ENZYMATIC DETERMINATION OF THE UNIT CHAIN LENGTH OF GLYCOGEN AND RELATED POLYSACCHARIDES

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Received 12 February 1971

1. Introduction

Enzymic methods for the determination of the average unit chain length ($\overline{CL}$) of branched amylaceous polysaccharides are superior to chemical methods on account of the precision, rapidity and specificity of the former. These methods employ one, two or three enzymes, i.e. α-amylase [1], β-amylase + pullulanase [2] and β-amylase + the amylo-1,6-glucosidase/oligo-1,4-1,4-transglucosylase complex of rabbit muscle or yeast [3], the last method being an adaptation of an earlier method [4] employing phosphorylase in place of β-amylase. The α-amylase method is not absolute since it depends on a calibration with polysaccharides whose $\overline{CL}$ have been determined by other methods. The second and third methods are absolute and rely on the release and specific assay of the glucose units at the reducing termini of the unit chains. We now describe a method, based on the use of a single, commercially-available enzyme, which is both absolute and rapid. The enzyme is *Cytophaga* isoamylase [5] which completely debranches glycogen and amylopectin. The assay is of the copper-reducing power of the reducing ends set free. The amount of polysaccharide required for an assay may be as little as 200 μg and there is the additional advantage that, unlike the other enzymic methods, the unit chains are not depolymerized. Therefore the distribution in length of the unit chains can be examined by gel filtration [6].

2. Materials and methods

Cytophaga isoamylase preparation was obtained from British Drug Houses, Poole, Dorset, U.K. The powder (25 mg/ml) was gently homogenised at 4° in 5 mM sodium citrate–phosphate buffer, pH 8.0 and the suspension was centrifuged at 40,000 *g* for 20 min. The supernatant solution was passed through a DEAE-cellulose column previously equilibrated with 5 mM sodium citrate-phosphate buffer, pH 8.0. The column was washed with the same buffer until the isoamylase activity had been completely eluted. The fractions containing enzyme activity were combined, dialysed overnight against distilled water at 4° and then freeze-dried. A large proportion of the inactive protein was retained on the DEAE-cellulose column to give a 10-fold purification of the isoamylase preparation. The specific activity of the freeze-dried preparation was 0.45 and it was stable at 4° over a period of two months. Appropriate amounts of the freeze-dried powder were dissolved in 10 mM sodium acetate buffer pH 5.5 as required. Isoamylase activity was measured at 37° as described by Gunja-Smith et al. [5] and protein concentration as by Lowry et al. [7].

Pullulanase was prepared from *Aerobacter aerogenes* by the method of Wallenfels et al. [8] as modified by Frantz [9]. Sweet-potato β-amylase was purchased from Worthington Biochemical Corporation and shellfish glycogen from Mann Research Laboratories. The remainder of the polysaccharides tested were kindly made available by Professor D.J.Manners.

Debranching of the polysaccharides was conducted at 37° in digests containing polysaccharide (2–5 mg/ml), isoamylase (0.06 units/ml) and 20 mM sodium acetate buffer pH 5.5. A drop of toluene was added to each digest to inhibit bacterial growth. Duplicate samples (0.2 ml) were withdrawn at intervals and the release of reducing sugars was measured.

When a maximum value was reached (18 hr for the slowest reaction) each digest was heated at 100° for 10 min to inactivate the isoamylase. Pullulanase (0.625 units in 10 μl of 20 mM sodium acetate buffer pH 5.5) was added to 0.5 ml of the inactivated digest containing amylopectin β-dextrin and the further release of reducing sugars determined. Reducing sugars were determined as by Nelson [10] except that all volumes were reduced by a factor of two to increase the sensitivity of the method. The method was calibrated using maltose monohydrate as a standard. Polysaccharide concentrations were determined as by Marshall and Whelan [11] and values of $\overline{CL}$ were determined from the equation:

$$\overline{CL} = \frac{\text{Total polysaccharide in digests (maltose equivalents)}}{\text{Reducing sugars released in digests (maltose equivalents)}}$$

To determine the extent of β-amylolysis of the debranched polysaccharide portions (0.4 ml) of the inactive isoamylase digests were treated with β-amylase as by Mercier and Whelan [12].

3. Results and discussion

The method depends on the inverse stoichiometric relation between copper-reducing power and degree of polymerization of the maltodextrins that constitute the unit chains of glycogen and amylopectin [13], the copper reagent being calibrated against maltose. The reproducibility of the isoamylase method was tested with shellfish glycogen. The average chain length of 9.9 (S.D. = 0.3), determined from four assays lies close to the value of 9.8 determined by the method of Carter and Lee [3]. Table 1 records the results obtained with 10 glycogens, two amylopectins and an amylopectin β-limit dextrin. The results are compared with values obtained by other workers,

Table 1
Determination of average chain lengths of glycogens and amylopectins.

