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PURIFICATION AND PROPERTIES OF A DEBRANCHING ENZYME FROM *ESCHERICHIA COLI*

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Summary

The debranching enzyme (EC 3.2.1.-) from *Escherichia coli* K12 was purified 312-fold with a 21% yield. DEAE-cellulose and DEAE-Sephadex chromatography were used for purification. The preparation was homogeneous and showed only a single band of protein and activity upon polyacrylamide gel electrophoresis. The enzyme hydrolyzed 1,6-α-glucosidic linkages in phosphorylase and β-amylase limit dextrins prepared from glycogen and amylopectin. Small branched oligosaccharides were also hydrolyzed. Amylopectin was also completely hydrolyzed but the enzyme showed only a very low activity with glycogen as the substrate. The enzyme cannot be classified as a pullulanase because it has practically no activity with pullulan. But it also differs from the bacterial isoamylases described in other studies because of its inability to hydrolyze glycogen. The optimal pH is about 5.6. The optimal growth conditions for the synthesis of the enzyme by *E. coli* were also examined in the present studies.

Introduction

Glycogen accumulation in bacteria generally occurs as a result of limited growth conditions in the presence of an excess carbon source [1]. When the exogenous carbon source has been completely utilized or when non-proliferating cells containing glycogen are placed in carbon free media, intracellular glycogen is degraded [2]. While the pathway by which glycogen is synthesized is well known, the enzymic pathway involved in glycogen utilization in bacteria has not yet been elucidated. Glycogen phosphorylase (EC 2.4.1.1., 1,4-α-D-glucan : orthophosphate α-glucosyltransferase) has been highly purified by Chen and Segel [3], but this enzyme cannot by-pass the 1,6-α-glucosidic linkages. Therefore, the complete degradation of glycogen also requires the involvement of an enzyme that is able to hydrolyze these linkages. An intracellular α-amylase (EC 3.2.1.1., 1,4-α-D-glucanglucanohydrolase) has also been found in *Escherichia*

coli K12 [4]. Two different types of debranching mechanisms are known. Direct debranching enzymes are able to attack unmodified glycogen or amylopectin by hydrolyzing 1,6-α-linkages. Indirect debranching enzymes attack these polysaccharides only after they are modified by other enzymes. The second mechanism has been found in yeast and mammals in which glycogen is debranched by a two-component system [5] (amylo-1,6-glucosidase/1,4-α-D-glucan : 1,4-α-D-glucan 4-α-glucosyltransferase; EC 3.2.1.33 : EC 2.4.1.25). In bacteria only direct debranching enzymes have been described and two classes have been distinguished: pullulanase (EC 3.2.1.41, pullulan 6-glucanohydrolase) and isoamylase (EC 3.2.1.68, glycogen 6-glucanohydrolase). Pullulanase, originally found in *Aerobacter aerogenes* [6] is able to hydrolyze the 1,6-α-glucosidic linkages in pullulan and amylopectin. Glycogen is hydrolyzed by pullulanase to a limited extent [7]. However with some glycogens, presumably these have not been degraded during isolation, pullulanase has no action [8].

Palmer et al. [9] suggested that this enzyme may be excreted by *E. coli* to debranch extracellular carbohydrates. Isoamylase has been detected in a soil *Pseudomonas* [10] and in a species of *Cytophaga* [11]. These isoamylases are the only known debranching enzymes which totally debranch glycogen and differ from pullulanase by their inability to attack pullulan. A debranching enzyme which hydrolyzes limit dextrins prepared from glycogen and amylopectin by treatment with phosphorylase and β-amylase has been detected in crude extracts of *E. coli* NCTC 5928 by Palmer et al. [9]. They proposed that the in vivo function of this enzyme was to degrade glycogen to maltodextrins which are subsequently hydrolyzed by the maltodextrin degrading system. In order to obtain experimental evidence for the role of this enzyme in *E. coli*, it was purified from crude extracts and its physical kinetic properties were examined. The results of the present study show that the properties of the enzyme isolated from *E. coli* differ from those of other debranching enzymes which have been described previously. The enzyme shows a lower affinity for glycogen and amylopectin than the limit dextrins prepared from these polysaccharides. Its classification in the group of isoamylases is discussed. A preliminary report of this work has been published [12].

