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ACTIONS OF *Aspergillus oryzae* ALPHA-AMYLASE, POTATO PHOSPHORYLASE, AND RABBIT MUSCLE PHOSPHORYLASE *a* AND *b* ON PHOSPHORYLATED (1→4)-α-D-GLUCAN

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ABSTRACT

Aspergillus oryzae alpha-amylase [(1→4)-α-D-glucan glucanohydrolase, EC 3.2.1.1] produced *O*-(6-phosphoryl-α-D-glucopyranosyl)-(1→4)-*O*-α-D-glucopyranosyl-(1→4)-D-glucopyranose (6^3-phosphorylmaltotriose) and *O*-α-D-glucopyranosyl-(1→4)-*O*-(3-phosphoryl-α-D-glucopyranosyl)-(1→4)-*O*-α-D-glucopyranosyl-(1→4)-D-glucopyranose (3^3-phosphorylmaltotetraose) from potato starch upon exhaustive hydrolysis. These products indicate that the enzyme hydrolyses the same linkages in the vicinity of the 6-phosphorylated residue as porcine-pancreatic alpha-amylase, but hydrolyses different linkages in the vicinity of the 3-phosphorylated residue when compared with *B. subtilis* and pancreatic alpha-amylases. Potato phosphorylase [(1→4)-α-D-glucan:orthophosphate α-D-glucosyltransferase, EC 2.4.1.1] and rabbit muscle phosphorylase *a* and *b* were unable to by-pass the phosphorylated D-glucosyl residue of 6-phosphorylated (1→4)-α-D-glucan, leaving three D-glucosyl residues attached to the 6-phosphorylated residue on the non-reducing side.

INTRODUCTION

The phosphate groups[1–4] and branch linkages[5–11] of amylopectin and the chemically modified D-glucosyl residues[12–14] of amylose are useful for the determination of the action specificities of amylolytic enzymes. The actions of alpha- and beta-amylases and phosphorylase in the vicinity of the branch linkage have been studied in detail, whereas little work in the vicinity of the modified residues has been done. We have investigated the actions of sweet-potato beta-amylase[2], *Aspergillus niger*[3,4] and *Rhizopus delemer*[15] glucoamylase, and *Bacillus subtilis* (liquefying type) and porcine-pancreatic alpha-amylases in the vicinity of the phosphate groups attached to positions 3[16] and 6[16–20] of the glucosyl residues, and suggested that they were characteristic of the type and source of the amylases. It was suggested that the phosphate groups are better for comparing the action specificities of various amylolytic enzymes than the branch linkages of amylopectin, because of the easy

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isolation of phosphorylated malto-oligosaccharides with ion-exchange resins, the characterisation of the action at 3- and 6-phosphate barriers, and the possible applications to enzymes which hydrolyse the branch linkage[4].

For detailed characterisation and comparison of the action specificities of various (1→4)-α-D-glucan-degrading enzymes, the actions of *Aspergillus oryzae* alpha-amylase and of potato and rabbit muscle phosphorylases on phosphorylated (1→4)-α-D-glucan have been examined.

EXPERIMENTAL

Materials. — Potato starch, obtained from a factory in Hokkaido, contained 334 p.p.m. of organic phosphate (80% at positions 6 and the remainder at positions 3). Crystalline rabbit-muscle phosphorylase *a* and *b*, alkaline phosphatase (Sigma), phosphoglucomutase, D-glucose 6-phosphate dehydrogenase (Boehringer), DEAE-Sephadex A-25, Sephadex G-75 (Pharmacia), Bio-Gel P-2 and P-150 (Bio-Rad), and DEAE-Toyopearl 650M (Toyo Soda Manuf. Co. Ltd.) were commercial products. *Aspergillus niger* glucoamylase was prepared as described previously[3].

