Journal of Chromatography, 213 (1981) 301–306
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROM. 13,883

AFFINITY COLUMN FOR SEPARATION OF PYRUVYLATED AND NON-PYRUVYLATED POLYSACCHARIDES

I. W. SUTHERLAND
Department of Microbiology, University of Edinburgh, Edinburgh EH9 3JG (Great Britain)
(Received April 14th, 1981)

SUMMARY

The possibility of separating pyruvylated polysaccharides into pyruvate-rich and pyruvate-poor fractions has been demonstrated using an affinity matrix. Thus in some polymers, a mixture of strand types exists. The affinity matrix was prepared by coupling antibodies to a *Rhizobium* polysaccharide to Sepharose gel. Elution was accomplished by the addition of pyruvate to the eluting buffer. Non-pyruvated polysaccharides were not adsorbed.

INTRODUCTION

There is currently considerable interest in polysaccharides such as that from *Xanthomonas campestris* (xanthan gum) which have commercially useful properties[1]. The physical characteristics of xanthan derive from its cellulosic backbone to which are attached trisaccharide side chains[2,3]. The presence of pyruvate (and acetate) in a molar ratio less than one per trisaccharide repeat unit is an unusual feature of this structure. Many, but not all, microbial exopolysaccharides possess structures in which each repeat unit carries an acetyl and/or a ketal group[4]. As several workers have reported variations in the pyruvate content of xanthan from different strains or prepared by different cultural procedures[5–7], the relationship of pyruvate to carbohydrate structure is of considerable interest. Although a method utilising fractional precipitation with ethanol has been employed to differentiate xanthan preparations into fragments with differing pyruvate content[8], it is most suitable for larger quantities of polysaccharide and is not satisfactory for small aliquots such as might be used in routine analysis or would be available from isotopic studies. It is also unlikely that it would be satisfactory for other partially pyruvylated polysaccharides.

As part of a programme to provide microanalytical procedures for defining microbial polysaccharides, a method was sought to determine whether pyruvate was present on all polymer strands, on alternate repeat units as was earlier found for both acetate and pyruvate[9,10] or whether pyruvate was present on some strands only and absent from others.

0021-9673/81/0000–0000/$02.50

EXPERIMENTAL

Organisms and growth conditions

Xanthomonas campestris strain 646 was grown routinely in a medium containing limiting nitrogen and excess carbohydrate[11] in 100-ml aliquots in 250-ml erlenmeyer flasks shaken at 30°C. The same conditions were used for *Enterobacter aerogenes* and *Escherichia coli* strains. Bacterial cells for preparation of radioactive polysaccharide were grown for 16 h in half-strength nutrient broth at 30°C.

Polysaccharide preparation and purification

Cultures were killed by the addition of 1% (v/v) formalin and bacteria deposited by centrifugation at 10,000 *g* for 30 min. The supernatant fluids were added to two volumes of cold (−40°C) acetone to precipitate polysaccharide. The precipitate was redissolved in a small volume of water, dialysed exhaustively against distilled water, ultracentrifuged at 50,000 *g* for 1 h and lyophilised. Radioactive polymer was prepared by transferring the cells grown in half strength nutrient broth to fresh nitrogen-deficient medium containing 0.1% (w/v) glucose and 0.1 mCi ^{14}C-labelled glucose. After 2–4 h incubation at 30°C, the polysaccharide was recovered in the normal way.

Analytical procedures

Total carbohydrate was assayed by the phenol–sulphuric acid method[12] or, if pyruvate was present, by a micro-modification of the anthrone procedure[13]. The latter method was adopted for column eluates containing pyruvate which interferes in the phenol-sulphuric acid assay. Micromethods for the assay for individual sugars in either intact polysaccharides or in hydrolysates and for acetate and pyruvate were those used in earlier studies[14]. Hydrolysis of the polysaccharides was performed at 100°C in 1 *M* trifluoracetic acid for 12 h. Hydrolysates were dried under vacuum, redissolved in distilled water and again dried. After solution of the hydrolysate in a known volume of distilled water, assays were made for the component sugars identified by paper chromatography. Chromatograms were on Whatman No. 1 paper with butan-1-ol–pyridine–water (6:4:3) as eluent. Paper electrophoresis was performed in pyridinium acetate buffer, pH 5.3 in Locarte (London, Great Britain) equipment. A current of 100 mA was applied for 3 h to 77 × 20 cm strips of Whatman 3MM paper.