Material and Methods

Materials

Oyster glycogen and amylose were obtained from Sigma; amylopectin (amylose free) was obtained from NBC, rabbit liver glycogen from BDH. DEAE-cellulose (DE-52) was purchased from Whatman; DEAE-Sephadex A-50 and Dextran T-10 and T-20 were purchased from Pharmacia; pullulan was a gift of Dr C. Mercier and Dr B. Enevoldsen; α-glucosyl Schardinger dextrin was kindly provided by Dr E.Y.C. Lee and branched oligosaccharides [13] were provided by Dr B. Enevoldsen. Glucostat was obtained from Worthington. Pre-coated thin-layer chromatography plates (cellulose F), layer thickness 0.10 cm, were obtained from Merck. Amberlite monobed resin MB3 was a product of BDH. Limit dextrins of glycogen and amylopectin were prepared by exhaustive treatment of solutions of the polysaccharide (20—30 mg/ml) in 100 mM acetate buffer (pH 4.8) with β-amylase (the enzyme was chromatographed on DEAE-

Sephadex A-50 to remove contaminating α-glucosidase activity as described by Marshall and Whelan [14]). Phosphorylase limit dextrins of glycogen and amylopectin were prepared by the procedure of Lee [15], phosphorylase *b* being repurified as described by Taylor et al. [16]. Phosphorylase *b* from rabbit muscle, β-amylase from sweet potato type I.B, non-crystalline deoxyribonuclease I from beef pancreas were from Sigma.

Analytical methods

Samples for chromatography were deionized with Amberlite monobed resin. Thin-layer plates were developed in water/ethanol/nitromethane (23 : 41 : 35, v/v) and sugar spots were visualized with the $AgNO_3$/NaOH dipping method as described by Trevelyan et al. [17]. Proteins were determined by the method of Lowry et al. [18] or by the absorbance at 280 nm. Total carbohydrate was determined by acid hydrolysis and the glucostat method as described by Sigal et al. [1]. The iodine staining powers of glycogen and amylopectin were measured by the addition of 5 ml of iodine reagent (iodine 0.02%/potassium iodide 0.2% in 0.05 M HCl) to 0.1 ml of solution containing 5—10 mg of polysaccharide per ml as described by Gunja-Smith et al. [11]. The absorbance of the solutions were read in a Zeiss spectrophotometer. The determination of the activity of the debranching enzyme on amylopectin and glycogen has been carried out by following the changes in the absorbance spectrum of the iodine complex between 400 and 600 nm.

Polyacrylamide gel electrophoresis

Protein samples (40 μg) were applied to a 7.5% porosity gel and submitted to electrophoresis as described by Ornstein and Davis [19]. Electrophoresis was carried out at pH 7.4 and 8.4, maintaining a current of 2 mA per gel at 4°C. The buffers used were respectively 0.096 M asparagine, 0.068 M imidazole (adjusted to pH 7.4 with 1 M KOH) and Tris/glycine buffer (0.025 M Tris/0.2 M glycine). Protein bands were stained with 10% Amido black in 7% acetic acid. Amylopectin 5% was incorporated in the gel; after electrophoresis, the gel was incubated in 0.02 M phosphate citrate buffer for 1 h. Then it was dipped in a solution of iodine 0.002%/potassium iodide 0.02% in 0.005 M HCl. The position of the debranching enzyme showed as a purple zone on a blue background.

Enzyme assay

Debranching enzyme activity in crude extracts and during purification was measured by the release of reducing power from oyster glycogen phosphorylase limit dextrin. A digest containing an appropriate amount of enzyme substrate (10 mg) and 100 mM sodium acetate buffer (pH 5.6) was incubated at 37°C. Samples were removed during the first 15 min and were heat inactivated. The reducing power was measured on 0.2 ml of the reaction mixture by the ferricyanide method of Park and Johnson [20] or by the method of Nelson [21]. In these conditions it has been checked that the amylase present in the crude extract does not interfere in the assay.

A unit of enzyme activity is defined as the amount of enzyme which releases one μmol of reducing end groups per min.

Column fractions were assayed for amylase activity in a 1.0-ml digest con-

taining 5 mg amylose, 25 μmol Tris/maleate buffer (pH 7.0) and 0.2 ml of fraction at 37°C for several hours. A 0.5-ml aliquot of the reaction mixture was added to 2 ml 0.1 M Tris (pH 8.0), 3.5 ml of water and 0.4 ml of 0.2—0.4% iodine/potassium iodide solution; the absorbance was measured at 640 nm. Glucosidase activity was determined by incubation of 0.3 ml of the fraction in a 1.0-ml digest containing 5 mg maltose, 100 μmol acetate buffer (pH 5.6) at 37°C for several hours; glucose is assayed by the glucose-oxydase (Glucostat Worthington Biochemical Corporation).

Microorganisms

The microorganisms used were *E. coli* K12 strain *Hfr G6* (455), a mutant of *Hfr G6*, lacking glycogen synthetase (193) and the strain JC 1553 KLF 41 (814) with an episome covering the region maltose A and glycogen.

Cultures and preparation of crude extracts

Strains were grown aerobically at 32°C on synthetic medium [1] supplemented by histidine and aneurine (32 mg/l) for strains 455 and 193 and by sucrose (20 g/l), aneurine, histidine, leucine (32 mg/l), methionine (46 mg/l) for the strain 814. Growth was limited by glucose for the carbon-limited culture of 814 or by ammonium chloride, glucose being in excess, for the nitrogen-limited cultures. The cells were harvested by centrifugation and disrupted in a French pressure cell at 12 000 lb/inches^2 in 5 vols. 0.05 M potassium phosphate buffer (pH 7.0).

