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Note

A new assay for mannuronan C-5-epimerase activity*

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An extracellular mannuronan C-5-epimerase can be isolated[1-3] from liquid cultures of the soil bacterium *Azotobacter vinelandii*. The enzyme is active during the biosynthesis of alginate by converting[4] D-mannuronic acid into L-guluronic acid residues in the polymer chain, provided the degree of polymerisation ($\overline{d.p.}_n$) of the substrate is greater than 10. The enzymic activity depends[2] upon the concentration of calcium ions, but does not appear to require NAD^+ or NADH.

The enzymic activity is accompanied by an exchange of protons with the medium[5]. By applying ^{1}H-n.m.r. spectroscopy to a polymer epimerised in D_2O, we have demonstrated[6] that the proton exchange takes place at C-5. Similar results have been obtained for the D-glucuronic acid C-5-epimerase that is active during the biosynthesis of heparin[7].

On the basis of this information, we have developed a method for determining the enzyme's activity, in which [5-^{3}H]alginate is used as a substrate, and the tritium released into the water is measured. A similar technique has recently been reported[7,8] for measuring the D-glucuronic acid C-5-epimerase in heparin biosynthesis.

The [5-^{3}H]alginate was produced by growing *Azotobacter vinelandii* in a medium consisting of D-glucose (20 g), K_2HPO_4 (1 g), $MgSO_4 \cdot 7\ H_2O$ (200 mg), $FeSO_4 \cdot 7\ H_2O$ (50 mg), $NaMoO_4 \cdot 2\ H_2O$ (5 mg), NH_4OAc (2.3 g), and $CaCl_2 \cdot 2\ H_2O$ (50 mg) diluted to one litre with water. The cells were grown at 30° with vigorous shaking. After 30 h, D-[5-^{3}H]glucose was added to a concentration of 0.6 mg/mL (specific activity, 0.7 μCi/mg), and the cells were allowed to grow for another 72 h. The culture was cooled in an ice-bath, and the cells were removed by centrifugation. The supernatant solution was dialysed against 0.05M sodium EDTA (3 × 5 litres) for 24 h followed by exhaustive dialysis against distilled water. The sodium alginate was then precipitated with ethanol in the presence of 0.2% of sodium chloride.

The composition and the nearest-neighbour frequencies of the polymer were

*Biosynthesis of Alginate, Part V. For Part IV, see ref. 6.

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TABLE I

THE COMPOSITION AND DIAD FREQUENCIES IN [5-^{3}H]ALGINATE FROM *Azotobacter vinelandii* GIVEN IN MOLE FRACTIONS

	F_M	F_G	F_{MM}	F_{MG}	F_{GM}	F_{GG}
Before fractionation	0.69	0.31	0.64	0.05	0.05	0.26
After fractionation	0.96	0.04	0.92	0.02	0.02	0.00

determined by high-resolution, ^{1}H-n.m.r. spectroscopy[9]. The results (Table I) indicate that the polymer had a high content of homopolymeric blocks. The alginate was further fractionated to obtain a polymer with a higher content of D-mannuronic acid. A solution of the alginate (80 mg) in distilled water (16 mL) was adjusted to pH 3.6 with 0.1M hydrochloric acid and then heated at 100° for 1 h. The pH was adjusted to 6.5 with 0.1M NaOH and the L-guluronic acid-rich fragments were precipitated by adding 0.1M $CaCl_2$ (19.8 mL). The calcium-soluble fraction was dialysed against 0.05M sodium EDTA (3 × 5 litres) for 24 h and exhaustively against water, and then freeze-dried and analysed by ^{1}H-n.m.r. spectroscopy. The results (Table I) showed that the content of L-guluronic acid had been decreased from 31% to <4% by this fractionation. There was no detectable resonance signal from GG-H-5, indicating that the few residues of L-guluronic acid still remaining in the polymer were

