A GLYCOGEN-DEBRANCHING ENZYME FROM *CYTOPHAGA*

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1. Introduction

Potato R-enzyme [1] and bacterial pullulanase [2,3] hydrolyse the α-1,6-linkages in pullulan and α-limit dextrins and also cleave the α-1,6-branch linkages in amylopectin [4]. The availability of highly purified preparations of pullulanase from *Aerobacter aerogenes* [5] has made this enzyme invaluable in the analysis of the fine structure of amylopectin [6]. Pullulanase, however, is of limited use in the analysis of glycogen structure because, although it is able to hydrolyse some branch linkages in degraded glycogen, it has little or no action on the undegraded macromolecule [7]. Yeast isoamylase [8] hydrolyses a limited proportion of the interchain linkages of amylopectin and glycogen but, unlike pullulanase, does not act on the 1,6-linkages of pullulan. Recently, an extracellular isoamylase from a new strain of *Pseudomonas* was reported to hydrolyse almost all the branch linkages of amylopectin and glycogen [9, 10]. The importance of this type of enzyme for the analysis of glycogen structure prompts us to report the discovery of an isoamylase in a species of *Cytophaga*. Studies of the partially purified enzyme indicate that its specificity of action is similar to that of the *Pseudomonas* enzyme and that it has the ability to hydrolyse the branch linkages of amylopectin and glycogen with the complete dismemberment of the branched macromolecules.

2. Materials and methods

The cell-free *Cytophaga* preparation was obtained from British Drug Houses Ltd, Poole, Dorset, England. The preparation (10 mg/ml), was homogenized at 4°in sodium acetate (100 mM) buffer, pH 5.5, and the suspension was centrifuged at 40,000 *g* for 20 min. The clear supernatant contained 85% of the total isoamylase activity and, when tested with oxidized amylose [11], was shown to be free of α-1,4-endoglucanase activity. This preparation, or a preparation purified 10-fold by DEAE-cellulose chromatography, at pH 8.0, was used where indicated. Isoamylase activity was assayed by release of copper-reducing power [12] from glycogen. A digest containing an appropriate amount of enzyme, glycogen (5 mg/ml) and sodium acetate (100 mM) buffer pH 5.5 was incubated at 37°. One unit is defined as that amount of enzyme that releases 1 μmole glucose equivalent per minute. The action of isoamylase on various substrates was tested at 37° in digests containing enzyme (as indicated), substrate (5 mg/ml) and sodium acetate (100 mM) buffer pH 5.5.

Pullulanase was prepared from *Aerobacter aerogenes* by the method of Wallenfels et al. [13] as modified by Frantz [14]. Sweet potato β-amylase was purchased from Worthington Biochemical Corporation. Shellfish glycogen was purchased from Mann Research Corporation, high molecular weight rabbit liver glycogen was isolated as by Mordoh et al. [15], sweet corn glycogen (phytoglycogen) as by Peat et al. [16] and waxy maize starch (amylopectin) as by Schoch [17]. T β-limit dextrins of glycogen and amylopectin were prepared as by Whelan [18], the phosphorylase (φ) limit dextrins as by Lee et al. [19] and the α-limit dextrins of amylopectin by the method of Whelan [20].

Total reducing sugars were determined as by Nelson [12] and glucose with glucose oxidase [21]. Concentrations of polysaccharide solutions were determined by hydrolysis with α-glucosidase [22] followed by estimation of copper-reducing sugars or glucose. The extent of β-amylolysis of polysaccharides was determined as by Walker and Whelan [23].

