STUDIES ON AMYLO-1,6-GLUCOSIDASE*

by

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Some properties and mechanism of action of the intestinal carbohydrases, maltase (α-glucosidase) and oligo-1,6-glucosidase have been presented[1,2]. In order to contrast these enzymes with an enzyme catalyzing the hydrolysis of α-1,6 linkages in polysaccharides, it has been of interest to investigate further the properties of amylo-1,6-glucosidase[3]. These studies form the basis of the present report.

METHODS AND MATERIALS

Enzymes

Rabbit muscle phosphorylase was prepared by the method of GREEN AND CORI[4] using 0.1 *M* NaF in the crystallization step and in the first recrystallization according to the procedure of E. W. SUTHERLAND, Jr.[5].

Amylo-1,6-glucosidase was prepared from pooled supernatant fluids collected after crystallization of rabbit muscle phosphorylase by a method previously described[6]. Several modifications were instituted as suggested by the work of BEIZENHERZ *et al.*[7]. Glass-redistilled water was used throughout. Saturated ammonium sulfate solutions were prepared from ammonium sulfate recrystallized at slightly alkaline pH from 0.0027 *M* versene. The pooled supernatant fluids were diluted to one per cent protein concentration with 0.0027 *M* versene pH 7.2. The ammonium sulfate fraction (0–0.3 saturation) was dissolved in 0.0027 *M* versene containing 0.003 *M* cysteine at pH 7.2.

To remove α-amylase two starch adsorptions with water-washed corn starch (Argo) were done as already described[3]. Amylo-1,6-glucosidase prepared under these conditions was considerably more stable than preparations prepared by the previous method[6] and withstood freezing and thawing 2 to 3 times with essentially no loss of activity. In some cases activity which had been lost after repeated freezing and thawing could be restored by the addition of glutathione.

It has been found that such preparations have specific activities ranging from 2600 to 5000 units per mg[3], but still contain phosphorylase and traces of α-amylase. Partial stabilization of the enzyme by versene has recently been reported by CORI[8].

Yeast hexokinase was prepared by an unpublished method of C. R. PARK and glucose-6-phosphate dehydrogenase by the method of KORNBERG[9].

Substrates, cofactors and inhibitors

Phosphorylase limit dextrin (LD_1)*** was prepared by two successive enzymic digestions of rabbit liver glycogen with repeatedly recrystallized phosphorylase (free of amylo-1,6-glucosidase) by the procedure already described[10]. Glucosidase-treated LD_1 (LD_1g) was prepared by incubating amylo-1,6-glucosidase (50 spectrophotometric units, see EXPERIMENTAL) with 60 mg LD_1 at 30° for 180 minutes. At the end of this time 112% of theoretical outer branch points were hydrolyzed as determined by increase in reducing power. Polysaccharide was isolated in the usual manner by several ethanol precipitations.

*Iso*maltose was a gift of Dr. A. JEANES, "panose" a gift of Dr. M. KILLEY, and corn and wheat

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*** For a discussion of terms see previous paper[10].

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amylopectins gifts of Dr. R. W. KERR. Maltose was a commercial product. Adenosine triphosphate (ATP) and triphosphopyridine nucleotide (TPN) were purchased from Sigma Chemical Company. ^{14}C uniformly labelled glucose was prepared by the method of WICK *et al.*[11] by submitting ^{14}C uniformly labelled starch to acid hydrolysis. *p*-chloromercuribenzoic acid and *o*-iodosobenzoic acid were obtained from Sigma Chemical Company. Iodoacetic acid was a gift of Dr. W. A. WOOD.

ANALYTICAL

Amylo-1,6-glucosidase activity was determined in the presence of excess phosphorylase, LD_1, and inorganic phosphate by increase of reducing power as previously described[3]. The method of NELSON[12] as modified[3] was used for reducing power determination. Deproteinization was accomplished either with 2.5 % $HgCl_2$ in 0.5 *N* HCl (Schenk) or by the method of SOMOGYI[13]. Protein was determined by a turbidometric method already outlined[1].

