Carbohydrate Research, 241 (1993) 237–244
Elsevier Science Publishers B.V., Amsterdam

End-labelled fluorescent polyguluronate and polymannuronate for the assay of alginate lyases

Frederick S. Wusteman * and Peter Gacesa

Department of Biochemistry, University of Wales College of Cardiff, P.O. Box 903, Cardiff CF1 1ST (United Kingdom)

(Received June 1st, 1992; accepted August 27th, 1992)

ABSTRACT

Alginate subfractions that approach homopolymers in mannuronate or guluronate have been end-labelled with the fluorescent dyes fluorescein and tetramethylrhodamine linked via putrescine. The putrescine–alginate intermediates were obtained in high yield and the labels were attached via the terminal amino group. The fluorescent alginates are suitable as substrates for the assay (simultaneous if necessary) or L-guluronate and D-mannuronate lyases by spectrophotometry or fluorimetry.

INTRODUCTION

Alginates are (1 → 4)-linked glycuronans composed of residues of β-D-mannosyluronic acid (M) and α-L-gulosyluronic acid (G). These residues are arranged in block structures which can be homopolymeric [poly(β-D-mannosyluronic acid) (MM) and poly(α-L-gulosyluronic acid) (GG)] or heteropolymeric, i.e., containing random blocks (MG). Enzymes that degrade alginate are specific for either L-guluronate- or D-mannuronate-rich regions and almost invariably act as lyases rather than simple hydrolases[1]. Thus, the action of alginate-degrading enzymes can be followed spectrophotometrically either by detecting the release of reducing ends or the appearance of unsaturated non-reducing residues.

In order to establish the specificity of a degrading enzyme, parallel experiments are necessary in which substrates enriched in one or other of the uronic acids are employed. These procedures are complex and time consuming and could be simplified if the substrates were specifically labelled in a way which distinguishes

* Corresponding author.

the bond being broken. This objective can be achieved if the labels are dyes with absorption maxima at distinct positions of the visible spectrum. It is relatively simple to achieve multiple labelling along the length of the polysaccharide chain. Thus, Remazol Brilliant Blue has been employed to label, for example, mannan[2] and xylan[3], to give chromogenic substrates for enzymes. However, alginate can be affected by the alkaline conditions required for the coupling with Remazol Brilliant Blue, presumably because of the carboxyl group[5]. Once coupled, this dye is readily lost during the assay[4]. Other workers[5] have also reported difficulties with the labelling of alginates with dyes. Furthermore, all products in which the dye molecule is attached along the length of the chain suffer from the disadvantage that some of the labels may interfere sterically with the active site of the enzyme. This effect appears in an extreme form with alginate labelled with Reactive Black which is totally resistant to attack by an epimerase[5].

We now describe a method by which alginate subfractions may be end-labelled with fluorescent compounds, to give products that can be used for the simultaneous assay of guluronate and mannuronate lyases in a rapid and simple procedure.

EXPERIMENTAL

Preparation of alginate block structures.—Alginate block structures that approached idealised polymannuronate and polyguluronate structures were derived by acid fractionation[7] of Manucol DH (Alginate Industries). Essentially, alginate was partially hydrolysed in mineral acid and fractions which corresponded to polymannuronate and polyguluronate were obtained as sodium salts. The composition and block structure of the various fractions was determined using Fourier-transform ^{1}H NMR spectroscopy[6]. The polymannuronate contained $> 89\%$ of D-mannuronate and the polyguluronate contained $> 93\%$ of L-guluronate (Table I).

Preparation of putrescine derivatives of alginate block structures.—The procedure required differs slightly for polymannuronate and polyguluronate because the latter is precipitated by putrescine and requires the presence of salt and a more dilute solution in order to ensure that it remains soluble during the reductive amination.