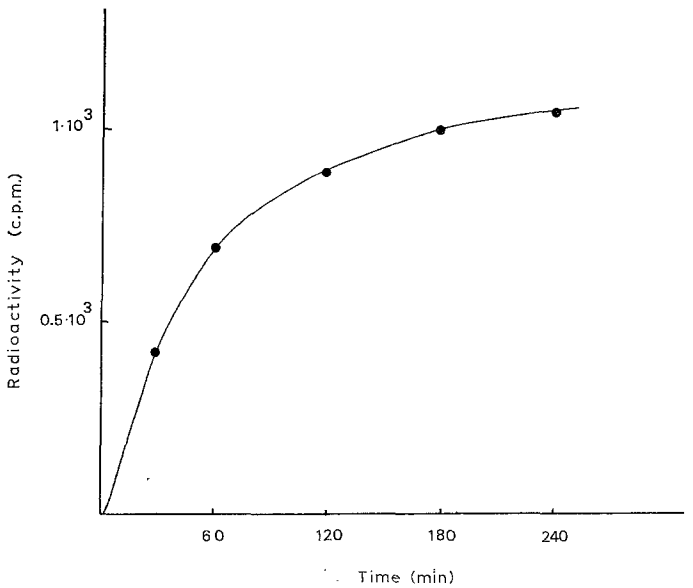

Fig. 1. Tritium release as a function of time: [5-^{3}H]alginate (8000 c.p.m.) was incubated with enzyme (0.24 mg of protein) in a total volume of 3.5 mL. The alginate was precipitated with 3.0 mL of ethanol, and the radioactivity in 2.0 mL of the supernatant was determined.

involved in heteroglycosidic linkages[9] (-M-G-M-). The intensity of the end-group resonance signal corresponded[9] to an average $\overline{d.p.}_n > 30$. The specific activity was determined by liquid scintillation (Isocap 300, Chicago Nuclear), using PPO (6 g) and Bis-MSP (0.5 g) in pseudocumene (1 litre). The enzyme was isolated from liquid cultures of *Azotobacter vinelandii* by ammonium sulphate precipitation[2] (50%), and desalted on a column of G-25 PD-10 (Pharmacia) equilibrated with 0.05M imidazole/HCl buffer (pH 6.8). Enzymic activity was assayed by the Dische carbazole reaction[2].

In the new assay system, the incubation mixture consisted of 0.5 mL of [^{3}H]alginate (2.5 mg/mL) (96% M; specific activity, 6400 c.p.m./mg), 2.0 mL of collidine/HCl buffer (pH 6.8), and $CaCl_2$ (added to give a concentration of 0.34mM), with the volume adjusted to 3.3 mL with distilled water. The mixture was incubated in centrifuge tubes (10 mL) with 0.2 mL of active enzyme (1.2 mg of protein/mL) at 20°. The reaction was stopped at intervals by adding sodium chloride to a concentration of 0.2%, and the alginate was precipitated with 3.0 mL of ethanol. The samples were centrifuged for 15 min, and the supernatant solutions were transferred to scintillation vials and counted for 20 min. The results (Fig. 1) demonstrated that label was gradually released, reaching ~40% of the total after 3 h. The background in control samples (containing radioactive substrate but no enzyme) was ~2.5% of the total label in the mixture. When the radioactive supernatant was distilled, >80% of the label was found in the distillate, indicating that tritium in the alginate had been exchanged with water, and that the substrate was labelled at position 5.

In order to rule out the possibility of interference by alginate lyase in the assay, the [5-^{3}H]alginate was replaced in the incubation mixture by the same concentration of [^{14}C]-labelled alginate. This substrate was prepared from *Azotobacter vinelandii*, as described above, by adding D-[^{14}C]mannose to the growth medium instead of D-[^{3}H]glucose. The alginate had a specific activity of 3.2×10^5 c.p.m./mg, and <2% was found in the soluble fraction after incubation with the enzyme for 2 h at 30° and precipitation with ethanol.

The only method available hitherto for determination of enzyme activity of the C-5 epimerase has been the carbazole method. Two major drawbacks, namely low reproducibility and low response, have precluded the use of this method for accurate studies of enzyme kinetics. In the present method, the accuracy may be improved by increasing the proportion of tritiated glucose in the growth medium, in order to obtain a substrate with higher specific activity. The method is well suited for determining V_{max} and K_m, a detailed report of which will be published elsewhere.

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