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Note

Automated, enzymic degradation of some oligoglucans

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A common method for the investigation of the anomeric configuration of interunit linkages in oligoglucans involves depolymerisation by α-D-glucosidases (*e.g.*, gamma-amylase) and β-D-glucosidases (*e.g.*, almond-emulsin β-D-glucosidase). Although limited by the specificity of the enzymes concerned, automation of this procedure provides a useful parameter in the structural characterisation of oligoglucans. With these enzymes, determination of released D-glucose by the D-glucose oxidase procedure[1] provides a convenient method for assessment of the extent of hydrolysis, provided that Tris buffer is used to inhibit extraneous glucosidase action. In an automated technique, prime consideration must be given to the time required for complete hydrolysis. In order to achieve complete conversion of maltose oligosaccharides (d.p. 2–6) into D-glucose, a mixture of beta- and gamma-amylase was required (Table I). Use of yeast β-D-glucosidase for degradation of laminarin oligosaccharides resulted in a 94% conversion into monomer. This could not be improved by variation of conditions.

TABLE I

INCUBATION OF MALTOHEXAOSE WITH GAMMA- AND BETA-AMYLASE

Gamma-amylase (μg)	*Beta-amylase (μg)*	*Temperature (degrees)*	*Conversion into D-glucose (%)*
2000	0	50	74.0
2000	200	50	87.2
2000	500	50	91.0
2000	1000	50	94.5
2000	10	37	100

Having established suitable conditions for the hydrolysis of oligosaccharides with these enzymes, the procedure was then automated. The sample solution was split into two streams, one of which was mixed with beta- and gamma-amylase, and the other with β-D-glucosidase. The automated D-glucose oxidase method was used for

determination of the D-glucose liberated. After incubation with the respective enzymes for one hour, D-glucose oxidase reagent was added. In order to keep the enzyme solution used to a minimum, a slow flow-rate (0.03 ml/min) was chosen for both the enzyme and substrate solutions. This was the minimum flow rate consistent with obtaining steady responses in the analysis system. The enzyme reagent solutions were kept in an ice bath to minimise their denaturation, the enzymes then being stable for at least 48 h. The D-glucose oxidase solution soon turned brown on standing, giving rise to increasing base lines. This was partially overcome by keeping this solution also in an ice bath. The browning of the solution, probably due to aerobic oxidation of the *o*-dianisidine, was further decreased by minimising contact of the solution with the air.

Maltose and maltohexaose were analysed by this automated procedure, using D-glucose as a standard. The flowing concentration of the oligosaccharide solutions was obtained by the automated cysteine–sulphuric acid assay. A schematic diagram of the combined enzyme-hydrolysis procedure and the cysteine–sulphuric acid assay is shown in Fig. 1. Conversion of maltose and maltohexaose into D-glucose by the automated procedure was 99% and 94%, respectively.

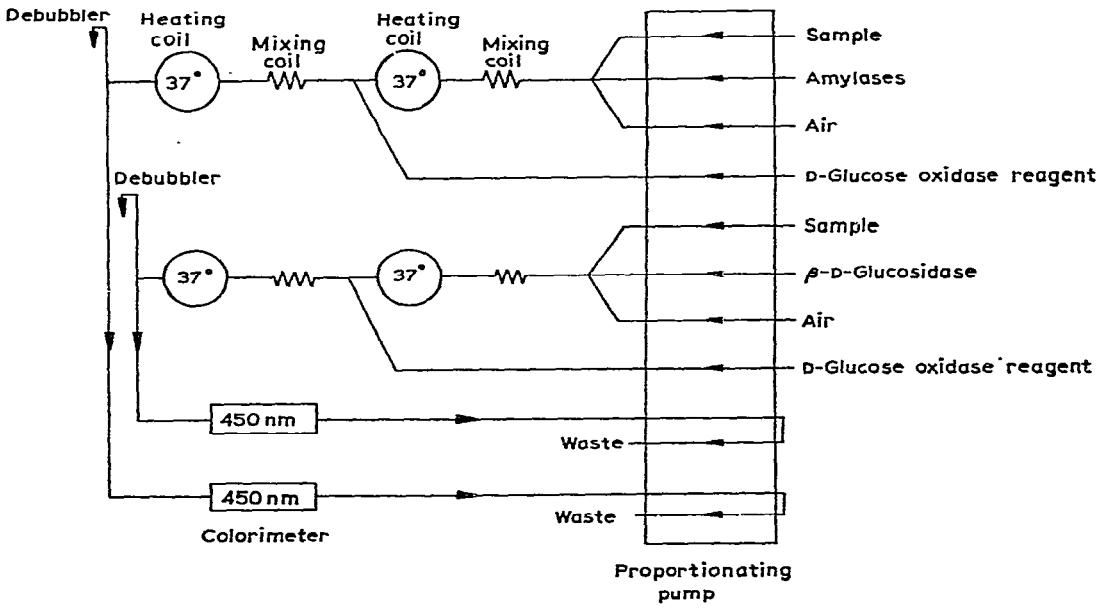

Fig. 1. Schematic representation of the automated system for enzyme hydrolysis. Reagent flow-rates: sample (0.03 ml/min), beta- and gamma-amylase (0.015 ml/min), air (0.05 ml/min), β-D-glucosidase (0.015 ml/min), waste-return line (0.16 ml/min).