Results

Influence of limited nitrogen and carbon sources on the levels of debranching activity in several strains of E. coli

Experiments were carried out to establish the optimal conditions for the synthesis of the debranching enzyme and the results were summarized in Table I. The amount of debranching enzyme was highest in the 814 strain. In fact the levels of many of the other enzymes involved in glycogen metabolism are much higher in this strain of *E. coli*. This result is probably caused by the presence of an episome which covers the maltose A and glycogen regions. The activity of the enzyme in the 814 and 455 strains increased after growth ceases due to nitrogen limitation and 3- or 4-fold higher levels were found after 24 h.

TABLE I

LEVELS OF DEBRANCHING ACTIVITY IN DIFFERENT STRAINS OF *E. COLI* DURING GROWTH.

Strains	Log phase	Specific activity (units/mg protein) after growth ceases		
		30—60 min	2 h	24 h
814 (N limited)	0.031	0.058	0.069	0.129
814 (C limited)	0.026	0.022	0.026	0.025
193 * (N limited)	0.009	0.008	—	0.012
455 (N limited)	0.0128	0.0148	0.0174	0.0366

* 193 is a mutant of *E. Coli* lacking glycogen synthetase.

This increase was not observed when glucose was the limiting factor (814, limited C, Table I). However, due to the lack of glucose, glycogen did not accumulate in this strain. In order to determine whether the increase in debranching activity was due to limited nitrogen or to the accumulation of glycogen, similar experiments were carried out with a mutant, 193, which lacks glycogen synthetase and therefore cannot accumulate glycogen. Comparing this mutant with 814 under conditions of limited nitrogen, there was no increase in debranching activity during the stationary phase. Therefore, it may be concluded that the increase in debranching activity during the stationary phase is related to the accumulation of glycogen in the cells. The levels of debranching activity during the logarithmic and stationary phase were similar when the cells were grown on glucose and maltose. This result suggests that maltose does not induce the synthesis of debranching enzyme in these cells.

Purification of debranching enzyme from E. coli 814

The procedure developed for the isolation of this enzyme is shown in Table II. The washed cells were disrupted in a French pressure cell. Deoxyribonuclease at a final concentration of 10 μg/ml and $MgCl_2$ 10 mM were added to the suspension and it was incubated for 15 min at room temperature. An inactive precipitate obtained by centrifugation at 3000 $\times g$ for 20 min was discarded. A second pellet which contained most of the debranching activity was collected by centrifugation at 70 000 $\times g$ for 90 min. This precipitate, which probably contained debranching enzyme bound to glycogen, was suspended in 0.02 M potassium phosphate (pH 8.0) and poured on to a DEAE-cellulose column (2 $\times$ 17 cm), which was previously equilibrated against the same buffer. The debranching enzyme was then eluted with a linear gradient prepared with 500 ml 0.02 M potassium phosphate (pH 8.0) in the mixing chamber and 500 ml of the same buffer and 0.3 M KCl in the reservoir. The debranching enzyme emerged from the column at approx. 0.15 M KCl. The active fractions were pooled and concentrated to about 17 ml (fraction 4) using an Amicon ultrafiltration cell fitted with a PM-10 membrane. The solution was dialyzed against 0.05 M Na_2HPO_4/0.75 mM citric acid buffer (pH 8.0) containing 0.1 M KCl and applied to a DEAE-Sephadex A-50 column (2 $\times$ 14 cm) previously equilibrated with the same buffer. The debranching enzyme was eluted as a single symetrical peak with the same buffer as shown in Fig. 1. Several other enzymes, including

TABLE II

PURIFICATION OF ENZYME

Fraction	Volume (ml)	Total activity (units)	Total protein (mg)	Specific activity (units/mg of protein)	Purification	Yield (%)
1 Crude extract	400	720	5952	0.121	1	100
2 3000 $\times$ *g* supernatant	390	655	5316	0.123	1.2	90
3 70 000 $\times$ *g* pellets	208	439	1058	0.415	3.9	61
4 DEAE-cellulose	17	320	51	6.3	52	44.4
5 DEAE-Sephadex A-50	4.9	152	4	37.8	312	21.1