Radioactivity was determined by counting in a gas flow counter. The samples were plated on metal planchettes (previously treated with a small amount of 1 % detergent) and dried under an infrared lamp. Glucose was counted in an infinitely thin film, polysaccharides at measured thickness. Corrections in the latter case were made by comparing the thickness of each sample with a self-absorption curve of ^{14}C wax. Conversion to absolute activities (disintegrations per minute, d.p.m.) was done using a polystyrene standard.

pH was determined with Beckman model G glass electrode.

EXPERIMENTAL

Spectrophotometric assay

Amylo-1,6-glucosidase activity has been determined by means of a spectrophotometric assay similar to that used for oligo-1,6-glucosidase[1]. Formation of glucose by the hydrolysis of LD_1 has been coupled with the reduction of TPN, catalyzed by the hexokinase glucose-6-phosphate dehydrogenase system. The linear relationship between rate of optical density change at 340 mμ (Beckman model DU spectrophotometer at room temperature) and amount of enzyme added is shown in Fig. 1, which also gives an indication of the reproducibility of the method. Under these conditions, a provisional spectrophotometric unit is defined as that amount of enzyme which catalyzes an optical density change of 0.001 per minute. As previously discussed[1] there is a short lag period before the maximum optical density change is achieved. The maximum rate of optical density change is the basis of the calculation of units. It is to be emphasized that spectrophotometric activity varies with conditions of assay (ratio of hexokinase to glucose-6-phosphates dehydrogenase). The present unit holds only under the specified conditions.

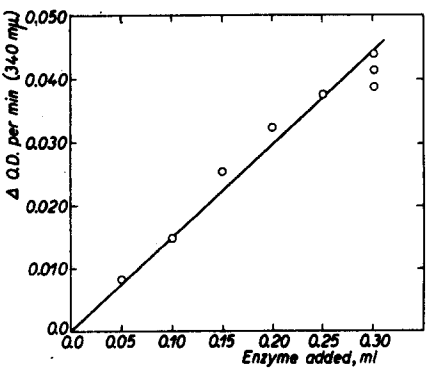

Fig. 1. Spectrophotometric determination of amylo-1,6-glucosidase activity. Reaction mixture contained glycylglycine buffer 0.25 *M*, pH 7.5, 1.0 ml; $MgSO_4$ 0.3 *M*, 0.1 ml; glucose-6-phosphate dehydrogenase 3 mg lyophilized powder per ml 0.2–0.4 ml (0.26–0.52 units[9]); hexokinase 0.1 % (30% pure) 0.03–0.05 ml; TPN 0.005 *M*, 0.1 ml; ATP 0.1 *M* 0.01 ml; LD_1 1 %, 0.1 ml; final volume 3.0 ml.

Amylo-1,6-glucosidase activity determined by the previous method of increase in reducing power[3] has been compared with activity determined by the spectrophotometric assay using the same enzyme preparations (Table I). An average value of 31 reduction units per spectrophotometric unit was obtained. No difference in the reducing power assay was noted with or without

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added Mg^{++}. For reasons of convenience and speed the spectrophotometric assay has been used wherever possible. In the experiments which follow, units refer to spectrophotometric units.

TABLE I

AMYLO-1,6-GLUCOSIDASE ACTIVITY DETERMINATION BY SPECTROPHOTOMETRIC AND INCREASED REDUCING POWER METHODS

Spectrophotometric activity (1)	*Reduction activity (2)*	*Ratio (2):(1)*
units/ml	*units/ml*	
400	(A) 13,800	34.5
281	(A) 7,680	27.3
522	(A) 17,800	34.1
599	(A) 16,000	26.8
501	(A) 16,000	31.9
268	(B) 8,050	30.0
148	(B) 4,670	31.5
	Average	31

A = Mg^{++} present; final concentration 0.012 *M*
B = Mg^{++} absent
(1) Reaction mixtures were as described in Fig. 1.

pH Activity curve

Variation of initial rate as a function of pH has been measured using the spectrophotometric assay (Fig. 2). Experiments were run in glycylglycine buffers previously adjusted to various pH's with dilute acid or alkali. In control experiments, it was shown that the hexokinase glucose-6-phosphate dehydrogenase system with glucose as substrate could operate at a rate greater than that found with amylo-1,6-glucosidase and LD_1 and therefore was not limiting. In contrast to oligo-1,6-glucosidase which has a pH optimum at 6.2 to 6.4 [1], amylo-1,6-glucosidase under these conditions has an optimum in the range 7.2 to 7.6.

