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Note

Re-examination of the action of sweet-potato beta-amylase on phosphorylated (1→4)-α-D-glucan*

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In a previous paper[1], it was reported that beta-amylase [(1→4)-α-D-glucan maltohydrolase, EC 3.2.1.2] hydrolyses a linear phosphodextrin ($\overline{d.p.}$ 6.25) until a D-glucosyl or maltosyl residue remains attached to the 6-phosphoryl-D-glucosyl residue. However, during a study of the location of the phosphate groups of potato amylopectin[2] it was noticed that its 6-phosphoryl-D-glucosyl residues appeared to be exposed at the non-reducing ends following treatment with a large excess of the enzyme. Therefore we have re-investigated the action of beta-amylase on linear, phosphorylated (1→4)-α-D-glucans focussing on the molecular size of the substrate and the enzyme concentration.

The potato amylopectin used in this study contained one phosphate group per 317 D-glucosyl residues. 69.4% of the phosphate was located[3,4] at C-6 and the re-

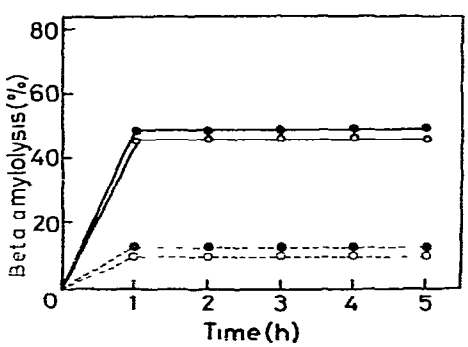

Fig. 1. Hydrolysis of phosphorylated (1→4)-α-D-glucans with two concentrations of beta-amylase. phosphorylated unit-chains (PUC) (———) and alpha-phosphodextrin (α-PD) ( ) were hydrolysed at pH 4.8 with low (2.5 units/mg of substrate) (○) and high (12.5 units/mg of substrate) (●) activities of beta-amylase at 37°. The degree of beta-amylolysis is expressed as the percentage conversion of the glucans into maltose.

*Studies on Starch Phosphate, Part 5. For Part 4, see ref. 1.

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mainder at C-3 The phosphorylated unit-chain of the amylopectin (PUC, $\overline{d\,p}$ 42 4) was prepared by debranching the amylopectin with *Pseudomonas* isoamylase[5], and alpha-phosphodextrin (α-PD, $\overline{d\,p}$ 5 03) was prepared by the method described previously[1] PUC and α-PD were hydrolysed with the low (2 5 units/mg of substrate) and high (125 units/mg of substrate) activities of beta-amylase and the exposed 6-phosphoryl-D-glucosyl residues at the non-reducing ends were determined by assaying glycerol before and after phosphatase treatment of the Smith-degradation products of the hydrolysates Both PUC and α-PD had one phosphate group per mole, and were hydrolysed to the respective limits in 1 h with the two concentrations of the enzyme, although the limits differed slightly with the enzyme activity, as shown in Fig 1

The analytical data for PUC and its beta-amylase-degraded products are given in Table I β-PUC and βL-PUC the degraded products of PUC with the low and high activities of beta-amylase had $\overline{d\,p}$ 19 5 and 18 2 respectively, suggesting that the phosphate is located randomly on the PUC Smith degradation of PUC yielded one mole of glycerol per mole and no additional glycerol from the non-reducing end after treating the Smith-degradation products with alkaline phosphatase, indicating that the PUC is a linear molecule and that no phosphate is located at C-6 of the non-reducing end Whereas Smith degradation of β-PUC and βL-PUC gave considerably lower amounts of glycerol, 0 87 and 0 70 mole/mole respectively the alkaline phosphatase treatment of their Smith-degradation products resulted in the complete recovery of glycerol βL-PUC was not further hydrolysed with 500 units of beta-amylase per mg of substrate, whereas β-PUC produced a small proportion of maltose suggesting that the maltosyl stub linked to the phosphoryl residue at the non-reducing side is hydrolysed with difficulty A similar situation has been reported by Lee[6] for the degradation of glycogen and amylopectin with beta-amylase, that is, the unbranched side-chains are shortened to three or four D-glucosyl residues and then the latter are degraded slowly to two D-glucosyl residues These results indicate that beta-amylase is capable of hydrolysing (1→4)-α-D-glucosidic linkages at the non-reducing sides of the 6-phosphoryl-D-glucosyl residues (linkage-$\overline{A}$, Fig 2) with varying difficulty However, these results are inconsistent with that of a previous report[1], which concluded that the enzyme could not hydrolyse linkage-$\overline{A}$