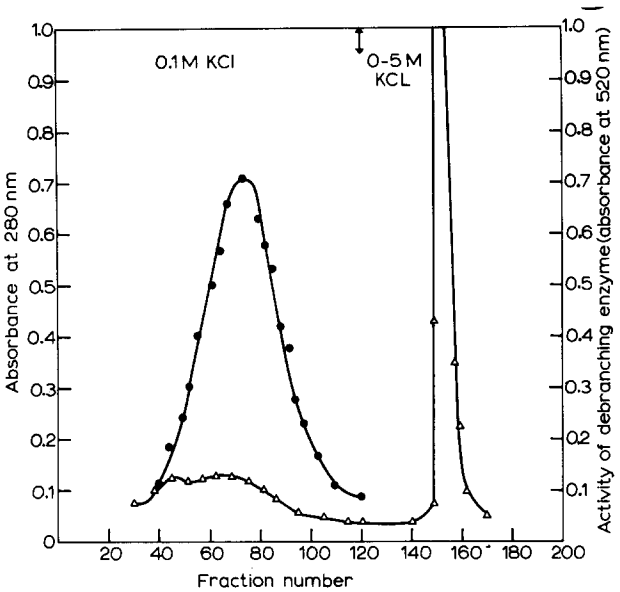

Fig. 1. Elution pattern of debranching enzyme from a DEAE-Sephadex A-50 column. Fractions of 3 ml were collected and assayed for enzyme activity as described under Materials and Methods. Debranching enzyme activity, ●——●; proteins, △——△.

α-amylase and maltase, remained bound to the column and could be eluted along with other proteins in 0.5 M KCl. The active fractions were collected and concentrated as described previously.

Evidence for homogeneity of the isolated enzyme

The final preparation was homogeneous and had a specific activity of 37.8 μmol/min per mg. An overall purification of about 300-fold of the crude extract was usually obtained. The preparation was completely free of isomaltase, maltase, dextranase, α-amylase, glycogen synthetase and glycogen phosphorylase. α-Amylase and maltase activity were removed during DEAE-Sephadex A-50 chromatography. No activity was observed with α-glucosyl-Schardinger dextrin, which indicates that the *E. coli* enzyme is different in this respect from mammalian 1,6-α-glucosidase.

Only a single well- defined protein band with debranching activity was found upon polyacrylamide disc gel electrophoresis at pH 7.4. A single protein band was also observed at pH 8.4. The purified enzyme was very unstable in the absence of KCl. However, the final preparation could be stored at 4°C in 0.05 M Na_2HPO_4/0.75 mM citric acid buffer (pH 8.0) containing 0.1 M KCl with little or no loss of activity.

Properties of the enzyme

Phosphorylase limit dextrin prepared from oyster glycogen was used to examine the properties of the enzyme. Under the standard conditions described in the methods, the rate of hydrolysis of 1,6-α-bonds was linear with time for at least 20 min. The verified rate of hydrolysis has been found to be proportional to the concentration of enzyme. The influence of pH on the rate of the

reaction is shown in Fig. 2. Maximum activity was observed in a pH range from 5.6 to 6.4. The effect of temperature is shown in Fig. 3. Maximum activity was observed at 45°—50° C and the activity was only 76% as high at 37° C, the temperature of the standard incubation mixture. The influence of the concentration of oyster glycogen phosphorylase limit dextrin was studied.

A K_m of 4.5 mg/ml was calculated from the Lineweaver-Burk plots. The reaction was carried out for short periods of incubation only since the only product formed under these conditions was maltotetraose.

Influence of various reagents on the debranching enzyme

As shown in Table III, the debranching enzyme was inhibited by iodoacetate, *p*-chloromercuribenzoate and $HgCl_2$. The enzyme was also strongly inhibited by cupric sulfate. Ammonium sulfate inhibited the activity (90% at 50 mM) but 0.1 M sodium sulfate had no effect. Sodium citrate, sodium chloride and EDTA did not inhibit the enzyme. However, complete inhibition was observed in the presence of 0.1 M Tris · HCl at pH 5.6.

Action of debranching enzyme on different substrates

The initial rates of hydrolysis of 1,6-α-bonds in several different polysaccharide substrates were compared (Table IV). The data obtained in these experiments are expressed as the percent of total 1,6-α-bonds cleaved for each sub-