Aspergillus oryzae *alpha-amylase.* — A commercial crude preparation (Sigma) was purified by the method of Yamakawa *et al.*[21], using DEAE-Toyopearl instead of DEAE-Sephadex. The eluate from a column of Sephadex G-75 was applied to DEAE-Toyopearl 650M and the enzyme was eluted with a linear gradient 0→0.2M NaCl. Two peaks showing alpha-amylase activity were observed. Acetone (−20°) was added to 60% to the combined fractions of the main peak. The resulting precipitate was collected by centrifugation at 11,000*g* at 4° for 10 min and dissolved in 0.1M acetate buffer (pH 6.0). The solution was again chromatographed as described above and gave a single peak for both activity and protein. The enzyme was precipitated by the addition of solid ammonium sulfate (75% saturation), recovered by centrifugation, suspended in aqueous 75% ammonium sulfate, and stored in a refrigerator. The purified enzyme (266 mg from 5 g of the crude preparation) had a specific activity of 102 U (μmol of reducing sugar equivalent to glucose per min, at 30° and pH 5.5) per mg of protein, and was homogeneous in electrophoresis on polyacrylamide gel[22] (7.5% gel, pH 7.0, activity and protein staining were examined) and SDS-polyacrylamide gel[23] (7% gel). Maltase activity was not detected in the preparation.

Potato phosphorylase. — This enzyme was purified by the method of Kamogawa *et al.*[24] with minor modifications to reduce a very weak amylolytic activity (ratio of alpha-amylase and phosphorylase activities was 3×10^{-3}), which was detected in a DEAE-Sephadex eluate on assaying by the method of Fuwa[25]. Solid ammonium sulfate was added to the eluate to 60% saturation, the resulting precipitate was collected by centrifugation, and a solution (89 mg of protein in 24 mL) in 5mM Tris–HCl buffer (pH 7.5) was applied to a column (3 × 30 cm) of Bio-Gel P-150 equilibrated and eluted with the buffer. The enzyme was precipitated by the addition of solid ammonium sulfate (55% saturation). The gel filtration

and ammonium sulfate precipitation reduced the amylolytic activity to a negligible level (ratio of alpha-amylase to phosphorylase activities was 6×10^{-5}). The purified enzyme (86 mg from 15 kg of potatoes) had a specific activity of 30.3 U (μmol of inorganic phosphate formed per min, at 30° and pH 6) per mg of protein.

Preparation of phosphorylated malto-oligosaccharides from potato starch with A. oryzae *alpha-amylase.* — A suspension of potato starch (150 g, dry weight) in 2mM $CaCl_2$ (300 mL) together with 1990 U of the enzyme was added to 2mM $CaCl_2$ (1.05 L) at 70–80° with stirring. The starch was gelatinised and liquefied simultaneously. The solution was kept for 15 min at 95–100°, and the pH of the solution was then adjusted to 5.5 with M HCl after cooling. Hydrolysis was resumed at 45° for 24 h by the addition of 6360 U of the enzyme. After termination of the hydrolysis by heating for 10 min at 95–100°, the coagulated protein was removed by centrifugation, and the supernatant solution was diluted to 5 L with water and applied to a column of DEAE-Sephadex A-25 (Cl^- type, 60 mL). Neutral sugars were eluted with water, and phosphorylated malto-oligosaccharides with 0.2M NaCl/0.01M HCl. Salts were removed by chromatography on Bio-Gel P-2 to give phosphorylated malto-oligosaccharides (1.64 g of carbohydrate as glucose) with an average degree of polymerisation ($\overline{d.p.}$) of 5.4.

Preparation of (1→4)-α-D-glucan phosphorylated at positions 6 (6-phosphorylglucan). — Potato starch with a water content of 20.7% was heated at 120° for 10 h in a sealed tube to increase the ratio of the 6-phosphate to organic phosphate[20]. The starch (3 g, dry weight) was washed with water and gelatinised with 5mM acetate buffer (pH 3.5, 400 mL), and the solution was incubated at 50° for 2.5 h with 100 U (μmol of reducing sugar equivalent to glucose per min, at 45°) of *Pseudomonas* isoamylase. 1-Butanol (40 mL) was then added and the mixture was kept at 30° for 2 h. The resulting precipitate was removed by centrifugation at 2500*g* for 10 min. The supernatant solution was diluted to 2 L with water and applied to a column (3 × 24 cm) of DEAE-Sephadex A-50 (Cl^- type). Neutral sugars were eluted with water. The 6-phosphorylglucan was eluted with 0.2M NaCl/0.01M HCl and precipitated with ethanol (final concentration, 75%). The material (62.3 mg) comprised linear molecules with a $\overline{d.p.}$ of 40 as judged by analyses of the reducing and non-reducing terminal residues, and 90% of the phosphate groups were attached to positions 6. Its glucoamylolysis limit was 48 ±1% (mean ±s.d., 6 experiments).