A solution of sodium polymannuronate (2 g) in water (50 mL) was mixed with 0.5 M putrescine hydrochloride (50 mL; adjusted to pH 7 with M NaOH), then

TABLE I

Composition of alginate subfractions

Fraction	F_M	F_G	F_{MM}	F_{GG}	F_{MG}	F_{GM}
Polyguluronate	0.066	0.934	0.033	0.901	0.033	0.033
Polymannuronate	0.894	0.106	0.807	0.019	0.087	0.087

treated with borane–pyridine complex (1 mL; Aldrich). This mixture was shaken vigorously in a sealed flask, then stored at room temperature for 14 days. The putrescine-linked alginate was precipitated by adding ethanol (100 mL, saturated with sodium acetate trihydrate) and collected as a sticky mass by centrifugation (700*g*, 30 min). A solution of this product in 0.2 M NaCl (100 mL) was applied to a column of Dowex 1-X2 (Cl^-) resin (30-mL bed volume, 200–400 mesh) previously equilibrated with 0.2 M NaCl. The column was washed with 0.2 M NaCl (the effluent was discarded) then eluted during 24 h with 0.6 M NaCl (100 mL). The product, precipitated by the addition of 1 vol of EtOH to the eluate and collected by centrifugation, was stirred with EtOH and then ether to give the putrescine-linked polymannuronate as a white powder (1.97 g, 97%, assuming a chain length of 20 residues[7] per putrescine residue).

A similar procedure was adopted for the preparation of putrescine-linked polyguluronate except that the reductive amination was effected in 200 mL of a solution that included NaCl at a final concentration of 0.5 M. Since the solution already contained enough Na^+ to ensure flocculation of the sodium alginate, the first precipitation step employed EtOH (200 mL) without added NaOAc. The resulting putrescine-linked polyguluronate was a white powder (1.68 g, 82%, assuming a chain length of 20 residues per putrescine residue).

Analysis of the putrescine–alginate samples.—Each sample had a negligible reducing-sugar content as assayed by the dinitrosalicylic acid method[8]. Primary amino groups were assayed by the 2,4,6-trinitrobenzenesulphonic acid method[9] with measurements taken at 413 nm before the final acidification step. It was essential to maintain a neutral or alkaline pH because alginates are precipitated at low pH. Using ϵ-aminocaproic acid as standard, putrescine–polyguluronate was found to have an amino content of 0.26 μmol/mg and putrescine–polymannuronate to have 0.37 μmol/mg.

Preparation of fluorescein isothiocyanate (FITC)-labelled polyguluronate.—A solution of putrescine-linked polyguluronate (0.4 g) in water (20 mL) was mixed with a solution of fluorescein isothiocyanate (40 mg, FITC; Isomer I, Aldrich) in pyridine (4 mL). The mixture was stored at room temperature overnight, EtOH (24 mL) saturated with sodium acetate trihydrate was added, the precipitate was collected by centrifugation (450*g*, 10 min) and washed with acetone to remove excess of dye, and a solution in 0.1 M NaCl (20 mL) was applied to a column of DEAE-cellulose (20 mL, DE52, Whatman; Cl^- form) previously equilibrated with 0.1 M NaCl. The column was washed with 0.1 M NaCl (100 mL, eluate discarded) then during 24 h with 0.4 M NaCl (60 mL). The eluate was diluted with 1 vol of EtOH and the yellow precipitate was allowed to flocculate out, then collected by centrifugation, washed on a sintered disc filter with EtOH, acetone, and ether, and air-dried. The resulting FITC-polyguluronate was a yellow powder (374 mg, 85%).

Preparation of tetramethylrhodamine isothiocyanate (TRITC)-labelled polymannuronate.—A solution of putrescine-linked polymannuronate (0.2 g) in water (4 mL) was mixed with a solution of tetramethylrhodamine isothiocyanate (10 mg,

TRITC, mixed isomers, Cl^- salt; Sigma) in pyridine (0.5 mL). The mixture was stored at room temperature overnight, then diluted with 1 vol of EtOH containing sodium acetate trihydrate. The precipitate was collected and subjected to chromatography on DEAE-cellulose (as described above). TRITC-polyguluronate was obtained as a pink powder (183 mg, 84%).