Radioactivity measurements

Radioactivity was measured in a Packard Tricarb liquid scintillation spectrometer. Aliquots (50 μl) were counted in a dioxane-based scintillant (NE250, Nuclear Enterprises, Edinburgh, Great Britain).

Preparation of the affinity material

The globulin fraction of antiserum prepared against *Rhizobium* strain TA1 polysaccharide was obtained by ammonium sulphate fractionation. After removal of the salt by dialysis, it was coupled to CNBr-activated Sepharose 6MB (Pharmacia, Uppsala, Sweden). The gel material (5 g) was swollen and washed for 15 min on a glass filter with 1 l 10 m*M* HCl. The protein fraction (50 mg) was dissolved in 25 ml 0.1 *M* $NaHCO_3$ buffer containing 0.5 *M* NaCl, mixed with the gel in a glass stoppered

bottle and rotated overnight at 4°C. To remove unbound protein, the gel was washed with coupling buffer and any residual active groups were blocked by treatment with 1 *M* ethanolamine at pH 8.0 for 2 h. The gel was finally washed alternately with acetate–saline buffer and with 0.1 *M* borate buffer (pH 8.0, containing 0.5 *M* NaCl), three times. It was stored until required in acetate buffer containing a few drops of 0.1 % (v/v) merthiolate solution as preservative at 4°C.

RESULTS

Separation of polysaccharides into pyruvate-rich and pyruvate-poor fractions

Although a fractional precipitation procedure has been described for separating *Xanthomonas campestris* polysaccharide into pyruvylated and poorly pyruvylated material[8], it requires relatively large amounts of material, of the order of 500 mg or more for successful results. It was also limited in its applicability, most other bacterial polysaccharides tested requiring different solvent concentrations from xanthan. There was clearly a need to develop a method for use with small amounts of polysaccharide, which could be applied to any polymer and not solely to those from a single bacterial species. The observation of Dudman and Heidelberger[15] that antibodies against the polysaccharide from *Rhizobium* strain TA1 were directed against the pyruvate ketals present in this polymer, indicated the possibility of developing an affinity procedure. Antiserum was fractionated to yield the globulin portion, which was then coupled to CNBr-activated Sepharose 6MB. The gel was employed in 5 or 10 cm × 1 cm columns. Radioactive *X. campestris* polysaccharide was applied to the column in 0.1 *M* acetate buffer (pH 4.0) containing 0.5 *M* NaCl. After thorough elution with this buffer, the eluent was changed to 0.1 *M* acetate buffer at the same pH, containing 0.25 *M* NaCl and 0.25 *M* sodium pyruvate. A typical elution pattern, as measured by the radioactivity in the eluate, is shown in Fig. 1. Clearly, some