Preparation of partially degraded 6-phosphorylglucan with phosphorylase. — The 6-phosphorylglucan (18 mg) was incubated in 0.18M phosphate buffer (pH 6.5, 18 mL) at 30° for 4 h with 47 U of potato phosphorylase and was degraded 35%. The reaction was terminated by heating and the coagulated protein was removed by centrifugation. The supernatant solution was concentrated to 5 mL and then applied to a column (2.6 × 100 cm) of Bio-Gel P-2 to remove D-glucose 1-phosphate and inorganic phosphate. The partially degraded 6-phosphorylglucan (7.6 mg) was precipitated with ethanol (final concentration, 75%).

Methods. — Organic phosphate was measured[26] as inorganic phosphate after

treatment with hot perchloric acid[27]. The 6-phosphate groups were determined[20] as D-glucose 6-phosphate and the remainder was assumed to be 3-phosphate. The reducing, terminal residue of the phosphorylated oligosaccharides was assayed[28] with minor modifications[4] as D-glucitol with sorbitol dehydrogenase. The $\overline{d.p.}$ was calculated from the reducing residue and total carbohydrate, determined by the anthrone–sulfuric acid method[29]. The non-reducing terminal residue was determined as glycerol after rapid Smith-degradation[30] with minor modifications[2]. The phosphorylated reducing and non-reducing terminal residues were determined as D-glucitol and glycerol, respectively, released from D-glucitol phosphate and glycerol phosphates with phosphatase[4]. D-Glucose was assayed by the D-glucose oxidase–peroxidase method[31]. H.p.l.c. was performed at room temperature on a column of Toyo Soda NH_2-60 in a liquid chromatograph (Toyo Soda HLC 803D) with a differential refractometer (Toyo Soda IR-8), with elution with acetonitrile–water (3:2) at 0.8 mL/min. Dephosphorylated samples for h.p.l.c. were prepared as reported previously[4]. The phosphorolysis was determined at 25° by the formation of NADPH, using the mixture containing 50mM phosphate (pH 7), 3mM EDTA, 10mM $MgCl_2$, 0.25mM NADP, 9μM D-glucose 1,6-diphosphate, 80mM AMP (when phosphorylase *b* was used), phosphoglucomutase (1.19 U/mL), and D-glucose 6-phosphate dehydrogenase (1.04 U/mL).

RESULTS

Structures of the phosphorylated malto-oligosaccharides produced with Aspergillus oryzae *alpha-amylase.* — The phosphorylated malto-oligosaccharide produced on hydrolysis of potato starch was further degraded with three concentrations (5.3, 7.9, and 10.6 U/mg of substrate) of the enzyme (Fig. 1). The same level of hydrolysis was attained after incubation for 21 h at the two higher concentrations. The phosphorylated malto-oligosaccharide, therefore, was degraded for 27 h with the enzyme at 7.9 U/mg of substrate, and the products were fractionated on Bio-Gel P-2 to give fractions F1–F3 in order of elution, based on the $\overline{d.p.}$ (Fig. 2a). F2 was further fractionated into F2a and F2b (Fig. 2b). All fractions except F1 were purified on Bio-Gel P-2. The h.p.l.c. elution curves for F2a, F2b, and F3, dephosphorylated with phosphatase, indicated the sugar components of F2a and F2b to be maltohexaose and maltopentaose, respectively, and that of F3 to be an ~1:4 mixture of maltotetraose and maltotriose (Fig. 3).