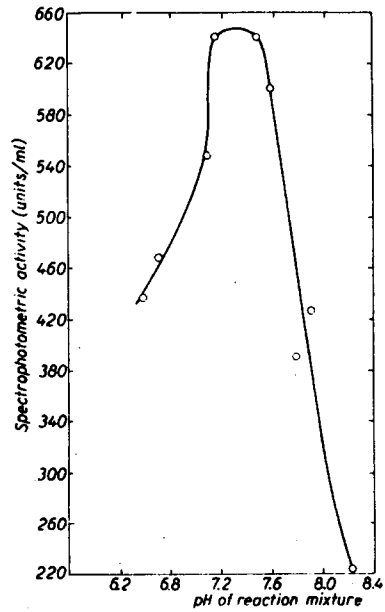

Fig. 2. pH Activity curve of amylo-1,6-glucosidase. Reaction mixture as described. pH of glycylglycine buffer adjusted with HCl or NaOH. pH of reaction mixture determined at end of run.

Sulfhydryl inhibitors

Because of the increased stability and activity of amylo-1,6-glucosidase in the presence of cysteine[3], versene, and glutathione (METHODS AND MATERIALS), the effect of sulfhydryl inhibitors on enzyme activity has been examined. Since the spectrophotometric assay system is inhibited by sulfhydryl reagents*, it was not used for these experiments. The reducing power assay with phosphorylase and inorganic phosphate was likewise not used because of the possibility that muscle phosphorylase also requires sulfhydryl groups for activity (cysteine activation). Accordingly, the hydrolysis of LD_1 by amylo-1,6-glucosidase was followed by increase in reducing power in the absence of accessory enzymes. Large enough amounts of LD_1 were used such that the glucose released was sufficient to be easily detectable by analytical methods employed. Under these conditions the reaction is essentially linear with time until approximately 30% hydrolysis of the available outer branch points. As shown in Table II, a concentration of *p*-chloromercuribenzoate of $5 \cdot 10^{-5}$ *M* completely inhibits activity.

* Unpublished experiments, J. LARNER AND R. E. GILLESPIE.

TABLE II

INHIBITION OF AMYLO-1,6-GLUCOSIDASE BY SULFHYDRYL REAGENTS*

Inhibitor	*Inhibitor concentration*	*Time of incubation***	*Glutathione concentration*	*Inhibition*
	M/l	*minutes*	*M/l*	*per cent*
p-chloromercuribenzoate	$1 \cdot 10^{-4}$	20	—	100.0
p-chloromercuribenzoate	$1 \cdot 10^{-4}$	20	$1.5 \cdot 10^{-2}$***	55.5
p-chloromercuribenzoate	$5 \cdot 10^{-5}$	20	—	100.0
p-chloromercuribenzoate	$1 \cdot 10^{-5}$	20	—	5.5
o-iodosobenzoate	$1 \cdot 10^{-4}$	20	—	61.8
o-iodosobenzoate	$1 \cdot 10^{-4}$	20	$1.5 \cdot 10^{-2}$§	0.0
o-iodobenzoate	$1 \cdot 10^{-5}$	20	—	2.5
iodoacetate	10^{-3}	10	—	0.0

* 100 units of enzyme used in all experiments.
** Incubations were at room temperature.
*** Added after 5 minutes.
§ Added after 5 minutes. Enzymic activity was restored to double that of control. This activation was to original level of activity before freezing and thawing.

o-Iodosobenzoate is a less effective inhibitor, while no inhibition with iodoacetate was noted. Glutathione reversed the inhibition by both *p*-chloromercuribenzoate and *o*-iodosobenzoate. Intestinal maltase and oligo-1,6-glucosidase, in contrast to amylo-1,6-glucosidase, are not inhibited by these sulfhydryl inhibitors[2]

Inhibition by carbohydrates

The following carbohydrates were found not to inhibit amylo-1,6-glucosidase in the spectrophotometric assay (27 units of enzyme used); *iso*maltose, 0.0012 *M*; "panose", 0.002 *M*; glucose-1-phosphate, 0.003 *M*.