Gel-permeation chromatography.—A sample (10 mg/mL) of FITC-polyguluronate was digested exhaustively (18 h at 30°C) with guluronate lyase (50 μL of a dialysed solution of the ammonium sulphate precipitate from *Klebsiella pneumoniae*[10]). Similarly, TRITC-polymannuronate was digested exhaustively with mannuronate lyase derived from an extract of Abalone acetone powder[11] (Sigma). Samples (10 mg in 1 mL) of alginate were applied to a column (410 × 12.4 mm) of Sephadex G-50 (fine grade; Pharmacia) and eluted with water at 20 mL/h. Fractions (2 mL) were collected and the absorbance at 490 nm (FITC-group) or 549 nm (TRITC-group) was measured.

Procedure for the assay of alginate lyases, using dye-linked substrate.—The dye-linked alginates were reprecipitated before use (recovery 95%) from solution in 0.3 M NaCl (6 mg/mL) by the addition of 0.7 vol of EtOH. This reduced the control values in the assays to negligible proportions.

A solution (2 mg/mL) of FITC-polyguluronate or TRITC-polymannuronate in 50 mM potassium phosphate buffer (pH 7.0) that contained 0.3 M NaCl was dispended in 1.4-mL aliquots. Enzyme solution (30 μL) was mixed with an aliquot using a vortex mixer. The mixture was incubated at 37°C, the reaction was terminated by vigorous mixing with EtOH (1 mL), and the precipitate (undigested alginate) was removed by centrifugation (450*g*, 5 min) supplemented, if necessary, by filtration of the supernatant solution through a small plug of cotton wool. The released dye-labelled oligosaccharides were determined spectrophotometrically at 496 nm (FITC-polyguluronate) or 547 nm (TRITC-polymannuronate). At the substrate concentration chosen, the increase in absorbance with time was linear up to values that indicated 10% solubilisation of substrate.

Both lyase activities could be measured simultaneously if the two substrates were dissolved in the same solution. The absorbance at 547 nm reflected digestion of the TRITC-polymannuronate, whereas that at 495 nm reflected degradation of FITC-polyguluronate with a small contribution from the absorbance of solubilised TRITC-polymannuronate. The relative concentrations of solubilised dye-labelled substrates may be determined as follows: solubilised TRITC-polymannuronate A_{547}, solubilised FITC-polyguluronate ($A_{496} - 0.25A_{547}$).

For assays at much greater sensitivities, the solubilised products were determined by fluorescence spectroscopy using a Perkin–Elmer LS-5 Luminescence Spectrometer (excitation/emission wavelengths of 488/517 nm for FITC-polyguluronate and 548/571 nm for TRITC-polymannuronate). This assay could be performed on a microscale as only 100 μL of the clarified supernatant solution was required for each measurement. The supernatant solution was diluted with 0.1 M NaOAc buffer (3 mL, pH 6.0) before measurements were made.

For purposes of comparison with established methods, a solution (2 mg/mL) of FITC-polyguluronate (extrapolated absorbance at 490 nm = 5.2) was digested with an extract of *Klebsiella pneumoniae* and the release of soluble fragments was compared with those of a similar digest that contained 2 mg/mL of the unlabelled polyguluronate. Compared with an increase of 0.007 absorbance unit/min of released FITC-labelled oligosaccharides, the absorbance (230 nm) of the 4,5-double bond increased at 0.005 unit/min, whereas the reducing sugar assays[7] were scarcely measurable. When the digestion was repeated with a 4-fold increase in enzyme concentration, the change in reducing sugar absorbance was 0.009 unit/min.

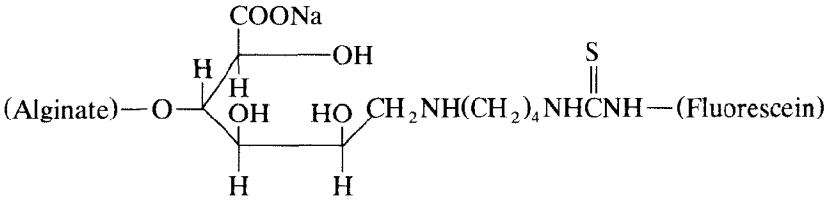
RESULTS AND DISCUSSION

Linkage of dyes to the ends of alginate chains.—In order to minimise interference between the dye label and the lyase, end-labelling of the polysaccharide chains was investigated. As this choice must necessarily limit the degree of substitution, it was essential to maximise the attachment of the chosen spacer arm (putrescine). Using a considerable molar excess of putrescine with a long exposure to the selective reducing agent borane–pyridine complex[12], amino-group analyses of the isolated product indicated that virtually every chain was terminated with an amino group.