Table I contains data on the phosphorylated malto-oligosaccharides. F1 was a mixture of linear molecules, with a $\overline{d.p.}$ of 7.4, carrying one or more phosphate groups attached to positions 3 and 6. F2a and F2b were linear molecules with a 3-phosphate group. No phosphate group was located at the non-reducing and reducing terminal residues of F2a and F2b which produced 1.09 and 0.46 mol of D-glucose, respectively, by limiting hydrolysis with glucoamylase[4]. Thus, F2a was *O*-α-D-glucopyranosyl-(1→4)-*O*-α-D-glucopyranosyl-(1→4)-*O*-(3-phosphoryl-α-D-glucopyranosyl)-(1→4)-*O*-α-D-glucopyranosyl-(1→4)-*O*-α-D-glucopyranosyl-

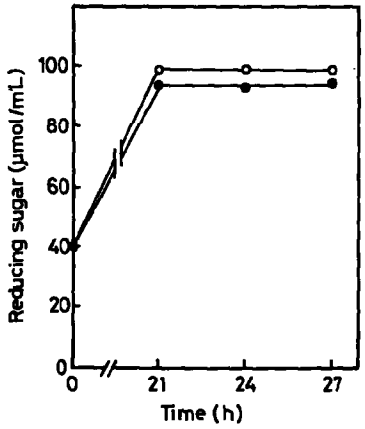

Fig. 1. Degradation of the phosphorylated malto-oligosaccharide with three concentrations of *Aspergillus oryzae* alpha-amylase. The phosphorylated malto-oligosaccharide prepared by the hydrolysis of potato starch, as described in the text, was degraded in 2mM $CaCl_2$ at pH 5.5 and 45° with 5.3 (●), 7.9 (○), and 10.6 (○) U of the enzyme per mg of substrate.

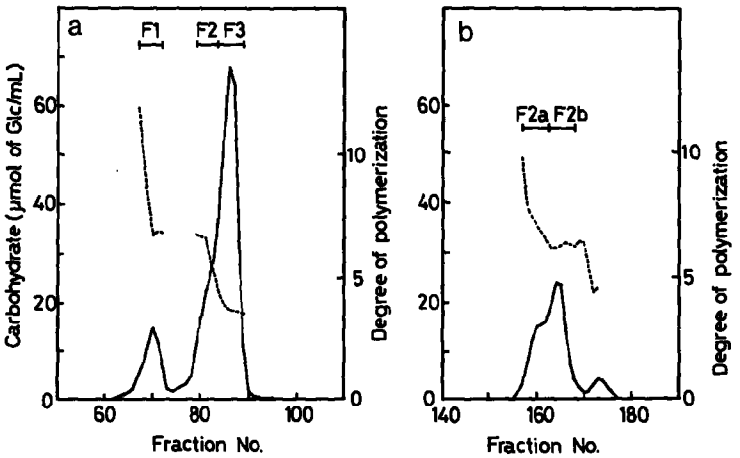

Fig. 2. Elution of (*a*) the products of degradation of the phosphorylated malto-oligosaccharide with a high concentration of *A. oryzae* alpha-amylase (7.9 U of the enzyme per mg of substrate for 27 h) from a column (2.6 × 100 cm) of Bio-Gel P-2 with 0.2M NaCl (3-mL fractions) (neutral sugars were eluted near fraction 100), and (*b*) fraction F2 from (*a*) (1.5-mL fractions); ——, carbohydrate concentration; ------, d.p.

(1→4)-D-glucopyranose (3^4-phosphorylmaltohexaose, and F2b was an ~1:1 mixture of *O*-α-D-glucopyranosyl-(1→4)-*O*-(3-phosphoryl-α-D-glucopyranosyl)-(1→4)-*O*-α-D-glucopyranosyl-(1→4)-*O*-α-D-glucopyranosyl-(1→4)-D-glucopyranose (3^4-phosphorylmaltopentaose) and *O*-α-D-glucopyranosyl-(1→4)-*O*-α-D-glucopyranosyl-(1→4)-*O*-(3-phosphoryl-α-D-glucopyranosyl)-(1→4)-*O*-α-D-glucopyranosyl-(1→4)-D-glucopyranose (3^3-phosphorylmaltopentaose), since glucoamylase left a D-glucose residue attached to the 3-phosphorylated residue[4] and h.p.l.c. of the