Glucose was tested as an inhibitor in the usual glucosidase reducing power assay in the presence of phosphorylase and inorganic phosphate. Since phosphorylase has been shown to be inhibited by glucose[14] enough phosphorylase was added in order that it would not prove limiting.

The results (Table III) with two concentrations of phosphorylase indicate that this condition was met. No inhibition was noted with 0.001 *M* and 0.0018 *M* glucose using 26 and 15 units of glucosidase during a 10 minute time period (Table III, experiments 1, 2). The ratio of added glucose to branch point glucose (assuming 11.4% branch points in LD_1) in experiments 1 and 2 was 2.2 and 3.5 respectively. Because of the possibility that phosphorylase might prevent inhibition of glucosidase by glucose, additional experiments were done in the absence of added phosphorylase and inorganic phosphate. With the concentrations of glucose employed, no inhibition of glucosidase activity was noted (Table III, experiments 3, 4). In experiment 3, the ratio of added glucose to available branch point glucose (outer tier) was 1, while in experiment 4, it was 3.5. Both oligo-1,6-glucosidase and maltase are inhibited by glucose[15].

Inhibition by LD_1g and K_m for LD_1

The other product of hydrolysis, the "debranched" limit dextrin (LD_1g), proved to be an inhibitor of the enzyme. As is shown in Fig. 3, a plot of $1/v$ versus $1/s$ for the inhibited and uninhibited system followed the kinetics of a reversible inhibition.

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TABLE III

EFFECT OF GLUCOSE ON ACTIVITY OF AMYLO-1,6-GLUCOSIDASE

Experiment Number	Phosphorylase added*	Glucose added	Amylo-1,6-glucosidase activity
	ml	μmoles	total units
1**	0.5	—	26.2
1**	0.5	2.78	26.6
1**	0.8	2.78	27.9
2**	0.2	—	15.1
2**	0.5	4.45	15.0
3***	—	—	77
3***	—	5.6	106
4***	—	—	106
4***	—	19.4	108

* A suspension of crystals recrystallized until free of amylo-1,6-glucosidase activity.

** Reaction mixture contained glucosidase, phosphate buffer 1.0 M pH 7.0, 0.3 ml; adenylic acid 0.01 M, 0.2 ml; LD_1, 2 mg; final volume 2.5 ml. Deproteinization was with Schenk reagent. Activity given in spectrophotometric units.

*** Reaction mixture contained glucosidase 70 units; glycylglycine buffer 0.25 M pH 7.4, 0.5 ml; LD_1 30 mg; final volume 2.3 ml. Deproteinization was with $Ba(OH)_2$-$ZnSO_4$. Activity given in Klett-Summerson colorimeter reading differences from zero time controls.

The Michaelis constant for LD_1 is 14 mg per cent. Expressed as outer tier branch point glucose (moles) the K_m is $2.7 \cdot 10^{-5} M$*. The K_I for LD_1g is 50.5 mg per cent or $9.8 \cdot 10^{-5} M$ if expressed as "originally available" outer tier branch point glucose (moles)**.

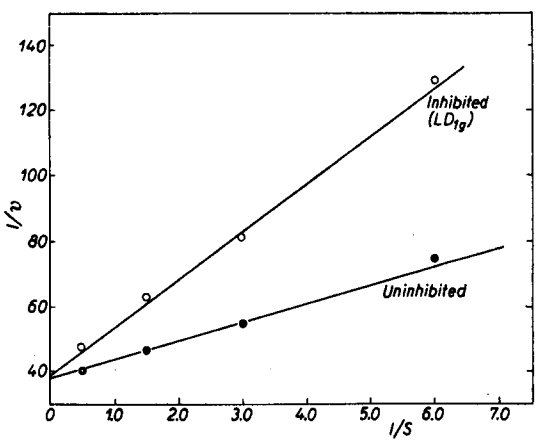

Fig. 3. Inhibition of amylo-1,6-glucosidase by LD_1g. Reaction mixture as described, containing in addition 0.1 ml LD_1g 2.75%; average pH 7.18.