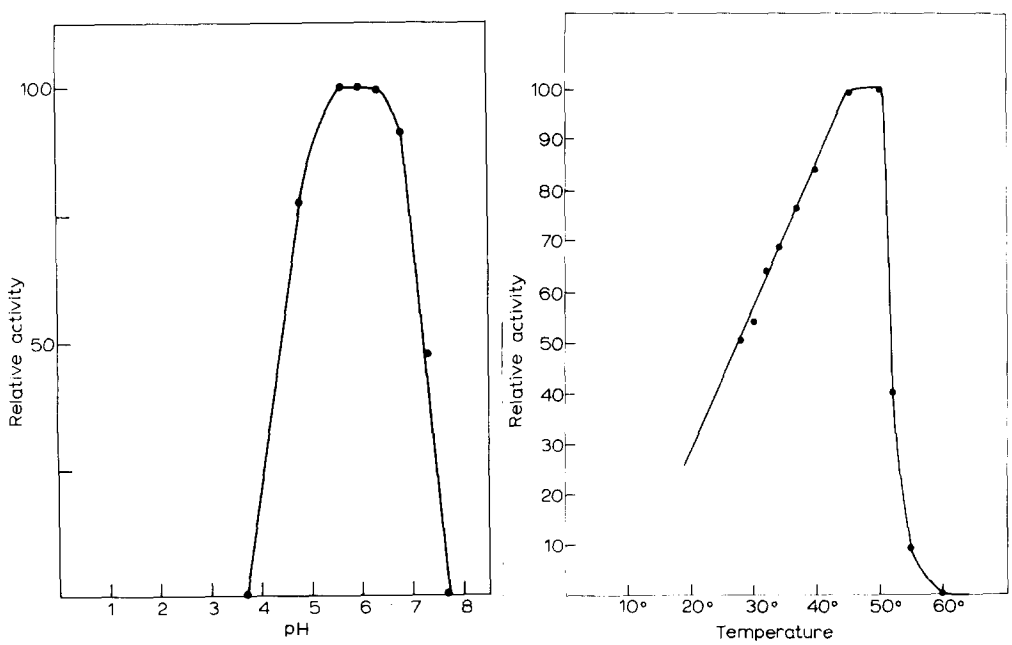

Fig. 2. Effect of pH on debranching enzyme activity. The activity was determined under standard assay conditions except that the buffer was 20 mM citrate/phosphate. The incubation mixture contained 0.4—0.8 μg of enzyme per ml.

Fig. 3. Effect of temperature. Debranching activity was determined under the standard assay conditions. The incubation mixtures contained 0.4—0.8 μg of enzyme per ml.

TABLE III

EFFECTS OF VARIOUS REAGENTS ON THE ACTIVITY OF THE ENZYME

The enzyme was assayed under the standard incubation conditions. Percent inhibition was calculated relative to activity found in a control.

Additions	Inhibition (%)					
	Final concentration (mM)					
	0.1	1.0	10	20	50	100
Sodium borate	11	—	97			
$(NH_4)_2SO_4$	—	—	—	25	90	100
NH_4Cl	—	—	—	—	37.5	84
Magnesium acetate	—	27	65	—	—	80
Potassium phosphate	—	—	—	—	27	80
Tris	—	—	—	20	61	100
p-Chloromercuribenzoate	100	100	—	—	—	—
Iodoacetate	—	15	100	—	—	—
$CuSO_4$	90	100	—	—	—	—
$HgCl_2$	100	100	—	—	—	—

strate, since direct measurements of reducing oligosaccharides released cannot be compared. All of the rates are compared to the rate obtained with phosphorylase limit dextrin which is taken as 100%.

The rates of hydrolysis of the limit dextrins prepared from glycogen and amylopectin by treatment with phosphorylase were 2-fold greater than that of the corresponding limit dextrins prepared with β-amylase. Intact amylopectin and glycogen were hydrolyzed very slowly in comparison with the rates obtained with limit dextrins and the enzyme showed very little activity with pullulan as substrate. The extent of hydrolysis of oyster glycogen phosphorylase limit dextrin was examined.

There was a very rapid rate of hydrolysis and only 25% of the 1,6-α-bonds were hydrolyzed even when a very high concentration of enzyme was used as seen in Fig. 4. Then a very slow rate was observed; more than 60 h and large amounts of enzyme were required for complete hydrolysis of the remainder of the molecule. Aliquots removed at various times from these reaction mixtures were analyzed by thin-layer chromatography. The major product released from

TABLE IV

INFLUENCE OF DEBRANCHING ENZYME ON THE INITIAL RATES OF HYDROLYSIS OF BRANCHED POLYSACCHARIDES

Substrate	Relative initial rate of hydrolysis
Oyster-glycogen phosphorylase limit dextrin	100
Amylopectin phosphorylase limit dextrin	117
Oyster-glycogen β-amylase limit dextrin	52.1
Amylopectin β-amylase limit dextrin	40.8
Amylopectin	2.14
Oyster-glycogen	0.38
Pullulan	0.15

