AN ENZYMIC METHOD FOR THE DETERMINATION OF THE DEGREE OF POLYMERISATION OF GLUCANS

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(Received July 20th, 1970; accepted for publication, September 3rd, 1970)

ABSTRACT

The number-average degree of polymerisation of glucans may be determined by measurement of the sorbitol (D-glucitol) content of an acid hydrolysate of the borohydride-reduced glucan, using sorbitol dehydrogenase. The method is applicable to both linear and branched polymers of either α- or β-D-glucose.

INTRODUCTION

Chemical methods for the determination of the number-average degree of polymerisation ($\overline{DP}_n$) of oligo- and poly-saccharides are based upon measurement of the relative proportion of terminal reducing groups. In certain methods, the terminal aldose residue is first reduced to the corresponding alditol by treatment with sodium or potassium borohydride. Peat and his co-workers[1] compared the reducing power of acid hydrolysates of an oligosaccharide with that of the borohydride-reduced oligosaccharide. These would contain x and $(x-1)$ molecules of monosaccharide, respectively, if x was the *DP*. Using the colorimetric anthrone–sulphuric acid reagent, the method is very satisfactory for the smaller oligosaccharides (*DP* 2–7), but the decrease in accuracy with increase in molecular size prevents the method being applied to polysaccharides.

Smith and his co-workers[2] have examined the periodate oxidation of borohydride-reduced polysaccharides. Terminal hexitol residues linked at C-2, C-5, or C-6 should give rise to one molecular proportion of formaldehyde, whilst residues linked at C-3 or C-4 should yield two molecular proportions. In practice, some cyclisation of the latter residues occurs during periodate oxidation, with the formation of pentose residues[3], so that the yield of formaldehyde is decreased by a variable amount, thus leading to uncertainty in the quantitation. Moreover, the experimental procedure is not simple, and requires the removal of the polysaccharide and periodate and iodate ions prior to the determination of formaldehyde.

The molecular size of some glucans has been determined by periodate-oxidation analyses. With linear, amylose-type molecules, the reducing and non-reducing end-groups yield two and one molecular proportions of formic acid, respectively, on simple Malapradian-type oxidation[4]. In practice, most samples of amylose are not

entirely linear[5], and it is difficult to ensure that some over-oxidation does not occur, with the production of additional formic acid. Hence, this method is of limited value. In an alternative method[6], glucans have been deliberately over-oxidised with periodate, and since the rate of over-oxidation is dependent on the number of reducing groups which were originally present, determination of the rate enables the molecular size to be calculated. This method is also restricted to linear molecules, since the over-oxidation process is affected by (1→6)-inter-chain linkages and other structural variations.

Two procedures involving the use of radioactive intermediates have been described. In the first[7], the polysaccharide is treated with sodium cyanide-[^{14}C], and the net radioactivity of the polysaccharide is measured by an appropriate counting technique. The method is unsuitable for alkali sensitive polysaccharides. Alternatively, reduction of a polysaccharide with sodium borohydride-*t*, followed by acid hydrolysis and measurement of the tritium content of a suitable aliquot, is a possible method[8], although variations in the quality of the sodium borohydride-*t* have prevented the widespread use of this procedure.

We now describe a method which is suitable for the routine estimation of the $\overline{DP}_n$ of polymers of D-glucose. After reduction with borohydride, the glucan is hydrolysed with acid, and the amount of sorbitol (D-glucitol) produced is estimated by an enzyme-catalysed oxidation reaction. The D-glucose content of the hydrolysate is determined by a reductometric method or use of D-glucose oxidase, and the $\overline{DP}_n$ is then calculated from the relative amount of D-glucitol.

EXPERIMENTAL

Sorbitol dehydrogenase (SDH: E.C. 1.1.1.14, also known as L-iditol:NAD oxidoreductase or L-iditol dehydrogenase) catalyses the following reversible reaction:

$$\text{Sorbitol} + \text{NAD}^+ \rightleftharpoons \text{D-fructose} + \text{NADH} + \text{H}^+$$

At pH values in the range 9–10 and in the presence of excess NAD^+, the oxidation of sorbitol is effectively quantitative[9]; the reduction of NAD^+ may be followed by measurement of the extinction at 340 nm. SDH may be prepared from sheep liver[10], or purchased from C. F. Boehringer. NAD was purchased from Sigma Chemical Company.

