INCOMPLETE CONVERSION OF GLYCOGEN AND STARCH BY CRYSTALLINE AMYLOGLUCOSIDASE AND ITS IMPORTANCE IN THE DETERMINATION OF AMYLACEOUS POLYMERS

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1. Introduction

Amyloglucosidase [EC 3.2.1.3.] hydrolyses both the $1 \rightarrow 4$- and $1 \rightarrow 6$-bonds of starch and glycogen, and is reportedly capable of causing a quantitative conversion of these polymers into glucose. It has become an agent of choice for the specific and quantitative determination of amylaceous polymers. Thus it was reported from our Laboratory that *Aspergillus niger* amyloglucosidase could be used for this purpose [1]. Recently we detected an α-amylase-like impurity in our preparation of *A. niger* enzyme and turned to the use of a crystalline preparation from *Rhizopus niveus*. The unexpected finding was then made that the amylase-free enzyme is unable in many instances to bring about a complete conversion of the starch components and glycogens into glucose. Adulteration of the glucamylase with α-amylase restores the conversion to a quantitative level.

2. Materials and methods

R. niveus amyloglucosidase (twice recrystallised) was purchased from Miles Laboratories Inc., and had an activity of 19 International Units per mg. The preparation was free from α-amylase activity when tested by the method of Smith et al. [2]. *A. niger* amyloglucosidase was prepared by the methods of Fleming and Stone [3] and Qureshi [4], the second enzyme peak from a DEAE-cellulose fractionation being used. The preparation had an activity of 10.5 IU per mg, and was shown to contain trace amounts of α-amylase [2]. α-Amylase was prepared from pig pancreas [5] and was twice crystallised.

Shellfish glycogen (Mann Research Labs.) was three times reprecipitated with ethanol before use. Waxy-maize starch was prepared as described by Schoch [6]. Rabbit liver glycogen was prepared as by Murdoh et al. [7] and the β-limit dextrins of glycogen and amylopectin as by Whelan [8]. Phytoglycogen and Floridean starch [9] were gifts from Dr. T.J.Schoch and Dr. J.R.Turvey, respectively. The potato amylose was from Nutritional Biochemicals Cpn. Other polysaccharide samples were obtained from Professor D.J.Manners.

α-Amylase activity was measured as by Robyt and Whelan [10], the incubation temperature being 30°. Amyloglucosidase activities were measured by the release of glucose from soluble starch at 30° [4]. Glucose was determined by the glucose oxidase method of Fleming and Pegler [11], as modified by Catley [12].

3. Experimental procedures

Determination of glucose released by acid hydrolysis: Polysaccharide (approx. 1 mg) was hydrolysed with 1.5 N sulphuric acid (0.5 ml) for 3 hr at 100° [1, 13]. The hydrolysate was neutralised with sodium hydroxide and the liberated glucose determined. A control was included to compensate for the glucose lost during the hydrolysis.

Determination of glucose liberated by enzymic hydrolysis: The glucose liberated from the poly-

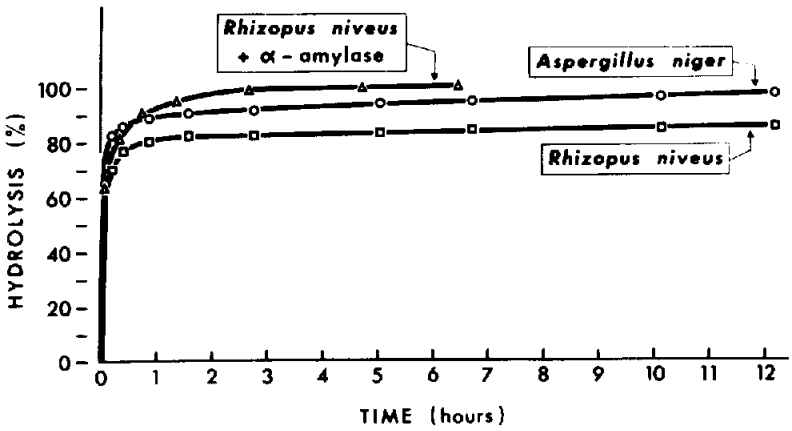

Fig. 1. Hydrolysis of cat liver glycogen by amyloglucosidase preparations ± α-amylase, as in table 1. Details are given under Experimental procedures. The degree of hydrolysis is expressed in terms of glucose released enzymically relative to glucose released by acid hydrolysis (cf. table 1)