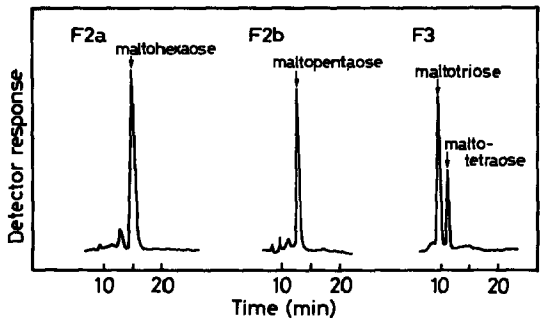

Fig. 3. H.p.l.c. of dephosphorylated F2a, F2b, and F3 (see Methods).

products of hydrolysis of F2b with glucoamylase revealed glucose, maltotetraose, and maltopentaose in the molar ratios 1:1:1.07 (Fig. 4). The results of analyses of the non-reducing terminal residue and phosphate group of F3 and h.p.l.c. indicated F3 to be a mixture of maltotetraose and maltotriose phosphorylated at positions 6 with ~70% of the non-reducing terminal residue phosphorylated. Treatment of F3 with glucoamylase increased the phosphorylated, non-reducing terminal residue from 0.69 to 0.90 mol and released 0.23 mol of glucose. These results implied that

TABLE I

PROPERTIES AND STRUCTURES OF PHOSPHORYLATED MALTO-OLIGOSACCHARIDES PRODUCED FROM POTATO STARCH WITH *Aspergillus oryzae* ALPHA-AMYLASE

	F1	*F2a*	*F2b*	*F3*
	(mol/mol)			
D.p.	7.4	6.7	5.7	3.5
Non-reducing terminal residue	1.08	1.01	0.94	0.92
Organic phosphate (Po)	1.76	0.92	0.90	0.87
6-Phosphate/Po	0.60	0.03	0.04	0.97
Phosphorylated, non-reducing terminal residue	0.09	0.05	0.06	0.69
Phosphorylated, reducing terminal residue		0	0	0
Glucoamylase treatment[a]				
D-Glucose formed		1.09	0.46	0.23
Phosphorylated, non-reducing terminal residue				0.90
Structure[b]		0-0-0-0-0-0-Ø | P-3	0-0-0-0-0-Ø | P-3 0-0-0-0-0-Ø | P-3	P-6 | 0-0-0 P-6 | 0-0-0-0

[a]The phosphorylated malto-oligosaccharides were incubated with glucoamylase (7.5 U/mg of substrate) at 40° for 24 h. [b]P-3 and P-6, phosphates at positions 3 and 6, respectively: 0, D-glucosyl residue; Ø, reducing D-glucose residue; -, (1→4)-α-D-glucosidic linkage.

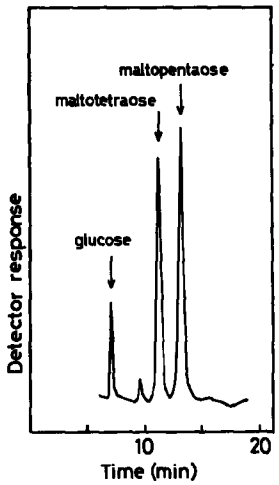

Fig. 4. H.p.l.c. of the products of hydrolysis of F2b with glucoamylase (7.5 U per mg of substrate at 40° for 24 h). The samples for h.p.l.c. were prepared as described in ref. 4.