With a maximum substitution of only one dye molecule per chain (in practice achieved only with wastefully high ratios of activated label to alginate chain), it was important to use a dye with a high molar extinction coefficient. Two such dyes were required with major absorption peaks widely spaced in order to permit their simultaneous use in mixed solutions. The fluorescein and Remazol[13] groups of dyes satisfied these conditions and, furthermore, they are available commercially in the form of derivatives which react with amino groups under mild conditions.

Fluorescein isothiocyanate is readily coupled[14] via the terminal amino group of putrescine to yield derivatives (1) with a strong yellow colour and an absorption spectrum that is sharply cut off above 530 nm. In order to obtain maximum resolution, a blue dye was used for the second label and the Remazol dye derivative Uniblue A (Aldrich) was coupled (results not shown) to the terminal amino group under mild conditions (pH 8, room temperature), in contrast to the potentially damaging conditions necessary for attachment of the unmodified dyestuff[2,3]. The product, which was satisfactory for qualitative studies, did not obey Beer's law when in aqueous or aqueous ethanol solution, presumably due to non-covalent interactions of the dye molecules. As all candidate blue dyes were possibly liable to this defect, a red dye of the fluorescein family, TRITC[14] was adopted as the alternative label. Although the absorption peak of the tetramethylrhodamine group is closer to that of FITC, it is resolved sufficiently for use in double-label assays. In aqueous ethanol solutions, the absorbance spectrum of the

TRITC group overlaps (25% of the peak TRITC absorbance) the maximum absorbance wavelength of FITC and so a simple correction factor is needed.



1

Possible interference of dye label with enzyme action.—The dye is attached to polyuronate residues via a reduced (ring-opened) uronate ring and the diaminobutane (putrescine) spacer. In order to assess the occurrence of any interference with the enzymes, samples of each substrate were digested exhaustively with an excess of the appropriate lyase and the products were fractionated by gel-permeation chromatography (Fig. 1). The dye co-elutes with the alginate before digestion but was found in a highly retarded peak that was well resolved from the small oligosaccharide fragments[1] after enzymic degradation of the chain. The fully retarded peak [fractions 29–31 in Fig. 1b] contained 90.2% of the eluted fluorescence associated with only 5.8% of the uronic acid. Since there are twenty residues in a chain and an unsaturated guluronic acid residue has an enhanced orcinol reaction[16], this finding is consistent with the bulk of the fluorescent fragments containing only one (unsaturated) uronic acid residue. Therefore, there is no evidence of indigestible stubs at the dye-labelled end of the chains.

Use of dye-labelled alginates in assay procedure.—The use of FITC-polyguluronate and TRITC-polymannuronate for the rapid assay of alginate lyases depends on the increasing solubility of these substrates in aqueous ethanol as the digestion proceeds. Vigorous addition of ethanol at the end of the incubation time both stops the reaction and precipitates the undegraded substrate. The coloured soluble fraction can be separated from the precipitate either by centrifugation or microfiltration. In order to obtain a linear release of product which is readily determined spectrophotometrically, it is necessary to use substrate concentrations of at least 1 mg/mL (ideally 2 mg/mL). Using the substrates labelled with the degree of substitution described in the text, the method described is not only more discriminating but also more sensitive than the reducing-sugar assay method employing dinitrosalicylic acid[7]. More sensitive reducing-sugar assays based on measurement of Cu(I) complexes are unsuitable, as the undegraded alginate binds the metal ion, thus causing considerable interference. It is of comparable sensitivity to methods[10] that involve changes in UV absorbance at 230 nm, but these assays are notoriously sensitive to UV-absorbing components present in crude enzyme extracts. Much greater sensitivity can be achieved if the assay is performed by fluorimetry which allows the volumes used to be decreased at least ten-fold. In addition to being more economical, the fluorimetric assay involves no cross-correc-