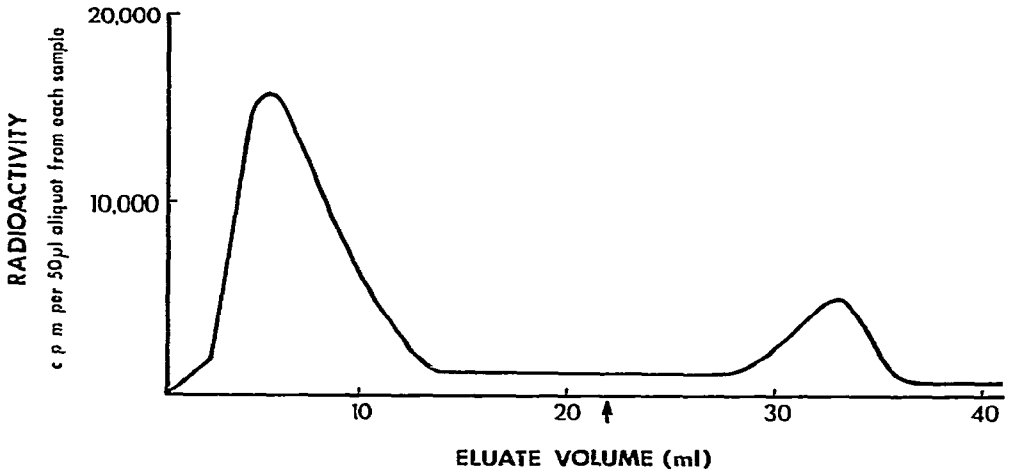

Fig. 1. Affinity chromatography of polysaccharide from *Xanthomonas campestris*. A preparation of ^{14}C-labelled polysaccharide from *X. campestris* strain 646, dissolved in 0.1 *M* acetate buffer (pH 4.0, containing 0.5 *M* sodium chloride) was applied to a column 5 × 1 cm) of Sepharose 6 MB to which *Rhizobium* strain TA1 antiserum had been coupled. The adsorbent had been equilibrated with the same buffer which was then used for elution. After 19 × 1 ml fractions had been eluted, buffer containing 0.25 *M* sodium chloride + 0.25 *M* sodium pyruvate was applied and elution continued.

material failed to adsorb and was eluted in the void volume. The remaining material adsorbed to the column and was eluted by buffer containing pyruvate. When the radioactive solutions were pooled as two separate fractions, the ratio of the radioactivity of the unadsorbed material to that adsorbed was approximately 3.24:1. Recovery of the radioactive material from the column was essentially complete.

After thorough washing with the initial buffer, the adsorbent was used repeatedly to prepare material from unlabelled polysaccharide. The pooled material from the non-radioactive polymer from the same *X. campestris* strain corresponded to 7.5 mg of "fraction 1" (unadsorbed) and 5.54 mg of "fraction 2" (eluted with pyruvate). Analysis of these fractions revealed the expected carbohydrate composition of glucose, mannose and glucuronic acid in the approximate molar ratio of 2:2:1. Both fractions contained about 4.5% acetate, but the pyruvate contents were 2.2% and 9.1% respectively. On treatment with a specific depolymerase enzyme, each fraction was converted to a mixture of two oligosaccharides corresponding to the pyruvylated repeating unit of xanthan and to the non-pyruvylated fragment respectively. These oligosaccharides were separated by paper electrophoresis, eluted and quantified by carbohydrate assay. The ratio of pyruvylated to non-pyruvylated oligosaccharide was 1:4.2 for "fraction 1" and 1:0.6 for "fraction 2". This together with the observed pyruvate content indicated that fraction 1 was "pyruvate-poor" while fraction 2 was "pyruvate-rich".

When materials from the unadsorbed fractions was re-applied to the column, a small amount of material was adsorbed and could be eluted with pyruvate-containing buffer. This was almost certainly due to overloading of the adsorbent in the initial preparative runs. When fraction 2 material was reapplied to the column, care being taken to ensure that it was not overloaded, it was completely adsorbed.

Similar results were obtained with a number of preparations of *Xanthomonas* polysaccharides from different strains or from strains grown under different culture conditions. Each yielded material eluting with the void volume and further material only eluting in the presence of pyruvate. Two exceptions were *X. campestris* polysaccharide from which the pyruvate groups had been removed by treatment with 10 m*M* trifluoracetic acid at 100°C for 90 min, and the polymer from *Xanthomonas phaseoli* strain 1128, a polysaccharide known to contain little, if any, pyruvate under the cultural conditions used for its preparation. Neither of these two preparations was adsorbed to the gel.