Using the integrated form of the Michaelis' equation with competitive product inhibition[16]

$$(S_0 - S_F)\left(1 - \frac{K_m}{K_I}\right) + K_m\left(1 + \frac{S_0}{K_I}\right)\ln\frac{S_0}{S_F} = Vmt$$

a calculated curve, Fig. 4, was constructed with values of K_m and K_I determined by the spectrophotometric method. For V_m, initial rate of hydrolysis with excess substrate as estimated by increased reducing power was used. V_m as determined

* Expressed in terms of outer tier rather than total branch point glucose, since only the outer tier is available to enzymic action in this assay. Outer tier branch points (moles) calculated as follows:

$$\frac{\text{concentration of polysaccharide (mg/ml)} \times 0.034}{180}$$

Molecular weight of 180 is used since polysaccharide concentration was determined as glucose after acid hydrolysis.

** K_I calculated from the expression $\text{slope} = \frac{K_m}{V_m}\left[1 + \frac{I}{K_I}\right]$.

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graphically by the spectrophotometric method at room temperature at this pH was on the average 15% lower than the experimental initial velocity obtained by hydrolysis at 30°. At pH 7.16, there is a good fit of the points determined experimentally by increased reducing power to the calculated curve, indicating that K_m and K_I as determined by the spectrophotometric or reducing power methods are in essential agreement. At pH 6.8 the K_m of muscle phosphorylase for glycogen in the direction of synthesis has been reported as 21 mg per cent[14]. Assuming an average of 7% branch points for glycogen[17] this value corresponds to $8 \cdot 10^{-5} M$.

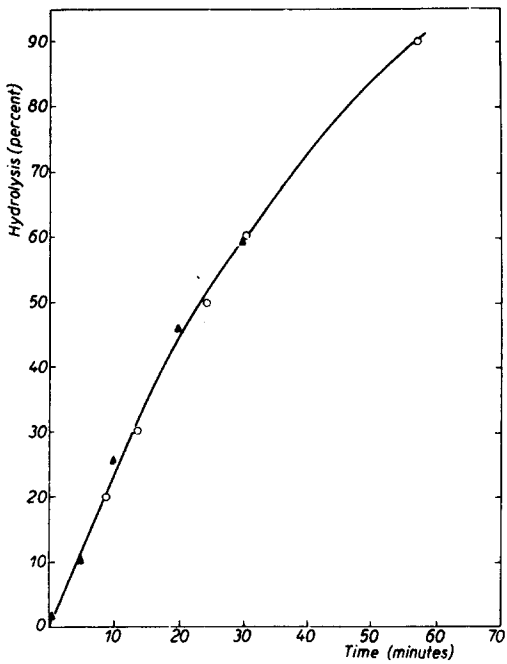

Fig. 4. Hydrolysis of LD_1 by amylo-1,6-glucosidase: ▲ Experimental. ○ Calculated using K_m $2.6 \cdot 10^{-5}$ M*, K_I $9.8 \cdot 10^{-5}$ M. $V_m = 0.16\gamma$ glucose/minute/unit enzyme. Reaction mixture contained glycylglycine buffer 0.25 M pH 7.3, 2.0 ml; $MgSO_4$ 0.3 M, 0.2 ml; glucosidase 74 units; LD_1 14.75 mg; final volume 6.0 ml.

Variation of Michaelis constant with pH

The variation of pK_m ($-\log K_m$) with pH has been studied, using the spectrophotometric assay method (Fig. 5). A biphasic curve is obtained when pK_m is plotted against pH similar in shape to that reported for intestinal maltase and oligo-1,6-glucosidase[2]. At acid pH pK_m appears independent of pH, while at alkaline pH the plot is best represented by a slope of −1. The change in slope at about pH 7.5–7.6 is somewhat higher than observed in the case of the intestinal enzymes (pH 7.0–7.1).

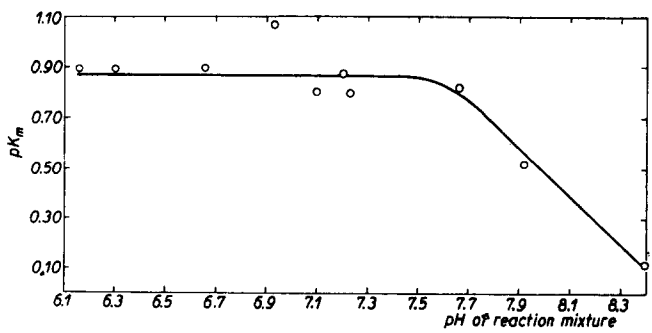

Fig. 5. pH Dependence of pK_m. Reaction mixtures as in Fig. 1. pH's are average values of each set of reaction mixtures. The values usually varied by several hundredths of one pH unit.