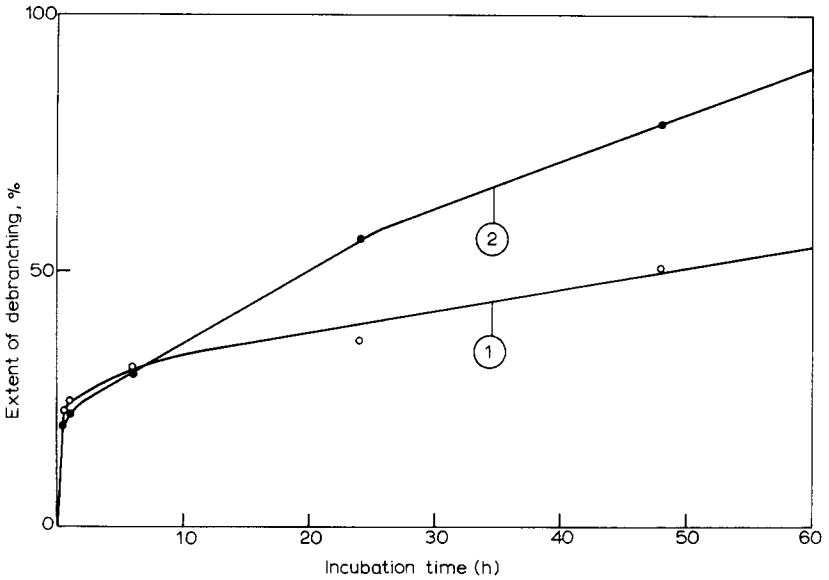

Fig. 4. Action of debranching enzyme on glycogen phosphorylase limit dextrin. 1. The reaction mixture contained 40 μg of enzyme per ml. 2. The reaction mixture contained 400 μg of enzyme per ml.

phosphorylase limit dextrin of oyster glycogen and amylopectin in the first hour was maltotetraose. Small amounts of matotriose, presumably due to the fact that the structure of the limit dextrin is not ideal [22], are also released from oyster-glycogen phosphorylase limit dextrin. After longer periods of time, more maltotriose and maltose as well as larger oligosaccharides were formed.

The principal product formed during the first hour of incubation with β-amylase dextrins was maltotriose. Later on, some maltose and very small amounts of maltotetraose and maltopentaose were also formed.

The hydrolysis of a number of short-chain branched oligosaccharides [13] was also examined by thin-layer chromatography (Table V). The products formed after 4 h of incubation with 40 μg of enzyme in 0.1 M sodium acetate buffer (pH 5.6) are shown in Table V. These results show that the enzyme is able to hydrolyze chains containing two and three glucose residues.

The simultaneous action of debranching enzyme and β-amylase were exam-

TABLE V

HYDROLYSIS OF SMALL BRANCHED CHAIN OLIGOSACCHARIDES

The first substrate was isolated from pullulan by partial hydrolysis with pullulanase; the others were isolated from beer. Hydrolysis was carried out with the standard assay system described in Methods. G_2, G_3, G_4 and G_5 represent linear maltosaccharides of degree of polymerisation 2, 3, 4, 5.

Substrate		Oligosaccharides formed
6^3-α-Maltotriosylmaltotriose		G_3
6-α-Maltosylmaltotetraose	95%	G_2, G_4
6-α-Glucosylmaltopentaose	4%	
6-α-Maltotriosylmaltotetraose	67%	G_2, G_3, G_4, G_5
6-α-Maltosylpentaose	33%	

TABLE VI

ACTION OF DEBRANCHING ENZYME ON AMYLOPECTIN, GLYCOGEN AND RELATED DEXTRINS AS MEASURED BY CHANGE IN THE EXTENT OF β-AMYLOLYSIS

Treatment	β-amylase alone	Successive action with β-amylase	Simultaneous action with β-amylase
Substrate	% conversion to maltose		
Amylopectin	54	102	102
Oyster glycogen	43	57	91
Amylopectin β-amylase limit dextrin	0	67	80
Glycogen Oyster β-amylase limit dextrin	0	63	96
Amylopectin phosphorylase limit dextrin	22	80	98
Oyster-glycogen phosphorylase limit dextrin	19	89	99

ined by incubating mixture of polysaccharide (2.5 mg) debranching enzyme and β-amylase in 0.04 M acetate buffer (pH 4.8) in a total volume of 0.5 ml for 20 h at 37° C.

To study the successive action of the debranching enzyme and β-amylase, polysaccharide (5 mg) was first treated with debranching enzyme (80—160 μg/