Polysaccharides from other bacterial species

Application of other bacterial polysaccharides to the affinity column yielded differing results. None of the polymers lacking pyruvate, which were tested, were adsorbed. Two polymers which, on the basis of their analysis, appeared to contain 1 mole of pyruvate per repeat unit, *Enterobacter aerogenes* type 1 and a pyruvylated form of *E. aerogenes* type 8, were completed adsorbed. So too were a number of preparations of colanic acid, the common product of *Escherichia coli*, *Enterobacter cloacae* and *Salmonella* species. These polysaccharides all eluted as single fractions with pyruvate-containing buffer.

When polysaccharide from *E. aerogenes* type 30 was applied to the column, an elution pattern similar to that obtained with the *X. campestris* polymers was seen (Fig. 2). Part of the material applied was eluted in the void volume and was found to

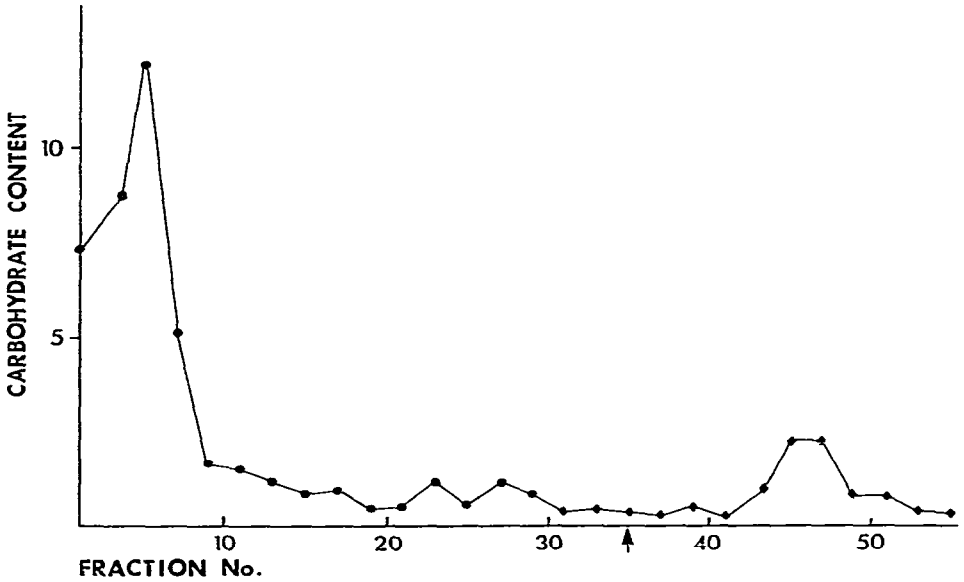

Fig. 2. Chromatography of *Enterobacter aerogenes* type 30 polysaccharide. A polysaccharide preparation (approx. 5 mg) was dissolved in acetate–saline buffer and applied to a column (10 × 1 cm) of adsorbent. The initial eluting fluid was the same buffer and 1 ml fractions were collected and aliquots (100 μl) assayed for carbohydrate by the phenol–sulphuric acid method. At the point indicated by the arrow, elution with pyruvate-containing buffer (0.25 *M*) was started and further assays of eluate made using the anthrone method. The carbohydrate content is given in μg/100 μl.

contain no appreciable pyruvate. The remaining material was adsorbed, but could be eluted with pyruvate-containing buffer. It contained approximately one mole pyruvate per pentasaccharide repeating unit.

DISCUSSION

Although fractionation of polysaccharides with ethanol or other water-miscible solvents is a recognised procedure[8], it has the disadvantage that the conditions must be determined for each polymer. A further disadvantage lies in the considerable variations in molecular weight which may be found in different polysaccharide preparations[16]. These greatly affect the precipitability of the polysaccharides independent of the degree of pyruvylation. In studies of the way in which polysaccharide composition is affected by different culture conditions, a method is needed to determine whether any single batch of material is composed of a mixture of strand types or is uniform in its composition having substituents such as pyruvate occurring irregularly on the molecules. The application of an affinity procedure avoids any molecular weight effects and provides a means of examining small quantities of any pyruvate-containing polysaccharide. A possible drawback might exist if polymers were closely related structurally to the *Rhizobium* TA1 polymer against which the antiserum was prepared. It is also possible that polysaccharides containing different pyruvate configurations[17] might react differently. This remains to be tested. It would

also be an advantage to have an adsorbent with higher capacity. For this, the preparation of an artificial antigen with pyruvate as the immunodominant group might be preferable to the use of *Rhizobium* polysaccharide.