The iodine-staining powers of glycogen and amylo-

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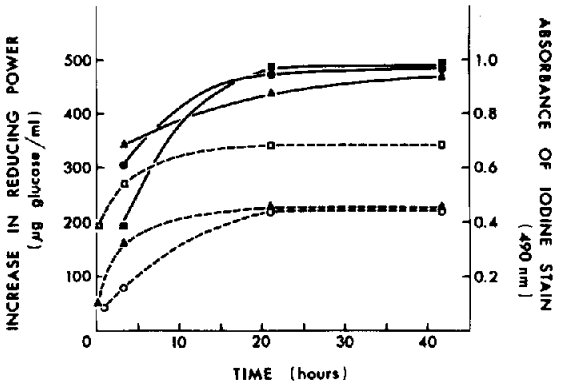

Fig. 1. Increase in reducing power and iodine-staining power of glycogens treated with isoamylase. Rabbit liver (□), sweet corn (○) and shellfish (△) glycogens were incubated with crude isoamylase (0.04 U/ml) under the conditions described in Materials and methods. Samples were removed at intervals to measure reducing power (solid line, closed symbols) and iodine stain (dotted line, open symbols).

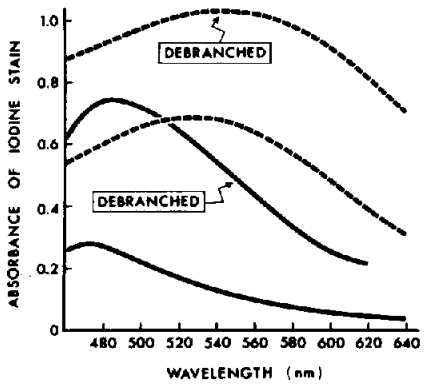

Fig. 2. Effect of isoamylase on the iodine stains of shellfish glycogen (——) and amylopectin (- - -). Incubation was as in fig. 1. Portions were stained with iodine at zero time and 24 hr("debranched") and the spectra scanned.

pectin were measured by addition of 5 ml of iodine reagent (iodine (0.02%)–potassium iodide (0.2%) in 0.05 N HCl) to 0.1 ml of solution containing 5.0 mg polysaccharide per ml. The absorbances of the solutions were read at 490 nm and 680 nm respectively in a Coleman Junior II spectrophotometer.

Protein was determined as by Lowry et al. [24]. Chromatography was conducted on Whatman No. 1 paper irrigated with propanol:ethyl acetate:water (14:2:7) or ethyl acetate:pyridine:water (10:4:3). Sugar spots were visualized with $AgNO_3$/NaOH [25].

3. Results

3.1. *Physical properties of isoamylase*

The optimum activity of the 10-fold purified enzyme was at pH 5.5, but activity was lost rapidly at pH values below 5.0. Maximum activity was observed at 40° but when the enzyme was incubated for 60 min at this temperature in the absence of substrate, almost all enzyme activity was lost. Gel electrofocussing indicated an isoelectric point between pH 5.0–5.5 and preliminary sucrose density gradient centrifugation studies showed that the isoamylase has a molecular weight in the region of 120, 000.

3.2. *Substrate specificity of isoamylase*

The following experiments served to characterize the action of isoamylase on glycogen, amylopectin and their ϕ- and β-dextrins.

1) The enzyme brings about large increases in the iodine-staining powers of glycogen and amylopectin (figs. 1, 2), accompanied by only small shifts in $E_{max.}$ (470→490 nm and 535→545 nm respectively).

2) The successive actions of isoamylase and β-amylase on glycogen and amylopectin result in an essentially complete conversion into maltose (table 1).

3) Paralleling the experiments in 1 and 2, paper chromatography of isoamylase-treated glycogen reveals a series of oligosaccharides extending back to the origin (fig. 3A). These disappear on β-amylolysis and maltose and glucose are formed (fig. 3B). (The glucose arises from chains containing an odd number of glucose units.) The trace of glucose present before β-amylolysis (fig. 3) corresponded to 0.4% of the glycogen (glucose oxidase).