* A K_m value of $2.6 \cdot 10^{-5} M$ rather than $2.7 \cdot 10^{-5} M$ was used because it is an average value at this pH (see Fig. 5).

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In control experiments the plot of pK_m against pH of the hexokinase glucose-6-phosphate dehydrogenase system for glucose between pH 6.4 and 8.1 had no change in slope[§]. Since the substrate is considered uncharged and since the pK_m pH plot of the hexokinase glucose-6-phosphate dehydrogenase system with glucose is without a change in slope in this pH range, this experiment suggests that an ionizable enzymic group with a pK_a of about 7.5–7.6 is involved in the combination of enzyme with substrate[18].

Reversibility

Previous experiments with the radioactive method had shown that crude glucosidase preparations are contaminated with small amounts of branching enzyme[19]. In order to obtain an active preparation of branching enzyme from muscle it was necessary to extract the powder obtained by pulverizing frozen muscle in a fitted stainless steel cylinder and plunger. Amylo-1,6-glucosidase, on the other hand, is extracted from fresh muscle with water. These differences in extractability pointed to the presence of two enzymes each catalyzing a separate reaction.

Reversibility of purified amylo-1,6-glucosidase was tested by two experimental approaches; first by using the iodine color assay developed for branching enzyme[20] (Table IV); and second by isolation and determination of radioactivity of the polysaccharide remaining after incubation of LD_1 with glucosidase in the presence of ^{14}C labelled glucose (Table V). Glucosidase (205 units—sufficient to completely "debranch" 187 mg LD_1 in 10 minutes) had essentially no action on 3 mg of corn amylopectin in 20 minutes as indicated by the constant value of the optical density of the iodine color complex at 570 mμ (Table IV). After 345 minutes the optical density had decreased to 57.5% of its original value. That this decrease was due at least in part to α-amylase action was shown in a separate experiment by measuring the increase in reducing power. As maltose, the increase in reducing power was 8.5% when the iodine color had decreased to 46% of the initial value. KUNG, HANRAHAN AND CALDWELL[21] have studied the decrease in iodine color as related to increase in reducing power with a series of ten α-amylases, hydrolyzing amylose. At 10% hydrolysis (as maltose) from 75.0 to 26.2% of the blue value remained, with most

TABLE IV

ACTION OF AMYLO-1,6-GLUCOSIDASE ON CORN AMYLOPECTIN

Incubation time	*Optical density at 570 mμ of iodine complex*	*Reducing power as maltose*
minutes	*per cent of initial value*	*per cent*
0	100*	
5	103	
10	105	
20	97	
345	57.5	
300	46.0**	8.5***

* Reaction mixture contained glycylglycine buffer 0.25 *M* pH 7.4, 0.5 ml; corn amylopectin 3 mg; glucosidase 205 units; final volume 1.8 ml.

** Reaction mixture as above except that 10 mg corn amylopectin used; final volume 3.5 ml.

*** Deproteinized with $Ba(OH)_2$-$ZnSO_4$.

§ Unpublished experiments, J. LARNER and R. E. GILLESPIE.

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TABLE V

INCORPORATION OF ^{14}C GLUCOSE BY AMYLO-1,6-GLUCOSIDASE DURING HYDROLYSIS OF LD_1*

Incubation time	*Outer branch points hydrolyzed*	*Analysis after ethanol precipitations*			*Analysis after dialysis*			*Incorporation after dialysis*
		Polysaccharide weight	*Radioactivity*	*Specific activity*	*Polysaccharide weight*	*Radioactivity*	*Specific activity*	
min	*per cent*	*mg*	*d.p.m.*	*d.p.m./mg*	*mg*	*d.p.m.*	*d.p.m./mg*	*per cent*
14	38.1							
15	—	11.5	162	14.1	10.1	327	32.4	0.072*
35	57.2							
36	—	14.9	633	42.5	12.7	430	33.8	0.11**
Control		15.3	68	4.4	17.7	60	3.4	

* Reaction mixture contained the following: Glycylglycine buffer 0.25 M pH 7.4, 1.0 ml; LD_1 84 mg; ^{14}C glucose $1.25 \cdot 10^6$ d.p.m.; glucosidase 146 units; final volume 3 ml. 1 ml samples taken for polysaccharide isolation; 0.5 ml samples for reducing power. For all samples except controls, counting was done for a length of time sufficient for a precision of $\pm$ 5%.