Determination of sorbitol. — The procedure is similar to that described by Williams-Ashman[9]. The following solutions were pipetted into a 1-cm silica cell:- 0.1M sodium pyrophosphate buffer (pH 9.5, 1.0 ml), 20mM NAD (0.2 ml), distilled water (0.75 ml), and sample containing less than 20μg of D-glucitol (1.0 ml). After mixing, the cell was inserted into an automatic ultraviolet spectrophotometer (Pye Unicam SP800) set to measure the extinction at 340 nm at intervals of 1 min. An external recorder and ten-fold scale expander were used to give a full-scale deflection of 0.20 extinction unit. The reaction was carried out at 25°.

After measurement of the initial extinction, the reaction was started by the

addition of Boehringer SDH (3.5 units, 0.05 ml), and readings were taken until the extinction became constant, usually within 10 min. Since the extinction coefficient of NADH at 340 nm is 6.22×10^6 cm^2/mole, the amount of D-glucitol (in μmole) is given by the increase in extinction $\times$ 3/6.22. Control experiments showed that there was a linear relation between the change in extinction and the D-glucitol concentration over the range 5–20 μg.

General procedure for determination of $\overline{DP}_n$. — The weights and volumes involved in the method should be adjusted to suit the quantities of D-glucitol and D-glucose being assayed. For an oligosaccharide, less than 2 mg is required, whereas for a large glucan, 25 mg may be necessary to give an adequate amount of D-glucitol.

Conditions for the total acid hydrolysis of the glucanitol should be determined in control experiments. For most water-soluble α- and β-D-glucans, 2M hydrochloric acid at 100° for 2–3 h is suitable.

For an estimation of a $\overline{DP}_n$ in the range 50–500, the following procedure is convenient. The glucan (10 mg) is dissolved in water (1–2 ml) and treated with potassium borohydride (25 mg) for 24–48 h at 18–20°. The excess borohydride is destroyed with hydrochloric acid (pH 4), and the solution evaporated to dryness at 40°. Borate is removed by five evaporations to dryness, using methanol (2 ml). The glucanitol is hydrolysed with 2M hydrochloric acid (1 ml) at 100° for 3 h, the hydrolysate is then cooled and neutralised to pH 9.5 (glass electrode) with M sodium hydroxide and the solution diluted to 10 ml with distilled water. Aliquots (1 ml) are removed for the direct estimation of D-glucitol, and after tenfold dilution, of D-glucose by the Nelson–Somogyi method[11]. The $\overline{DP}_n$ is given by the formula: $\overline{DP}_n$ = (μmoles of D-glucose/μmole of D-glucitol) +1. In control experiments, there was no selective destruction of either D-glucose or D-glucitol during treatment with 2M hydrochloric acid for 2 h at 100°

RESULTS

Determination of $\overline{DP}_n$ *of standard glucans.* — In the first control experiments, the maltosaccharides used were isolated from a partial, acid hydrolysate of amylose by Dr. J. R. Stark, Maltohexaose (2.0 mg) gave a $\overline{DP}_n$ of 6.1, and maltodecahexaose (2.5 mg) a DP_n of 14.7.

In later experiments, β-D-glucans were examined. In laminarin, some of the molecules are terminated by a reducing D-glucose residue, and other molecules by mannitol[12]. Treatment of a sample of soluble laminarin (10.0 mg) gave a solution having a D-glucitol content of 0.7%. Previous analysis[12] of this glucan gave a mannitol content of 3.0%. The total proportion of end groups is therefore 3.7%, equivalent to a $\overline{DP}_n$ of 27.0; by periodate-oxidation analysis[12], the sample gave a $\overline{DP}_n$ of 25.5. Analysis of a β-(1$\rightarrow$6)-D-glucan (10.0 mg) isolated from yeast cell-walls[13] gave a $\overline{DP}_n$ of 141. On gel filtration from a column of agarose (Bio-Gel A 0.5 m), the elution volume of this glucan (determined by Dr. W. L. Cunningham) indicated a molecular weight in the range of 23,000–26,000, equivalent to a $\overline{DP}_n$ of 142–160. The column

had been calibrated with Procion-stained dextrans covering the molecular weight range 1×10^4–2×10^6.

Effect of pH on enzyme activity. — The effectiveness of the oxidation is very sensitive to small changes in pH. Variation in the pH from 9.5 to 9.3 may cause a 10% decrease in extinction. Careful adjustment of the samples to pH 9.5 (using a glass electrode) is essential prior to analysis. SDH activity may also be measured at pH 9.5, using 50mM Tris–hydrochloric acid buffer or 50mM glycine–sodium hydroxide buffer, instead of the 0.1M sodium pyrophosphate buffer.

