discussed later.

A value of 6·26% represents 1 mole of acetate per repeat unit of xanthan, this value being reported by a number of workers. In all cases the values obtained by the HPLC procedure were higher (by 10–14%) than those found by colorimetry. As acetate was found to be stable to the alkaline conditions used, and because conditions were optimized for the release of acetate, the authors believe the HPLC values to be the more accurate. Table 1 also shows that both pyruvate and acetate levels are lower in samples which have been subjected to

oxidative/reductive depolymerization by ascorbic acid (Herp, 1980). On the other hand, sonicated xanthan retains the pyruvate, but loses some acetate.

3.2 Lability of the pyruvate ketal

Literature values for pyruvate vary considerably and reflect the lability of the pyruvate as well as real variability in the samples (Jeanes *et al.*, 1976; Sandford *et al.*, 1978; Sutherland, 1981*b*; Rinaudo *et al.*, 1983).

Release of pyruvate during autoclaving is substantial (25%) in 0·1 M sodium chloride (Table 1) and almost complete in water (not shown).

Rinaudo *et al.* (1983) observed by n.m.r. spectroscopy that heating xanthan in 0·1 M NaCl (48 h, 90°C) removed all but 10% of the pyruvate, whereas similar heating in water completely removed the pyruvate. Rinaudo *et al.* (1983) interpret this as higher stability in the ordered, helical form (in salt) than in the more disordered coil form (in water). The ordered form is likely to persist, at least in part, at 90°C (Morris *et al.*, 1977) in salt solution.

It is therefore likely that some of the work in the literature has been carried out on xanthan samples which are much lower in pyruvate content than expected. The results emphasize the need to report the method of dissolution.

3.3 Optimization of conditions

The acid conditions for removal of pyruvate were optimized using time studies. The amount of pyruvate removed increased up to about 90 min, and remained constant to $\simeq$ 120 min, after which the peak diminished in size, presumably due to decomposition of the pyruvate. Standard solutions of pyruvate were prepared on the day of use. Samples of standard pyruvate were subjected to the hydrolysis conditions, and no losses were detected up to $\simeq$ 2 h, after which the peak size decreased gradually.

The conditions for acetate removal were determined similarly. No loss of released acetate was noted up to 24 h.

Acetate standards were stored frozen and showed no deterioration after several weeks. Xanthan concentrations were determined by use of the phenol/sulphuric procedure (Dubois *et al.*, 1956) which had been

calibrated against a standard curve prepared by careful purification and drying of a xanthan sample.

Xanthan samples, which had been subjected to acid or alkali treatment as described above, were dialysed against distilled water, freeze dried and subjected to colorimetric analysis. No residual acetate or pyruvate was detected. The conditions used here for removal of acetate and pyruvate for analysis are more severe than those used when the object is to recover and study the modified xanthan (Sutherland, 1981*b*).

4. CONCLUSION

The HPLC reported is straightforward and direct. It lends itself to rapid checking of samples which might have been subjected to conditions conducive to the loss of the labile pyruvyl group.

As with all HPLC procedures, equipment and columns are an initial expense, but it is evident that they are capable of more specific and often simpler assays than are colorimetric methods. Colorimetry, however, may be more sensitive.

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