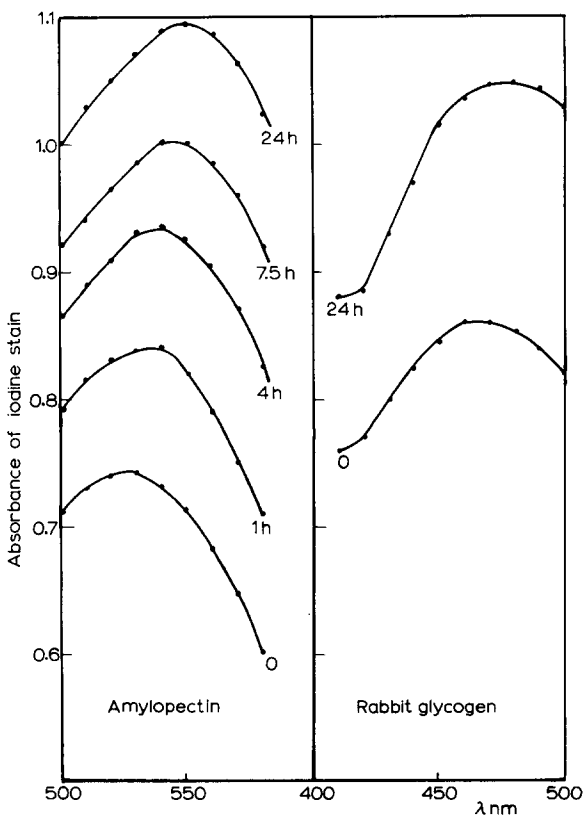

Fig. 5. Increase of iodine coloration of amylopectin and glycogen on treatment with the debranching enzyme. 5 mg of amylopectin in 0.02 M citrate/phosphate buffer (pH 5.5) was treated with 80 μg of enzyme in a total volume of 1 ml; 10 mg of rabbit glycogen was treated in the same buffer with 160 μg of enzyme. Samples of 0.1 ml were removed at intervals and added to 5 ml of iodine reagent, as described in Material and Methods, for the determination of the spectra of the iodine complex.

ml) at pH 5.6 in a total volume of 1 ml. After heat inactivation, 100 µl of the reaction mixture was incubated with β-amylase (50 units) in 0.04 M acetate buffer (pH 4.8) for 4 h and the maltose formed was measured with Nelson's reagent. The effect of preincubation with debranching enzyme on the β-amylolysis of amylopectin, glycogen and related dextrins is shown in Table VI. The debranching enzyme helped in the complete degradation of amylopectin both by simultaneous and successive action with β-amylase. Complete hydrolysis of glycogen and amylopectin phosphorylase limit dextrin was observed when debranching enzyme and β-amylase acted simultaneously and 80—90% of these substrates were hydrolyzed when they acted successively. Although the debranching enzyme aided in the complete degradation of glycogen by simultaneous action with β-amylase, successive treatment caused only a small increase in the degree of hydrolysis (43—57%). With glycogen and amylopectin β-amylase limit dextrins the debranching enzyme caused extensive β-amylolysis (63.5 and 67.3%) by successive action with the β-amylase. The simultaneous action of these two enzymes caused the complete hydrolysis of glycogen β-amylase limit dextrin and 88% hydrolysis of amylopectin β-amylase limit dextrin.

Further evidence for the hydrolysis of longer chains was obtained by the use of very large amounts of enzyme and examination of iodine coloration. As show in Fig. 5, a 50% increase in iodine staining of amylopectin was observed after complete hydrolysis of 1,6-α-bonds by the debranching enzyme. The absorbance maximum shifted from 530 to 550 nm. When glycogen was treated in a similar manner, a 20% increase in iodine coloration was observed.

Discussion

The isolated enzyme was homogeneous as judged by disc polyacrylamide gel electrophoresis at pH 8.4 and pH 7.4. It had an optimum pH at 5.6 with glycogen phosphorylase limit dextrin as substrate. It was strongly inhibited by SH-group reagents such as iodoacetate and *p*-chloromercuribenzoate. The enzyme from *Pseudomonas* was only slightly inhibited [23] by these reagents. Inhibition by Tris and NH_4^+ has not been reported for the isoamylase of *Pseudomonas* and *Cytophaga*. Glycogen and amylopectin phosphorylase limit dextrins were debranched and maltotetraose was the major product liberated until 30% of the 1,6-α-bonds were hydrolyzed. No glucose was formed. These results show that the enzyme has a direct action on 1,6-α-glucosidic bonds and therefore it may be classified in the group of direct debranching enzyme. The fact that the purified preparation was unable to attack α-glucosyl-Schardinger dextrin is further evidence of its difference from the indirect system occuring in yeast and mammals. Two classes of direct debranching enzyme have been distinguished by their ability to attack α-glucan, pullulan [24]: pullulanase and isomylase. The purified enzyme isolated from *E. coli* does not readily release maltotriose from pullulan. Yokobayashi et al. [23] have also reported that *Pseudomonas* isoamylase showed only a slight activity towards pullulan. Isoamylases of *Pseudomonas* and *Cytophaga* hydrolyzed all of the 1,6-α-bonds in glycogen and amylopectin with formation of relatively long maltodextrin chains. The debranching enzyme of *E. coli* is able to hydrolyze all the 1,6-α-bonds in amylopectin but the initial rate with this substrate is much slower than that ob-

served with phosphorylase and β-amylase limit dextrins. The 1,6-α-bonds in native glycogen were hydrolyzed at a slower rate than those present in amylopectin. This could be due to the difficulty of the enzyme to reach the 1,6-α-linkages buried inside the packed molecule.

In this repect, the *E. coli* enzyme is more similar to *A. aerogenes* pullulanase [25] and yeast isoamylase [26].