The recognition from the results presented here, that polysaccharides from batch culture, may comprise a mixture of ketalated and non-ketalated molecules indicates that variations in the pyruvate content may depend on the age of the culture, etc. It has already been observed that in continuous culture using different nutrient limitations and other growth conditions, considerable variation in *X. campestris* and related polysaccharides can exist[5,6]. The method described here would enable more accurate examination of such material, particularly when it is possible to use it in conjunction with specific depolymerase enzymes. Recent results suggest that there is much greater inherent variability in polysaccharide structures than has hitherto been recognised[6,18].

ACKNOWLEDGEMENTS

This work would not have been possible without the generous gift of antiserum, polysaccharide and cultures from Dr. W. F. Dudman. Mrs. Lynn Kennedy is thanked for her technical assistance.

REFERENCES

1 I. W. Sutherland and D. C. Ellwood, in A. T. Bull, D. C. Ellwood and C. Ratledge (Editors), *Microbial Technology, Current State and Future Prospects*, Cambridge University Press, Cambridge, 1979, pp. 107–150.
2 P.-E. Jansson, L. Kenne and B. Lindberg, *Carbohydr. Res.*, 45 (1975) 275–282.
3 L. D. Melton, L. Mindt, D. A. Rees and G. R. Sanderson, *Carbohydr. Res.*, 46 (1976) 245–257.
4 I. W. Sutherland, *Biochem. J.*, 104 (1967) 278–285.
5 I. W. Davidson, *FEBS Lett.*, 3 (1978) 347–349.
6 C. Evans, R. G. Yeo and D. C. Ellwood, in R. C. W. Berkeley, G. W. Gooday and D. C. Ellwood (Editors), *Microbial Polysaccharides and Polysaccharases*, Academic Press, London, New York, San Francisco, 1979, pp. 51–68.
7 M. C. Cadmus, S. P. Rogovin, K. A. Burton, J. E. Pittsley, C. A. Knutson and A. Jeanes, *Can. J. Microbiol.*, 22 (1976) 942–948.
8 P. A. Sandford, P. R. Watson and C. A. Knutson, *Carbohydr. Res.*, 63 (1978) 253–256.
9 G. G. S. Dutton and T. E. Folkman, *Carbohydr. Res.*, 78 (1980) 305–315.
10 G. G. S. Dutton and K. L. Mackie, *Carbohydr. Res.*, 62 (1978) 321–335.
11 I. W. Sutherland and J. F. Wilkinson, *J. Gen. Microbiol.*, 39 (1965) 373–383.
12 M. Dubois, K. A. Gillies, J. K. Hamilton, P. A. Rebers and F. Smith, *Anal. Chem.*, 28 (1956) 350–356.
13 N. J. Fairbairn, *Chem. Ind. (London)*, (1953) 86.
14 I. W. Sutherland, *Biochem. J.*, 115 (1969) 935–945.
15 W. F. Dudman and N. Heidelberger, *Science*, 164 (1969) 954–955.
16 B. A. Nisbet and M. Kerr, unpublished results.
17 P. J. Garegg, P.-E. Jansson, B. Lindberg, F. Lindh, J. Lönngren, I. Kvarnström and W. Nimmich, *Carbohydr. Res.*, 78 (1980) 127–132.
18 I. W. Sutherland, in A. Rose and J. G. Morris (Editors), *Advances in Microbial Physiology*, Vol. 24, Academic Press, London, New York, San Francisco, 1981.