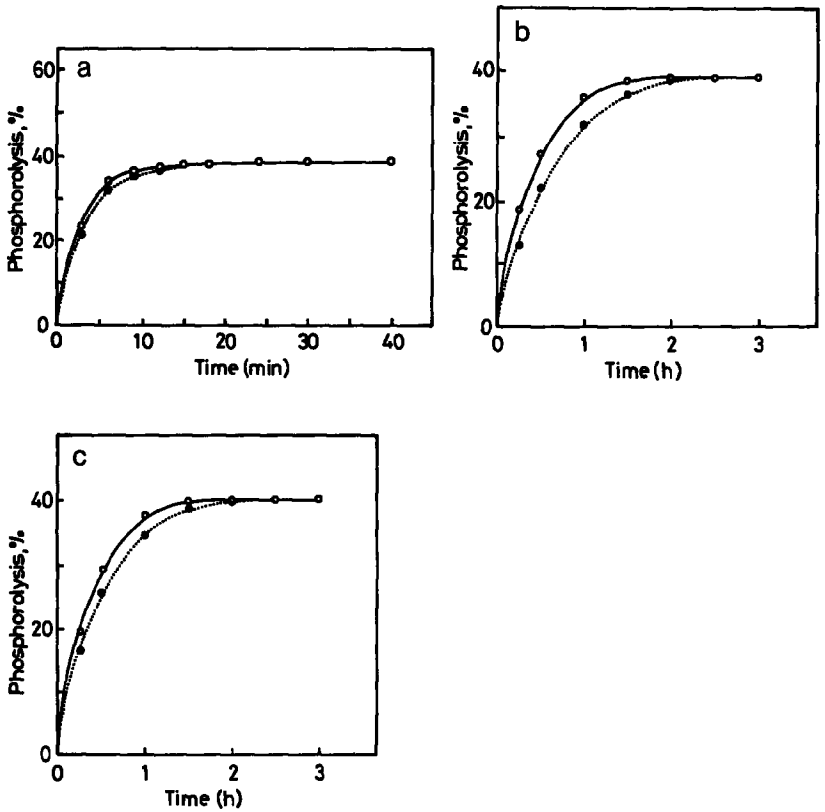

Fig. 5. Degradation (see Experimental) of the 6-phosphorylglucan with (a) potato phosphorylase, 42 (●) and 84 U/mg (○); (b) rabbit muscle phosphorylase *a*, 2100 (●) and 4200 U/mg (○); (c) rabbit muscle phosphorylase *b*, 550 (●) and 1100 (○) U/mg of substrate.

TABLE II

HYDROLYSES OF F2A AND F2B WITH A LARGE EXCESS OF *Aspergillus oryzae* ALPHA-AMYLASE[a] AND GLUCOAMYLASE

Order of hydrolyses with enzymes	*F2a*	*F2b*
	(glucose formed, mol/mol)	
Alpha-amylase	0.77	0.33
Alpha-amylase → glucoamylase	1.01	0.48
Linkage hydrolyzed with alpha-amylase (↑)	$\bar{B}\bar{A}ABC$ 0–0–0–0–0–Ø ↑ | P-3	$\bar{A}ABC$ 0–0–0–0–Ø | P-3 $\bar{B}\bar{A}AB$ 0–0–0–0–Ø ↑ | P-3

[a]The phosphorylated malto-oligosaccharides were incubated with the enzyme (76 U/mg of substrate) at 45° for 72 h. After termination of the digestion by heating, the mixture was incubated with glucoamylase (7.5 U/mg of substrate) at 40° for 24 h.

F3 was an ~4:1 mixture of 6^3-phosphorylmaltotriose and *O*-α-D-glucopyranosyl-(1→4)-*O*-(6-phosphoryl-α-D-glucopyranosyl)-(1→4)-*O*-α-D-glucopyranosyl-(1→4)-D-glucopyranose (6^3-phosphorylmaltotetraose). A large excess of *A. oryzae* alpha-amylase (76 U/mg of substrate) hardly hydrolysed F3, but hydrolysed F2a and F2b with difficulty (Table II) to give 0.77 and 0.33 mol of glucose, respectively, after 72 h. Subsequent limiting-hydrolysis of the alpha-amylase digests with glucoamylase increased the amount of glucose to 1.01 and 0.48 mol from F2a and F2b, respectively. These values accorded with those obtained using glucoamylase alone (Table I) and indicated that the alpha-amylase cleaved the same linkage of the non-reducing terminus as did glucoamylase[3], producing 3^4-phosphorylmaltopentaose from F2a and 3^3-phosphorylmaltotetraose from F2b.