4) The action of isoamylase on glycogen is accompanied by an increase in copper-reducing power (fig. 1). The fact that debranching goes to completion, as evidenced by β-amylosis [see 2], means that the average chain length ($\overline{CL}$) of the glycogen can be calculated from the reducing power. For shellfish, liver and sweet-corn glycogens, the values found were 10.5, 14 and

Table 1
Action of isoamylase on poly- and oligosaccharides

Substrate	Glucosidic bonds hydrolysed by isoamylase (%)	Glucosidic bonds subsequently hydrolysed by pullulanase (%)	Degree of β-amylolysis after isoamylase action (%)
Phytoglycogen	8.7	0	109
Shellfish glycogen	9.45	0	108
Shellish glycogen β-dextrin	12.7	3.8	78
Shellfish glycogen φ-dextrin	12.3	0	100
Amylopectin	5.1	0	106
Amylopectin β-dextrin	9.3	2.2	70
Amylopectin φ-dextrin	9.4	0	102
Pullulan	1.8	29	9.5
α-Limit dextrins	6.7	10.7	81

Substrates (5 mg/ml) were incubated with purified isoamylase (0.15 U/ml) under the conditions described in Materials and methods. Samples (0.2 ml) were removed after 18 hr for measurement of reducing power, the remainder of the digest being heated at 100° for 3 min. A sample of the heated digest (0.2 ml) was treated with pullulanase (0.1 ml, 0.5 units) for 1 hr 37° and the increase in reducing power measured. A second sample (0.4 ml) was treated with β-amylase (0.1 ml, 500 U/ml) for 24 hr at 37°. The reducing power was then measured.

11.5, respectively, corresponding to values determined by other methods.

5) Isoamylase has no action on pullulan and a limited action on α-limit dextrins (table 1), though a small but rapid initial increase in reducing power of the latter indicated that the mixture may contain a proportion of highly susceptible molecules.

6) Glycogen φ-dextrin is completely debranched but the β-dextrin is not (table 1). Pullulanase action on isoamylase-treated β-dextrin brings about a marked increase in reducing power, but not so on isoamylase-treated glycogen, glycogen φ-dextrin and amylopectin (table 1). Glycogen β-dextrin was debranched with isoamylase and pullulanase to about the same extent (75% measured by release of reducing power) and subjected to paper chromatography. Pullulanase liberated about equal amounts of maltose and maltotriose (fig. 3D). Isoamylase liberated maltotriose, but only a barely detectable amount of maltose (fig. 3E). β-Amylolysis and chromatography of isoamylase-treated glycogen β-dextrin reveals the presence of undegraded oligosaccharides (fig. 3F), whereas none remain when isoamylase and pullulanase are first used to debranch the β-dextrin (fig. 3G).

4. Discussion

The partially purified debranching enzyme of *Cytophaga* exhibits properties similar to the isoamylase of *Pseudomonas* [10]. Thus, both enzymes have a pH optimum and an isoelectric point on the acid side of neutrality, are rather heat-labile, and have molecular weights near to 100,000. More striking, however, is the similarity in the specificity of action of the two enzymes. Like the *Pseudomonas* isoamylase, the debranching enzyme of *Cytophaga* is unable to hydrolyse the α-1, 6-glucosidic linkages of pullulan, but readily hydrolyses all the α-1, 6-branch points of glycogen and amylopectin (table 1). On this basis the *Cytophaga* enzyme may be classed as an isoamylase. A further similarity is seen in the failure of the *Cytophaga* enzyme to hydrolyse all α-1, 6-branch points of the β-limit dextrins of glycogen and amylopectin. This may be attributed, as in the case of *Pseudomonas* isoamylase [10], to the inability of the enzyme to remove the 1→6-bonded maltosyl residues formed by β-amylase, and serves further to distinguish the enzyme from pullulanase (fig. 3D, E).

The increase in iodine staining power to constant values on treatment of several glycogens with the

