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Note

An enzymic method for the assay of D-mannuronan C-5 epimerase activity

ANDREW J. CURRIE AND JAMES R. TURVEY
School of Physical and Molecular Sciences, University College of North Wales, Bangor, Gwynedd LL57 2UW (Great Britain)

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During investigations on D-mannuronan C-5-epimerase from bacterial and algal sources, sensitive methods for assaying enzyme activity were required. The usual method for such assays depends on measuring the absorbance of the complexes formed by uronic acids with a carbazole reagent under conditions where D-mannuronic acid residues give a lower absorbance than an equivalent of L-guluronic acid residues[1]. The production of L-guluronic acid by epimerase action on D-mannuronic acid therefore results in an increase in the absorbance with carbazole[2], but the procedure is time consuming and the relatively high blank given by the substrate alone detracts from the sensitivity of the method. For the related glycuronic acid C-5 epimerase of mouse microsomal preparations, an assay has been developed which is based on the release of tritium into the medium from a (D-[5-^{3}H]glucosyluronic acid)-labelled heparin precursor[3]. However, this method requires stocks of a suitable ^{3}H-labelled precursor, which were not available for our enzyme. We wished not only to detect low levels of epimerase activity but also to follow the progress of the reaction when only small amounts of epimerisation had occurred.

The L-guluronan lyase from *Klebsiella aerogenes*[4] has no action on D-mannuronan, but readily cleaves L-gulosiduronic acid linkages in L-guluronan and in the regions of alginate where both uronic acids occur in mixed sequences[5]. Such action is readily followed by the increase in the absorbance at 235 nm due to the 4,5-unsaturated uronic acid produced by the lyase. We have made this the basis of a sensitive procedure for detecting C-5-epimerase activity. The substrate used for epimerase action was the alginate fraction rich in D-mannuronic acid (95%) isolated from fruiting bodies of *Ascophyllum nodosum*[6]. An epimerase preparation from *Azotobacter vinelandii*[2] was incubated with this substrate and, at suitable intervals (0.5–4 h), aliquots were transferred to a phosphate buffer and the lyase was then added. At zero time and at various times thereafter, the absorbance was measured at 235 nm. The absorbance at zero time was deducted from all subsequent readings on that aliquot. Fig. 1 shows the changes in absorbance that occurred for several aliquots when incubated with lyase.

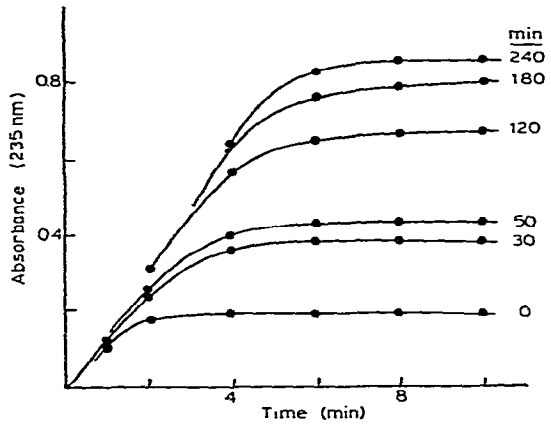

Fig. 1. Action of L-guluronate lyase on aliquots removed at various times from an epimerase digest of D-mannuronan.

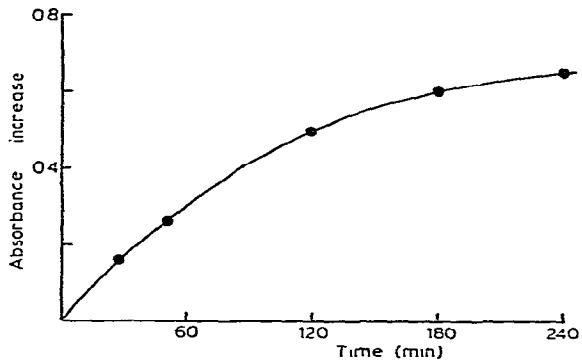

Fig. 2. Progress of C-5 epimerase action on D-mannuronan, as assayed by the action of L-guluronate lyase.

It can be seen from Fig. 1 that, under the conditions used, action of the lyase was complete within 10 min. The increase in absorbance when lyase acted on the aliquot removed from the epimerase digest at zero time represents action of the lyase on the small amount (5%) of L-guluronic acid present initially in the substrate. When the increase in absorbance read at 10 min for each aliquot was corrected for this initial action and then plotted against the time of incubation with epimerase, the curve shown in Fig. 2 was obtained. From this, it is seen that the progress of epimerase action can be followed from an early stage of reaction. For routine work, it is not necessary to follow the course of lyase digestion, but to take the absorbance at zero time and again at 20 min when all lyase action is complete.