** Calculated as follows: Specific activity of ^{14}C glucose $2.14 \cdot 10^6$ d.p.m./mg

$$\text{mg } ^{14}C \text{ glucose per mg } LD_1 = 33.8 \frac{\text{d.p.m.}}{\text{mg}LD_1} \times \frac{1}{2.14 \cdot 10^6} \frac{\text{mg glucose}}{\text{d.p.m.}} = 15.8 \cdot 10^{-6}$$

$$\text{mg outer branch point glucose remaining/mg } LD_1 = 0.428 \times 0.034 = 14.6 \cdot 10^{-3}$$

$$\text{per cent reincorporation} = \frac{15.8 \cdot 10^{-6}}{14.6 \cdot 10^{-3}} \cdot 10^2 = 0.11.$$

of the amylases giving values about 55%. The present value of 46.5% is well within this range*.

For the radioactive experiment (Table V), control, and experimental reaction mixtures were prepared which were identical except for the absence of enzyme in the control. Per cent hydrolysis was determined by increase in reducing power in aliquots deproteinized by the $Ba(OH)_2$-$ZnSO_4$ method and calculated assuming that complete hydrolysis is equivalent to the liberation of 3.4% reducing power (as glucose). A correction was applied for the reducing power of ^{14}C labelled glucose initially present.

Polysaccharides were precipitated from reaction mixtures by ethanol. Supernatant fluids from the first ethanol precipitation were set aside for isolation of ^{14}C glucose. Precipitates were taken up in water and digested with alkali (10% NaOH) at 100° for 2 minutes. Five additional ethanol precipitations were done. After the last ethanol precipitation polysaccharides were dissolved in water, aliquots plated out on metal planchettes, weighed and counted. After counting, the material was dissolved from the planchettes, dialyzed at 3° for 84 hours against multiple changes of distilled water, replated and recounted.

Significant radioactivity in the two experimental samples (greater than control) is present both after alkali treatment and extensive dialysis (Table V). The lower specific activity of the 15 minute sample before dialysis was probably due to uneven plating. In terms of per cent reincorporation, the radioactivity found was 0.072 in the 15 minute sample, and 0.11% in the 35 minute sample. The enzyme does not under these conditions catalyze reincorporation of labelled glucose into polysaccharide to any appreciable extent.

* This does not rule out some action by branching enzyme in addition to α-amylase

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DISCUSSION

The properties of amylo-1,6-glucosidase clearly differentiate this enzyme from oligo-1,6-glucosidase. Previous work[1] has indicated the differing substrate requirements of the two enzymes. The present work indicates that amylo-1,6-glucosidase operates at a higher pH optimum than oligo-1,6-glucosidase, and that amylo-1,6-glucosidase is inhibited by sulfhydryl reagents and not inhibited by glucose. Other properties studied here include K_m, and competitive inhibition by LD_1g. It is of some interest that muscle phosphorylase and amylo-1,6-glucosidase have K_m's in the same general range. Evidence has been presented which indicates that a charged enzymic group of pK_a 7.5–7.6 participates in combining with substrate. Reversibility has been tested and no evidence for significant reversibility could be found. Further studies with regard to mechanism of action of amylo-1,6-glucosidase and other carbohydrases are in progress.

SUMMARY

Additional properties of amylo-1,6-glucosidase have been studied using a rapid spectrophotometric assay. The pH optimum is in the region between 7.2 to 7.6.

The enzyme is inhibited by *p*-chloromercuribenzoate and *o*-iodosobenzoate. The inhibition is reversed by glutathione.

No inhibition has been observed with glucose but the "debranched" phosphorylase limit dextrin proved an inhibitor of the competitive type with a K_I of 50.5 mg per cent.

The variation of K_m with pH has been determined and indicates that an ionizable enzymic group with a pK_a of 7.5–7.6 is involved in combining with substrate.

No evidence for significant reversibility of the reaction could be found under the conditions tested.

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