The phosphorylase limit dextrins prepared from glycogen and amylopectin were the best substrate for the *E. coli* enzyme. These substrates probably contained only 20—30% short outer chains and these were rapidly released. The reaction then slowed down; a long time and large amount of enzyme were necessary to reach completely debranching of the molecule. The fact that simultaneous action with β-amylase led to 100% β-amylolysis in 24 h further indicates that the enzyme is more active with substrates that contain short chains. The β-amylase limit dextrins prepared from glycogen and amylopectin were poorer substrates than the phosphorylase limit dextrins. However a partial debranching does occur, since β-amylolysis did increase from 0 to 60—70%. The presence of both enzymes resulted in the hydrolysis of almost all the 1,6-α-bonds. At the start of the reaction, maltotriose was the major oligosaccharide released, but later on maltose and small amounts of higher oligosaccharides appeared. The *E. coli* enzyme is less active in cleaving the α-maltosyl residues in β-amylase limit dextrins. However, in contrast to *Pseudomonas* and *Cytophaga* isoamylases, it is also able to liberate maltose residues and by simultaneous action with β-amylase, it hydrolyzes almost all the 1,6-α-bonds. It has been reported that the isoamylases of *Cytophaga* and *Pseudomonas* debranch β-amylase limit dextrins of glycogen and amyloptectin with the formation of maltotriose and a barely detectable amount of maltose, but *A. aerogenes* pullulanase liberates equal amounts of maltose and maltotriose. This observation has served to distinguish isoamylase from pullulanase even though *S. mitis* pullulanase [27] has the same action as isoamylase. Branched oligosaccharides were also hydrolyzed by the *E. coli* enzyme. A mixture of 6-α-matotriosylmaltotetraose and 6-α-maltosylpentaose was the best substrate. Maltose was released from 6-α-maltosylmaltotetraose and 6-α-maltosylpentaose; this further indicates that the enzyme is active against maltose residues.

In conclusion, the *E. coli* enzyme is a direct debranching enzyme with a high affinity for limit dextrins obtained by action of phosphorylase or amylase. We are not certain that the enzyme can be classified as an isoamylase, in view of the fact that it shows very little activity with unmodified glycogen.

Differences in the initial relative rates of hydrolysis of various substrates containing residues with different chain lengths indicate that those containing four glucose units are the best substrates.

Maltotriose and maltose residues are released at slower rates. From the results of the debranching action on glycogen and amylopectin which show that the maltodextrins having more than four glucose units are not easily hydrolyzed, it seems that the debranching enzyme of *E. coli* does not act directly in vivo on glycogen, but only after it has been converted to a phosphorylase limit dextrin by glycogen phosphorylase. The phosphorylase limit dextrin obtained is a good substrate for debranching enzyme which liberates maltotetraose and branched dextrins. Maltotetraose is a poor substrate for glycogen phosphor-

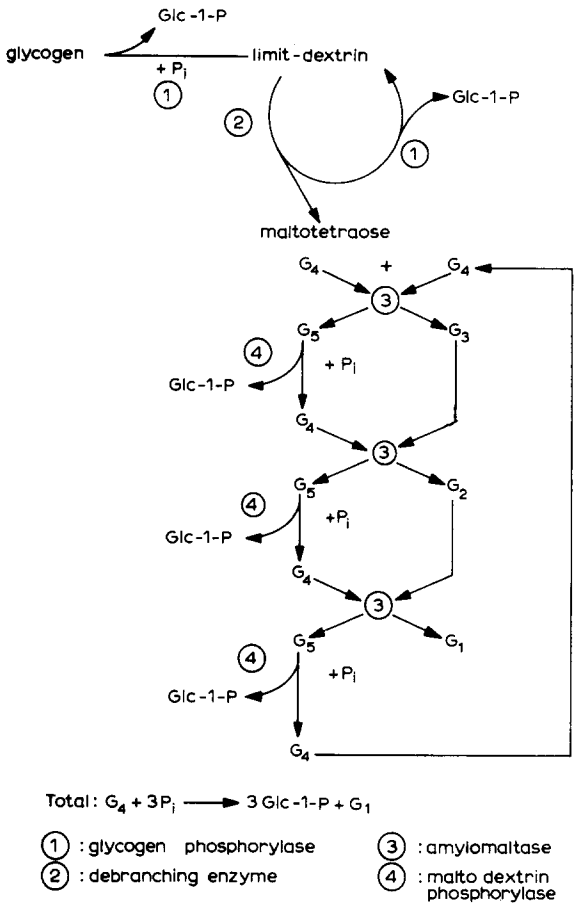

Fig. 6. Proposed scheme for the catabolism of glycogen bacteria.

ylase [28], but is has been shown to be a substrate for transglucosylase [29]. Glucose and glucose-1-*P* can be formed in a series of reactions in which maltotetraose behaves catalytically. As shown in Fig. 6, the maltotetraose may be converted to glucose-1-*P* and glucose by the successive action of amylomaltase and maltodextrin phosphorylase or other enzymes which act on maltodextrin chains. In this scheme, a maximum conversion of the glucosyl residues in maltodextrins to glucose-1-*P* can occur.

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