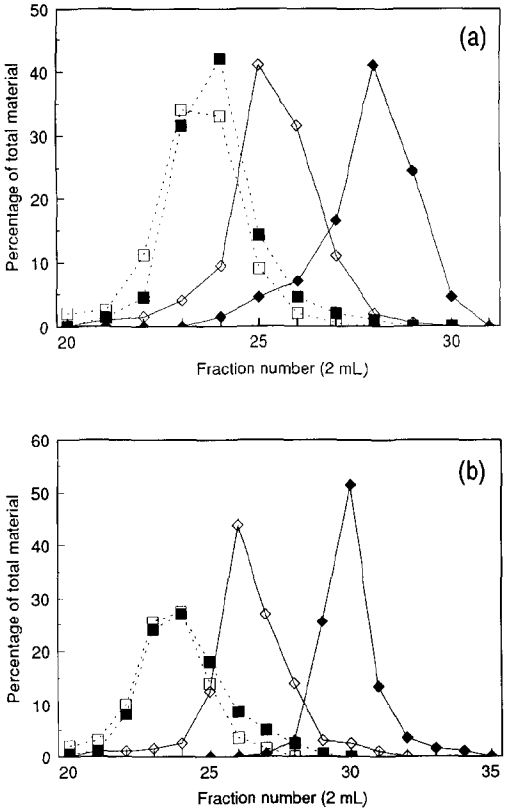


Fig. 1. Gel-permeation chromatography of the end products of digestion with lyase (see Experimental). Alginate, before (·····) and after (———) digestion, as determined by the orcinol reaction[14] (□, ◇) and absorbance of the fluorescent dye (■, ◆). (a) TRITC-polymannuronate incubated overnight at 30°C with 50 μL of an extract of Abalone powder [10 mg/mL in 25 mM Tris/acetate (pH 7.0)]. (b) FITC-polyguluronate treated as in (a) after digestion overnight with an extract of *Klebsiella pneumoniae*.

tions when both substrates are present in the same solution. Unfortunately, it is not possible to convert ethanol-precipitability directly into the number of bonds broken. In order to achieve this result, each substrate would have to be calibrated using one of the traditional assay procedures.

The assay described above is not only more sensitive than conventional assay procedures, but also allows simultaneous determination of the substrate specificity.

REFERENCES

1 P. Gacesa, *Int. J. Biochem.*, 24 (1992) 545–552.
2 B.V. McCleary, *Carbohydr. Res.*, 67 (1978) 213–221.
3 P. Biely, D. Mislovicova, and R. Toman, *Anal. Biochem.*, 144 (1985) 142–146.
4 P. Gacesa, unpublished results.

5 M.A. Ragan, *Hydrobiologia*, 204/205 (1990) 415–417.
6 H. Grasdalen, B. Larsen, and O. Smidsrød, *Carbohydr. Res.*, 68 (1979) 23–31.
7 A. Haug, B. Larsen, and O. Smidsrød, *Acta. Chem. Scand.*, 21 (1967) 691–704.
8 G. Noelting and P. Bernfeld, *Helv. Chim. Acta*, 31 (1948) 286–290.
9 Z. Yosizawa, T. Kotoku, F. Yamauchi, and M. Matsuno, *Biochem. Biophys. Acta*, 141 (1967) 358–365.
10 J. Boyd and J.R. Turvey, *Carbohydr. Res.*, 57 (1977) 163–171.
11 F. Haugen, F. Kortner, and B. Larsen, *Carbohydr. Res.*, 198 (1990) 101–109.
12 J.C. Cabacungan, A.I. Ahmed, and R.E. Feeney, *Anal. Biochem.*, 124 (1982) 272–278.
13 I.P. Griffith, *Anal. Biochem.*, 46 (1972) 402–412.
14 K. Wang, J.R. Feramisco, and J.K. Ash, *Methods Enzymol.*, 85 (1982) 514–562.
15 A.H. Brown, *Arch. Biochem.*, 11 (1946) 269–278.
16 F.S. Wusteman, unpublished results.