For the assay of epimerase activity, a standard incubation time of 0.5 h for enzyme and substrate was chosen. After this time, aliquots were removed and treated with lyase as above, the increase in absorbance after 20 min being used as a measure of epimerase activity. A convenient unit of enzyme activity is defined as the amount

of enzyme that causes an increase in absorbance of 0.1 under these digest conditions. Using various amounts of enzyme, it was established that there was a linear relationship between the increase in absorbance and the amount of enzyme used between 1 and 10 units of activity.

The assay described above is about ten times more sensitive than the carbazole assay and is, therefore, more useful for following the early stages of epimerase action in a digest or for measuring low levels of enzyme activity. A possible limitation to the use of this method might arise if the epimerase preparation contained high levels of endogenous lyases as impurities. Although such lyase action occurring during the initial digestion with epimerase would merely result in a high blank (at the start of the second digestion), further lyase action during the second digestion with the *Klebsiella* enzyme would give high figures for apparent epimerase activity. We have not yet encountered this problem, as all our epimerase preparations, from either bacterial or algal sources, contained negligible lyase activity; where an appreciable error might arise, the problem could be overcome by heating the aliquot taken from the epimerase digest, to inactivate the lyase before digesting with the *Klebsiella* enzyme, and taking a zero reading at the beginning of this second digestion.

EXPERIMENTAL

Substrates and enzymes. — The lyase from *Klebsiella aerogenes* type 25 was prepared as described previously[4], and the freeze-dried preparation had an activity of 20 units/mg. An alginate fraction rich in D-mannuronic acid was isolated from fruiting bodies of *Ascophyllum nodosum*[6] and analysis of this fraction by p.m.r. spectroscopy[7] gave a D-mannuronic acid content of 95%.

Crude D-mannuronan C-5-epimerase was isolated from a culture of *Azotobacter vinelandii*, strain LC, essentially by the method of Haug and Larsen[2]. The growth medium contained D-glucose (20 g), K_2HPO_4 (1 g), $MgSO_4 \cdot 7\ H_2O$ (0.2 g), $FeSO_4 \cdot 7\ H_2O$ (0.05 g), $NaMoO_4 \cdot 2\ H_2O$ (5 mg), NH_4OAc (2.3 g), and 0.1mM $CaCl_2$ in water to 1 L. The sterile medium was inoculated with a growing culture of the organism and was then shaken at 30° for 30 h. The culture was cooled to 0°, and cells were removed by centrifugation at 6000*g*. Ammonium sulphate was added to 50% saturation and, after 16 h at 4°, the precipitate was collected by centrifugation at 15000*g*. The precipitate was redissolved in water and dialysed against a solution containing the same concentrations of inorganic constituents as the growth medium, but without $CaCl_2$. The solution was then freeze-dried.

Action of lyase on epimerase-digested D-*mannuronan.* — The substrate solution contained D-mannuronan (5 mg/mL) in 0.1M Tris-HCl buffer (pH 7.54) containing 3.4mM $CaCl_2$. Freeze-dried epimerase was dissolved in water, to give a stock solution containing 5 mg/mL. Substrate solution (2 mL) was incubated with epimerase (0.4 mL) at 30°. At zero time and at intervals up to 4 h, aliquots (0.25 mL) were withdrawn and added to 50mM phosphate buffer (2 mL, pH 7.0) in 1-cm u.v. cells maintained at 30°. Lyase solution (5 mg/mL, 0.25 mL) was added and the absorbance

was followed at 235 nm in a Unicam SP800 spectrophotometer. The curves for the aliquots removed at various times, corrected for initial absorbance, are shown in Fig. 1.

Assay for epimerase activity. — The substrate, buffers, and lyase solutions were as described above. Substrate solution (0.5 mL) and epimerase solution (0.1 mL), previously equilibrated at 30°, were mixed and incubated at 30° for 30 min. An aliquot (0.25 mL) was then withdrawn and diluted with phosphate buffer (2 mL), and lyase solution (0.25 mL) was added. The digests were incubated at 36° for 20 min, and the absorbance was then read in a 1-cm cell at 235 nm on a Unicam SP500 spectrophotometer. The blank contained substrate (0.5 mL) and water (0.1 mL) treated in the same way. Epimerase solutions containing 5–25 mg/mL were assayed by this procedure, and the results showed a linear relationship between the increase in absorbance and the amount of enzyme used.

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