Sample	$\overline{CL}$[a] by periodate	$\overline{CL}$ by other enzymic methods[b]			$\overline{CL}$ by isoamylase	Degradation by β-amylase after isoamylase treatment (%)
		I	II	III		
Glycogens						
1) Cat liver I	13	–	15	14	13	101
2) Human muscle II	11	–	11	12	11	102
3) Rabbit muscle III	13	13	13	14	11	112
4) Ascaris lumbricoides I	11	12	12	13	12	100
5) Human liver type III glycogenosis	6	–	5	5	7	90
6) Skate liver I	13	–	11	12	11	108
7) Trichomonas foetus	15	–	14	15	14	103
8) Rabbit liver VII	14	14	13	15	14	104
9) Horse diaphragm (pre-rigor)	17	16–17	14	16	13	108
10) Shellfish (commercial)	–	–	10	–	10	101
Amylopectins						
1) Waxy-maize starch I	20	–	20	21	20	101
2) Waxy-sorghum starch II	22	–	20	23	20	100
3) Potato amylopectin β-dextrin[c]	9–10	–	10	10	10	–

[a] All $\overline{CL}$ in table have been converted to the nearest whole number of glucose units.
[b] Methods I, α-amylase [1]; II, β-amylase and yeast glucosidase-transferase [3]; III, phosphorylase *a* and yeast glucosidase-transferase [3].
[c] Pullulanase was added to this assay as described in the text.

but using the same specimens, by periodate oxidation [14] and enzymic methods [1, 3]. Good agreement is seen in all cases except for one glycogen (horse diaphragm). In the experiment with potato amylopectin β-dextrin, pullulanase was added to the isoamylase because of the inability of the latter to detach the α-maltosyl residues that constitute half the A chains of this polymer [5]. It is only with a β-dextrin that supplementation with pullulanase is necessary.

That debranching was complete was shown by the degrees of β-amylolysis after debranching (table 1). That values of more than 100% conversion are recorded reflects the fact that the results are calculated in terms of theoretical maltose, while the actual products include glucose, arising from chains with odd number of glucose units [2].

The commercial availability of the isoamylase and the relatively simple purification procedure required to give a stable enzyme preparation free from hydrolytic activities that attack α-1,4-glucosidic bonds, are advantages not shared by the earlier enzymic methods that provide an absolute $\overline{CL}$ determination and therefore should ensure the routine use of this analytical method.

Acknowledgements

This work was supported by grants from the National Science Foundation (GB 8342) and the National Institutes of Health (AM 12532). We are grateful to Professor D.J.Manners for his generous gift of polysaccharide samples and to Professor W.J. Whelan for helpful discussion of this work.

References

[1] D.J.Manners and A.Wright, J. Chem. Soc. (1962) 1592.
[2] E.Y.C.Lee and W.J.Whelan, Arch. Biochem. Biophys. 116 (1966) 162.
[3] J.H.Carter and E.Y.C.Lee, Anal. Biochem. (1971) in press.
[4] G.T.Cori and J.Larner, J. Biol. Chem. 188 (1951) 264.
[5] Zeenat Gunja-Smith, J.J.Marshall, E.E.Smith and W.J. Whelan, FEBS Letters 12 (1970) 96.
[6] Zeenat Gunja-Smith, J.J.Marshall, Christiane Mercier, E.E.Smith and W.J.Whelan, FEBS Letters 12 (1970) 101.
[7] O.N.Lowry, N.J.Rosebrough, A.L.Farr and R.J.Randall, J. Biol. Chem. 193 (1951) 265.
[8] K.Wallenfels, H.Bender and I.R.Rached, Biochem. Biophys. Res. Commun. 22 (1966) 256.
[9] B.M.Frantz, Ph.D.Thesis, Univ. of London, 1968.
[10] N.Nelson, J. Biol. Chem. 153 (1944) 375.
[11] J.J.Marshall and W.J.Whelan, FEBS Letters 9 (1970) 85.
[12] Christiane Mercier and W.J.Whelan, European J. Biochem. 16 (1970) 579.
[13] J.F.Robyt and W.J.Whelan, Biochem. J. 95 (1965) 10P.
[14] D.J.Manners and A.Wright, J. Chem. Soc. (1961) 2681.