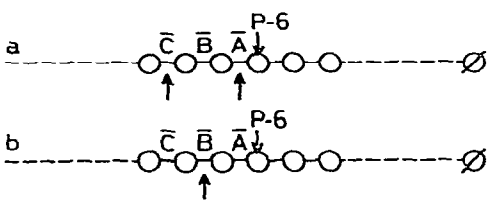

Fig 2 The action of beta-amylase on linear phosphorylated (1→4)-α-D-glucans having an even number (a) and an odd number (b) of D-glucosyl residues on the non-reducing-side chain ○ D-glucosyl residue, Ø, reducing-end D-glucose, —, α-(1→4) bond, P-6, the phosphate group attached to C-6 of the D-glucosyl residue, ↑, point of cleavage by beta-amylase

TABLE I

PROPERTIES OF THE PHOSPHORYLATED UNIT-CHAIN (PUC) AND ITS DEGRADED PRODUCTS (β-PUC AND βL-PUC) WITH BETA-AMYLASE

	PUC (mole/mole)	*β-PUC*	*βL-PUC*
Carbohydrate (as Glc), $\overline{d\,p}$	42 4	19 5	18 2
Glycerol, produced by Smith degradation			
Before phosphatase treatment	1 00	0 87	0 70 (0 62)[a]
After phosphatase treatment	1 01	1 04	1 02 (0 98)[a]
Maltose formed by beta-amylase treatment[b]	11 4	0 14	0 0

[a]βL-PUC was prepared by using beta-amylase purified on DEAE-Sephadex A-50 [b]PUC, β-PUC, and βL-PUC were incubated with 500 units of beta-amylase per mg of substrate for 2 h at 37°

TABLE II

PROPERTIES OF ALPHA-PHOSPHODEXTRIN (α-PD) AND ITS DEGRADED PRODUCTS (β-PD AND βL-PD) WITH BETA-AMYLASE

	α-PD (mole/mole)	*β-PD*	*βL-PD*
Carbohydrate (as Glc), $\overline{d\,p}$	5 03	4 49	4 43
Glycerol, produced by Smith degradation			
Before phosphatase treatment	1 01	0 99	0 92 (0 92)[a]
After phosphatase treatment	1 01	0 99	1 00 (1 02)[a]

[a]βL-PD was prepared by using beta-amylase purified on DEAE-Sephadex A-50

To determine the reason for these contradictory results, the above examination was performed on α-PD, and the results are presented in Table II The α-PD was prepared[1] by hydrolysing potato starch with liquefying alpha-amylase of *B subtilis*, but the α-PD was a somewhat smaller molecule than the previous preparation The reason is not clear The α-PD and its degraded product (β-PD) with the low activity of beta-amylase had no 6-phosphoryl-D-glucose at their non-reducing ends as reported earlier[1] However, a small but definite amount of 6-phosphoryl-D-glucose (0 08 mole per mole of βL-PD) was found at the non-reducing end of the α-PD hydrolysed with the high activity of the enzyme (βL-PD), and this was missed previously[1] Thus, in this case also, a large excess of the enzyme hydrolysed linkage-$\overline{A}$

The possibility that the hydrolysis of linkage-$\overline{A}$ may be effected by a trace of α-D-glucosidase in the preparation of beta-amylase was examined by using the enzyme further purified by DEAE-Sephadex chromatography, as described by Marshall *et al* [7]. The results were negative, as indicated in Tables I and II

Thus, it is concluded that beta-amylase is capable of hydrolysing amylose

phosphorylated at C-6 of the D-glucosyl residue from the non-reducing end and that one or no D-glucosyl residues remain attached to the 6-phosphoryl-D-glucose as illustrated in Fig. 2. This behaviour is in accord with the action of the enzyme on branched oligosaccharides having a D-glucosyl stub as reported by Kainuma and French[8].