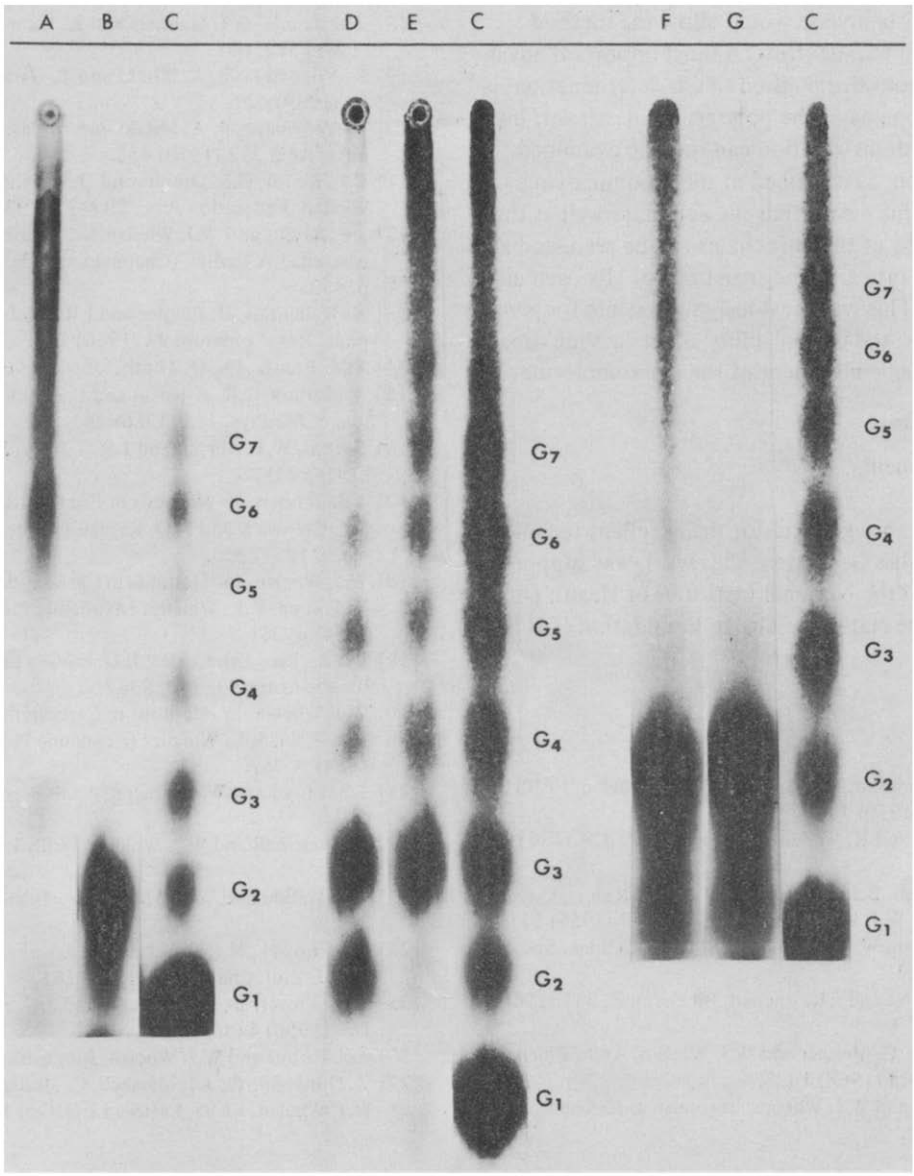

Fig. 3. Comparative actions of isoamylase and pullulanase on shellfish glycogen and its β-dextrin. (C=control series of glucose (Gl), maltose (G2) etc.). (A) Glycogen + isoamylase; (B) as A, followed by β-amylase; (D, E) β-dextrin + pullulanase or + isoamylase, respectively; (F) β-dextrin + isoamylase, followed by β-amylase; (G) β-dextrin + isoamylase + pullulanase, followed by β-amylase. In (D) and (E) the extents of 1→6-bond hydrolysis were equal, at about 75%. Where β-amylase was used, the debranching enzyme(s) were first heat-inactivated. Conditions of hydrolysis as in fig. 1.