saccharides by (a) *A. niger* amyloglucosidase, (b) *R. niveus* amyloglucosidase and (c) *R. niveus* amyloglucosidase and pancreatic α-amylase acting together was determined as follows. Polysaccharide (approx. 0.1 mg), amyloglucosidase (1.05 units), 100 mM acetate buffer pH 5.0 (0.4 ml) and, in (c) 2.1 units of α-amylase, in a total volume of 1.0 ml were incubated at 30° for 2 hr together with polysaccharide-free digests. Samples (200 μl) were then removed for glucose determination. Digests of similar composition were used in the experiments in fig. 1, where the progress of hydrolysis was followed.

4. Results

The results of the hydrolyses of a selection of starches and glycogens are collected in table 1. It is first of all confirmed that the *A. niger* preparation releases amounts of glucose comparable with those yielded by acid*. Conversions by the *Rhizopus* enzyme, however, are for the most part very significantly lower than those achieved by acid or by *A. niger* amyloglucosidase. The "missing" material was found in one case to be an apparently high molecular weight limit dextrin, excluded on passage of the hydrolysis products through Sephadex G-50.

On the basis that the *Rhizopus* enzyme was free from α-amylase, while that from *A. niger* was contaminated with amylase-like activity, we adulterated the former preparation with α-amylase. The conversions into glucose then attained essentially quantitative levels, even superior to those found with *A. niger* (table 1).

The conditions used to obtain the foregoing results were essentially those previously determined to be appropriate for the *A. niger* preparation [1] (see footnote*). It was checked whether the low conversion by *R. niveus* amyloglucosidase was simply due to an insufficient time of incubation. Cat liver glycogen was chosen as substrate since its conversion had been particularly low (table 1). Fig. 1 shows that similar dif-

* If the degrees of conversion are not so nearly quantitative as reported earlier [1], this could be for three reasons. (1) Only one of the samples is from the same source as before. (2) The temperature of incubation was lowered from 35° to 30°. (3) The method used to correct the acid hydrolysis values for loss of glucose was different. Pirt and Whelan [13] found that the loss of copper reducing power of glucose under the conditions we have used in the hydrolysis was 1.5% and this value was used earlier [1] to correct the acid hydrolysis values. However, since the glucose is being determined here not with a copper reagent but with glucose oxidase, which will not react with copper-reducing products of acid-treated glucose such as oligosaccharides [14], or hydroxymethylfurfural [15], the correction factor is likely to be higher. This proves to be the case. The loss of glucose oxidase positive material corresponds to 4% of the glucose used in a control acid hydrolysis.

Table 1
Determination of glucose released enzymically from samples of starch and glycogen.

	Glucose measured by enzymic hydrolysis expressed as % of glucose released by acid*		
	A. niger amyloglucosidase	*R. niveus* amyloglucosidase	*R.niveus* amyloglucosidase + α-amylase
Potato amylose	97.0	90.1	101.0
Waxy-maize starch	100.0	97.6	103.0
Waxy-sorghum starch	98.0	92.6	100.0
Floridean starch	90.8	77.8	97.5
Waxy-maize starch β-dextrin	97.7	91.1	100.0
Shellfish glycogen	99.2	98.1	99.3
Sweet-corn phytoglycogen	99.9	96.2	100.3
Rabbit liver glycogen	98.9	98.4	100.1
Skate liver glycogen	95.3	89.3	99.3
Human muscle glycogen	96.3	87.8	99.4
Rabbit muscle glycogen	98.2	93.2	99.3
Cat liver glycogen	93.1	82.2	98.8
Shellfish glycogen β-dextrin	94.5	92.7	97.0

* Acid hydrolysis figures corrected for glucose destroyed (see text). Experimental details are given under Experimental procedures.

ferences to those noted in the 2-hr incubations recorded in table 1 for *R. niveus* and *A. niger* amyloglucosidases, and the former supplemented with α-amylase, are still to be seen after incubation times up to 12 hr. The *A. niger* preparation plus α-amylase reached 100% conversion in 2 hr and with waxy-maize starch the unsupplemented *A. niger* and *R. niveus* preparations reached the same limiting conversions noted in table 1 after 7 and 65 min respectively.