An application of this technique was illustrated by classification of oligosaccharides in column eluates. A mixture of starch, maltotetraose, maltotriose, laminaribiose, and D-glucose was fractionated according to molecular size on a

column of cation-exchange resin (AG 50W x2, Li^+ form, 200–400 mesh; Fig. 2*a*). Starch was degraded by gamma- and beta-amylase to D-glucose. Maltotetraose and maltotriose were also degraded by these enzymes to give D-glucose. Conversely, laminaribiose was degraded by β-D-glucosidase, but not by gamma- and beta-amylase, to D-glucose. D-Glucose responded to the D-glucose oxidase assay on both channels in the automated procedure. Fractionation of laminaripentaose, laminaritetraose, laminaribiose, and D-glucose, on a similar column of smaller dimensions, showed that these β-(1→3)-linked oligosaccharides gave D-glucose on incubation with β-D-glucosidase, but not with alpha- and beta-amylase (Fig. 2*b*).

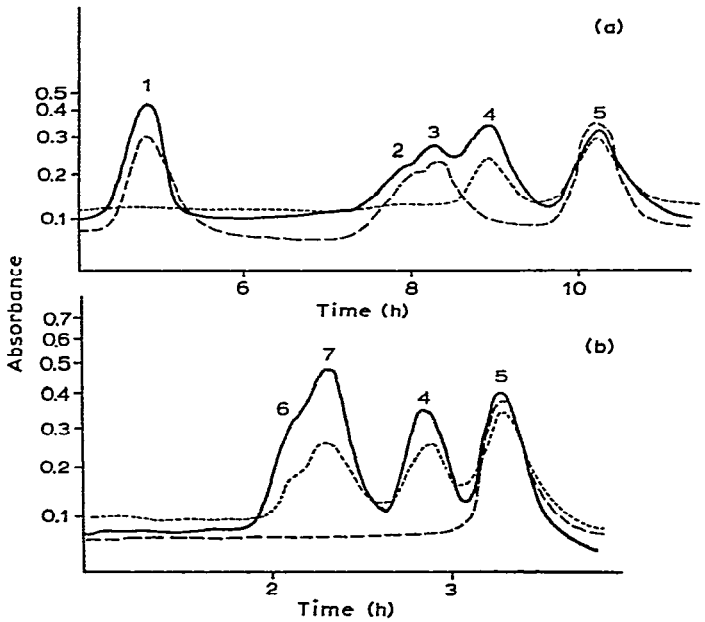

Fig. 2. Automated enzyme hydrolysis of column eluates. Starch (1), maltotetraose (2), maltotriose (3), laminaribiose (4), D-glucose (5), laminaripentaose (6), laminaritetraose (7). D-glucose oxidase assay after beta- and gamma-amylase incubation, — — —; D-glucose oxidase assay after β-D-glucosidase incubation, ...; cysteine–sulphuric acid assay, ———.

The results obtained demonstrated the feasibility of an automated scheme of enzyme degradation for establishing the anomeric configuration of certain interunit linkages. The method is limited in application by the specificity of the enzymes employed. Selection, for example, of an α-D-glucosidase with less specificity for the position of substitution of the penultimate sugar unit would extend the range of oligo-D-glucans susceptible to hydrolysis. The application of this scheme, in conjunction with other automated systems, to provide structural information will be discussed in a subsequent report.

EXPERIMENTAL

Incubation of maltohexaose with beta- and gamma-amylase. — To a solution of maltohexaose (0.5 mg/ml, 1 ml), gamma-amylase (2 mg/ml) in ammonium acetate–acetic acid buffer (M, pH 5; 1 ml) and beta-amylase (1 mg/ml, 0.01 ml) were added. The solution was incubated at 37° for 1 h. The enzymes were denatured by heating at 100°, and the solution was analysed by the D-glucose oxidase procedure for D-glucose. Quantitative conversion into D-glucose was achieved.

Incubation of cellobiose with β-D-glucosidase. — Solutions of cellobiose (0.5 mg/ml, 1 ml) and β-D-glucosidase (2 mg/ml; 400 units*/mg; 1 ml) in potassium dihydrogen orthophosphate buffer (0.05M, pH 6.5) were incubated at 37° for periods up to 2 h. Analysis of D-glucose by the D-glucose oxidase assay showed that 94.5% conversion into D-glucose was achieved after 1 and 2 h, respectively.

Automation of the enzyme hydrolysis and the D-glucose oxidase assay. — The sample solution was continuously mixed with gamma-amylase (2 mg/ml) and beta-amylase (5 μg/ml) in acetate buffer (M, pH 5.0) and air, over a period of 1 h at 37°. D-Glucose oxidase reagent was added and the solution was passed for 1 h through a coil maintained at 37°. The solution was debubbled and the absorbance of the chromophore was recorded at 420 nm. An identical procedure was employed for the hydrolysis with β-D-glucosidase. A schematic diagram is presented in Fig. 1.

Analysis of oligosaccharides in column eluates. — A mixture of starch, maltotetraose, maltotriose, laminaribiose, and D-glucose (20 mg, each component 1 ml) was fractionated on a column (90 × 5 cm) containing AG 50 x2 (Li^+) resin[2] (200–400 mesh). The column was eluted with water at 2.0 ml/min. A portion of the eluate (0.03 ml/min) was degraded continuously by the automated enzyme hydrolysis system. The experiment was repeated for a mixture of D-glucose, laminaribiose, laminaritetraose, and laminaripentaose. The mixture (200 μg of each oligosaccharide) was fractionated on a column of smaller dimensions (140 × 0.8 cm). The column was eluted with water at 0.23 ml/min. Elution profiles are reproduced in Figs. 2*a* and 2*b*.

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*1 unit liberates 1 mg of D-glucose from salicin per min at pH 5.0 and 35°.