EXPERIMENTAL

Preparation of phosphorylated unit-chains (PUC) — A solution of potato amylopectin (920 mg in 92 ml of 5mM acetate buffer, pH 3.5) was incubated with 15 units of *Pseudomonas* isoamylase[5] for 2.5 h at 50°. The $\overline{dp}$ of the digest reached 23.3 within 1 h and there was no further decrease during the prolonged incubation. The reaction was terminated by heating, and the digest was diluted immediately to 920 ml with water to prevent precipitation. The solution was applied to DEAE-Sephadex A-50 (Cl^- form, ~100 ml), and neutral dextrin was removed by elution with water. PUC was eluted with 0.2M NaCl in 0.01M HCl and precipitated with ethanol (final conc. 75%). The precipitate was collected by centrifugation, and dissolved in water and the solution was kept in a freezer until use. The yield of PUC was 90 mg and the recovery of phosphorus was 90%.

Preparation of β-PUC and βL-PUC — PUC (30 mg) in 3 ml of 25mM acetate buffer (pH 4.8) was incubated with 75 units (low) and 3750 units (high) of beta-amylase respectively at 37° for 4 h. No further reducing-sugar was produced after incubation for 1 h in either of the digests (Fig. 1). The degraded PUCs were isolated by the same procedure as for the preparation of PUC. The yield of phosphorus in the degradation was ~60% in both cases.

Preparation of β-PD and βL-PD — γ-PD was obtained by the methods described previously[1] and desalted by gel filtration with Bio-Gel P-2. β-PD and βL-PD were obtained by degradation of γ-PD with the low and high activities of beta-amylase respectively, as described above. No further decrease in $\overline{dp}$ of either of the digests was observed after 1-h incubation. The resulting β-PD and βL-PD were isolated by DEAE-Sephadex chromatography as described above, and freed from salts by gel filtration with Bio-Gel P-2 instead of alcohol precipitation. The recoveries of phosphorus in β-PD and βL-PD were 96 and 92%, respectively.

Assays — Reducing sugar and total carbohydrate were determined by the methods described previously[3]. Organic phosphate was measured as inorganic phosphate by the method of Itaya *et al.*[7] after treatment with hot perchloric acid.

The non-reducing end of phosphodextrins was determined by rapid Smith-degradation[10], with minor modifications. The acid hydrolysis involved in Smith degradation was performed with 0.5M H_2SO_4 for 1 h, instead of 0.2M H_2SO_4 for 20 min, for the phosphorylated glucans which were resistant to acid hydrolysis. Glycerol phosphates in the Smith-degradation products were determined as glycerol after treatment with alkaline phosphatase (0.25 unit/ml) for 6 h at 37°.

Materials — *Pseudomonas* isoamylase was a gift from Professor T. Harada

(Osaka University) Crystalline, sweet-potato beta-amylase was prepared by the method described previously[11] and recrystallised from $(NH_4)_2SO_4$ solution The activity was assayed[12] at 37° in the presence of Triton X-100, and a unit of activity was defined as the liberation of 1 μmol of maltose per min α-D-Glucosidase activity in this preparation was hardly detectable by the method of Marshall *et al*[7] the activity ratio of α-D-glucosidase to beta-amylase was $< 1\ 5 \times 10^{-8}$ Potato amylopectin was prepared by the method of Schoch[13], but in an atmosphere of N_2 Crystalline, *B subtilis* alpha-amylase (liquefying type), the specific activity of which was 563 μmol of reducing sugar (equivalent to glucose)/min/mg (30°, pH 5 8), was obtained from Daiwa Kasei Co Glycerol kinase glycerol 3-phosphate dehydrogenase and alkaline phosphatase (*E coli*) were purchased from Boehringer

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