Cytophaga enzyme (fig. 1) suggests that there is no hydrolysis of linkages other than the 1,6-branch points. Since the maltodextrins react stoichiometrically with the Nelson copper reagent [26], the reducing power set free by isoamylase can be used to calculate the proportion of branch points, and hence the value of $\overline{CL}$. This promises to be the most rapid and facile method yet described for measuring $\overline{CL}$. The inclusion of pul-

lulanase with isoamylase would allow the method to be extended to β-dextrins. A most important advantage, arising from this method of $\overline{CL}$ determination, is that the unit chains of the polysaccharide are left intact. The length distribution can then be examined by gel filtration, as described in the accompanying paper [27]. This means that the actual, as well as the average, lengths of the unit chains can be seen, and an insight gained into the fine structures of glycogen and amylopectin. This was previously impossible for glycogen owing to the unavailability of an enzyme capable of the total dismemberment of the macromolecule.

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References

[1] G.S. Drummond, E.E. Smith and W.J. Whelan, FEBS Letters 9 (1970) 136.
[2] H. Bender and K. Wallenfels, Biochem. Z. 334 (1961) 79.
[3] M. Abdullah, B.J. Catey. E.Y.C.Lee, J.F.Robyt, K.Wallenfels and W.J. Whelan, Cereal. Chem. 63 (1966) 111.
[4] P.N. Hobson, W.J. Whelan and S. Peat, J. Chem. Soc. (1951) 1451.
[5] K. Wallenfels and I.R. Rached, Biochem. Z. 344 (1966) 524.
[6] E.Y.C. Lee, C. Mercier and W.J. Whelan, Arch. Biochem. Biophys. 125 (1968) 1028.
[7] C. Mercier and W.J. Whelan, European J. Biochem. 16 (1970) 579.
[8] Z.H. Gunja, D.J. Manners and K. Maung, Biochem. J. 81 (1961) 392.
[9] K. Yokobayashi, A. Misaki and T. Harada, Agr. Biol. Chem. 33 (1969) 625.
[10] K. Yokobayashi, A. Misaki and T. Harada, Biochim. Biophys. Acta 212 (1970) 458.
[11] E.E. Smith, G.S. Drummond, J.J. Marshall and W.J. Whelan, Federation Proc. 29 (1970) 930.
[12] J.F. Robyt and W.J. Whelan, in: Starch and its Derivatives, ed. J.A.Radley (Chapman and Hall, London, 1968) p. 432.
[13] K. Wallenfels, H. Bender and I.R. Rached, Biochem. Biophys. Res. Commun. 22 (1966) 254.
[14] B.M. Frantz, Ph. D. Thesis, Univ. of London, 1968.
[15] J. Mordoh, C.R. Krisman and L.F. Leloir, Arch. Biochem. Biophys. 113 (1966) 265.
[16] S. Peat, W.J. Whelan and J.R. Turvey, J. Chem. Soc. (1956) 2317.
[17] T.J. Schoch, in: Methods in Enzymology, Vol. 111, eds. S.P. Colowick and N.O. Kaplan (Academic Press, New York, 1957) p. 5.
[18] W.J. Whelan, in: Methods in Carbohydrate Chemistry, Vol. 4, ed. R.L. Whistler (Academic Press, New York, 1964) p. 261.
[19] E.Y.C. Lee, J.H. Carter, L.D. Nielsen and E.H. Fischer, Biochemistry 9 (1970) 2347.
[20] W.J. Whelan, in: Methods in Carbohydrate Chemistry, Vol. 4, ed. R.L. Whistler (Academic Press, New York, 1964) p. 252.
[21] J.B. Lloyd and W.J. Whelan, Anal. Biochem. 30 (1969) 467.
[22] J.J. Marshall and W.J. Whelan, FEBS Letters 9 (1970) 85.
[23] G.J. Walker and W.J. Whelan, Biochem. J. 76 (1960) 264.
[24] O.N. Lowry, N.J. Rosebrough, A.L. Farr and R.J. Randall, J. Biol. Chem. 193 (1951) 265.
[25] W.E. Trevelyan, D.P. Procter and J.G. Harrison, Nature 166 (1950) 444.
[26] J.F. Robyt and W.J. Whelan, Biochem. J. 95 (1965) 10P.
[27] Z. Gunja-Smith, J.J. Marshall, C. Mercier, E.E. Smith and W.J. Whelan, FEBS Letters 12 (1970) 101.