Effect of various carbohydrates and ions on enzymic activity. — D-Fructose had a zero extinction at 340 nm and did not therefore affect the SDH assay. D-Glucose, in an excess ranging from 100- to 1000-fold, had no effect on the assay of D-glucitol. When D-glucitol was assayed in the presence and absence of mannitol (2 mg), a change in extinction corresponding to the presence of 10 μg of D-glucitol was noted. After three recrystallizations of the mannitol from ethanol, no such change was observed. It was concluded that commercial samples of D-mannitol may contain traces of D-glucitol, and that pure D-mannitol does not interfere with the SDH assay.

D-Glucitol was estimated in the presence and absence of 0.2M sodium chloride; the changes in extinction were identical, showing that the salt which would be produced during the neutralisation of acid hydrolysates had no effect. D-Glucitol (100 μg) and potassium borohydride (25 mg) were dissolved in water (1 ml), and the borohydride was then destroyed by the addition of 2M hydrochloric acid. The solution was then neutralised with sodium hydroxide to pH 9.5, and diluted to 10 ml. The solution completely inhibited SDH, showing the necessity of removing borate at this concentration prior to analysis. However, when smaller proportions of borohydride are used for the reduction, removal of borate may not be essential, and a control experiment showed that a final concentration of 1 mM borate did not interfere with the SDH assay.

Certain glucans[13] are insoluble in mineral acid, and require a preliminary hydrolysis with formic acid to effect dissolution. When D-glucitol (10 μg) was estimated in the presence of formic acid (0.5 ml) and 2M sulphuric acid (0.25 ml) which had been neutralised with sodium hydroxide, SDH activity was not apparent. However, when the formate was first removed by repeated evaporation with water, normal enzymic activity was observed.

Specificity of enzymic oxidation. — The following carbohydrates (2 mg) were incubated with SDH under the standard conditions; no effect was observed with D-glucose, D-mannitol, D-xylose, galactitol, D-arabinitol, L-arabinitol, erythritol, L-rhamnitol, and 2-deoxy-D-*arabino*-hexitol. Xylitol and ribitol were oxidised at a significant rate; for example, 0.66 μM ribitol reduced 0.17 μM NAD.

DISCUSSION

The method described in this paper has been in routine use in this laboratory for two years, and has given satisfactory results provided that samples are essentially

free from formate and borate, and that the pH is carefully adjusted to 9.5. Examples of its use include measurement of the $\overline{DP}_n$ of (*a*) a stubbed glucan prepared by acidic and enzymic degradation of amylopectin[14], (*b*) the maltosaccharide chains liberated from amylopectin by the debranching enzymes isoamylase and pullulanase[15], (*c*) various samples of β-(1→3)-D-glucan from yeast cell-walls[13]. The method is equally satisfactory with both linear and branched polymers of either α- or β-D-glucose, whereas the enzymic method developed by Banks and Greenwood[16], using beta-amylase and D-glucose oxidase, is applicable only to linear α-(1→4)-D-glucans.

Since the SDH method was developed, and a preliminary account published[17], Dutton and his co-workers have described[18] a related chemical method for the determination of the $\overline{DP}_n$ of polysaccharides. An acid hydrolysate of a borohydride-reduced polysaccharide was treated with chlorotrimethylsilane, and the TMS derivatives of the reducing sugars and alditols were separated and estimated by gas–liquid chromatography. The method gave satisfactory analysis for synthetic mixtures containing a D-glucose–D-glucitol ratio of 150:1, although results with standard polysaccharides were not described. This method is clearly applicable to almost any homopolysaccharide.

Although the SDH assay is relatively highly specific, the fact that xylitol is also oxidised means that glucan preparations must be free from any contaminating xylan. The reaction with xylitol forms the basis of a similar method for the measurement of the $\overline{DP}_n$ of xylans, details of which will be given in a later communication. It should be emphasised that these conclusions on specificity refer only to sheep-liver SDH. It is known that SDH preparations from other sources, *e.g.*, ram spermatozoa[9], guinea pig[19], and *Bacillus subtilis*[20] are not as specific as the sheep-liver enzyme, and will oxidise a variety of pentitols and hexitols. Moreover, these latter three enzymes have pH optima at 8.6, 8.9, and 10.0, respectively, so that for the determination of the $\overline{DP}_n$ of glucans, the experimental conditions described in this paper refer only to sheep-liver SDH.

ACKNOWLEDGMENT

We are indebted to the Eda, Lady Jardine Charitable Trust for the award of a research fellowhip (to A.J.M.).

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