5. Discussion

Two conclusions may be drawn from these results. There would appear to be an obstacle within most of the starch and glycogen molecules that effectively blocks the progress of the amylase-free exo-acting amyloglucosidase. This obstacle is bypassed by α-amylase, which is a contaminant of the *A. niger* preparation and which may be presumed to contaminate the amorphous preparations of amyloglucosidase that have earlier been used in methods for the quantitative determination of starch and glycogen. The finding of an incomplete conversion of an amylaceous polymer by a crystalline exo enzyme has its parallel in the observation that while crude preparations of β-amylase had been found largely to convert amylose into maltose, the crystalline enzyme gives conversions that are far from complete [16]. It was subsequently shown that amorphous β-amylase is contaminated with α-amylase [17]. It might be further noted that in reviewing the literature on amyloglucosidases of different origin, Fleming [18] has documented more examples of incomplete than of quantitative starch conversions.

As to the nature of the obstacle to the amyloglucosidase, one can refer to the claims that starch and glycogen contain 1 → 3-bonds [18]. These might block the enzyme. Attention is drawn to Floridean starch, having a relatively high proportion of such

linkages [9], which had the lowest degree of conversion of any polysaccharide tested (table 1). There is also the possibility of chemical modification of glucose residues during isolation procedures [20]. Yet another possibility, not invoking new linkages or artefactual structural features, is that there may be present arrangements of 1 → 6-bonds that act as obstacles. Regard must also be had to the possible introduction of barriers by an inherent or contaminating transglucosylase activity in the *Rhizopus* enzyme preparation. This seems unlikely in view of the fact that waxy-maize starch and two of the glycogens were almost quantitatively converted into glucose without the need for addition of α-amylase (table 1).

An important practical point that emerges from these findings is that if this very useful method of glycogen and starch determination is to remain in vogue, the precaution must be taken of employing an amyloglucosidase either contaminated with sufficient endogenous α-amylase or supplemented with added amylase. Furthermore, the variations noted in the time taken to achieve hydrolysis of different substrates require that the duration of incubation be designed for the particular polysaccharide under determination.

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References

[1] E.Y.C.Lee and W.J.Whelan, Arch. Biochem. Biophys. 116 (1966) 162.
[2] E.E.Smith, G.S.Drummond, J.J.Marshall and W.J.Whelan, Federation Proc. 29 (1970) 930.
[3] I.D.Fleming and B.A.Stone, Biochem. J. 97 (1965) 13 P.
[4] M.Y.Qureshi, Ph.D.Thesis, University of London (1967).
[5] E.H.Fischer and E.A.Stein, Arch. Sci. 7 (1954) 131.
[6] T.J.Schoch, in: Methods in Enzymology, Vol. III, eds. S.P.Colowick and N.O.Kaplan (Academic Press, New York, 1957) p. 5.
[7] J.Murdoh, C.R.Krisman and L.F.Leloir, Arch. Biochem. Biophys. 113 (1966) 265.
[8] W.J.Whelan, in: Methods in Carbohydrate Chemistry, Vol. 4, ed. R.L.Whistler (Academic Press, New York, 1964) p. 261.
[9] S.Peat, J.R.Turvey and J.M.Evans, J. Chem. Soc. (1959) 3223.
[10] J.F.Robyt and W.J.Whelan, in: Starch and Its Derivatives, ed. J.A.Radley (Chapman and Hall, London, 1968) p. 430.
[11] I.D.Fleming and H.F.Pegler, Analyst (London) 88 (1963) 967.
[12] B.J.Catley, Ph.D.Thesis, University of London (1967).
[13] S.J.Pirt and W.J.Whelan, J. Sci. Food Agr. 2 (1951) 224.
[14] I.J.Goldstein and T.L.Hullar, Advan. Carbohydrate Chem. 21 (1966) 431.
[15] F.H.Newth, Advan. Carbohydrate Chem. 6 (1951) 83.
[16] S.Peat, S.J.Pirt and W.J.Whelan, J. Chem. Soc. (1952) 705.
[17] W.L.Cunningham, D.J.Manners, A.Wright and I.D.Fleming, J. Chem. Soc. (1960) 2602.
[18] I.D.Fleming, in: Starch and Its Derivatives, ed. J.A. Radley (Chapman and Hall, London, 1968) p. 498.
[19] M.L.Wolfrom and A.Thompson, J. Am. Chem. Soc. 78 (1956) 4116; 79 (1957) 4212.
[20] W.Banks and C.T.Greenwood, Staerke 19 (1967) 197.