Degradation of 6-phosphorylated (1→4)-α-D-glucan (6-phosphorylglucan) with potato and rabbit muscle phosphorylases. — Fig. 5 shows the degradation of the 6-phosphorylglucan with low and high concentrations of potato phosphorylase and rabbit muscle phosphorylase *a* and *b*. With both concentrations, the limits (39–40%, Table III) of phosphorolysis were attained after 15-min incubation for potato phosphorylase and after incubation for ~2 h with muscle phosphorylase *a* and *b*. No further phosphorolysis occurred on prolonged incubation even though the enzyme was still active. Thus, the phosphorylases could not by-pass the 6-phosphorylated residue. The phosphorolysis limits (Table III) were lower than the glucoamylolysis limit (48 ±1%), implying that the limiting action of phosphorylases left several D-glucosyl residues attached to the 6-phosphorylated residue on the non-reducing side since hydrolysis by glucoamylase continued until no D-glucosyl

TABLE III

ACTIONS OF POTATO AND RABBIT MUSCLE PHOSPHORYLASES ON 6-PHOSPHORYLATED (1→4)-α-D-GLUCAN (6-PHOSPHORYLGLUCAN)

	Potato phosphorylase	*Rabbit muscle phosphorylase*	
		a	b
6-Phosphorylglucan			
Phosphorolysis limit (%)	39 ±1 (7)[a]	39 ±0.5 (4)	40 ±1 (9)
Number of D-glucosyl residues per molecule	3.6 ±0.8	3.6 ±0.6	3.2 ±0.8
Partially degraded 6-phosphorylglucan			
Phosphorolysis limit (%)	3.4 ±0.2 (6)	3.3 ±0.1 (4)	4.1 ±0.2 (7)
Number of D-glucosyl residues per molecule	3.2 ±0.2	3.2 ±0.2	3.0 ±0.2

[a]Mean ±s.d. (number of experiments)

residue remained attached to the 6-phosphorylated residue[3]. The number of D-glucosyl residues attached to the 6-phosphorylated residue after the limiting action of phosphorylases is given by

$$[(\text{glucoamylolysis limit, \%}) - (\text{phosphorolysis limit, \%})] \times \overline{\text{d.p.}} \text{ of the 6-phosphorylglucan})/100.$$

The values for potato phosphorylase and rabbit muscle phosphorylase *a* and *b* were 3.6 ±0.8, 3.6 ±0.4, and 3.2 ±0.8 (mean ±s.d.), respectively, suggesting that the phosphorylases left three or four D-glucosyl residues attached to the 6-phosphorylated residue. These somewhat indefinite results appear to be due to the high phosphorolysis limits of the 6-phosphorylglucan and their relatively high standard deviations. Therefore, the 6-phosphorylglucan was partially degraded with a small amount of potato phosphorylase, and the resulting degraded 6-phosphorylglucan was used as a substrate to minimise experimental errors. The material had a $\overline{\text{d.p.}}$ of 28, carried one phosphate group, and had a glucoamylolysis limit of 15 ±0.5% (6 experiments). The phosphorolysis limits and their standard deviations of this material (Table III) were lower than those of the original material, indicating that three D-glucosyl residues remained attached to the 6-phosphorylated residue with the limiting action of each phosphorylase.

DISCUSSION

A high concentration (7.9 U/mg of substrate) of *A. oryzae* alpha-amylase produced 3^4-phosphorylmaltohexaose, 3^4-phosphorylmaltopentase, and 3^3-phosphorylmaltopentaose (Table I) by hydrolysing the $\bar{C}$ and D, $\bar{B}$ and D, and $\bar{C}$ and C linkages, respectively, of 3-phosphorylated (1→4)-α-D-glucan (P3-glucan) (Fig. 6). The enzyme appeared to cleave these linkages at similar rates since it yielded similar amounts of these maltopentaoses. The enzyme produced two phosphoryl-

ated maltopentaoses, whereas porcine-pancreatic and *B. subtilis* alpha-amylases produced only one, namely, 3^2-phosphorylmaltopentaose and 3^3-phosphorylmaltopentaose, respectively[4], and the latter was a product also with *A. oryzae* alpha-amylase. A large excess of *A. oryzae* alpha-amylase hydrolysed the $\bar{B}$ linkages of 3^4-phosphorylmaltohexaose and 3^3-phosphorylmaltopentaose with difficulty (Table II), producing 3^4-phosphorylmaltopentaose and 3^3-phosphorylmaltotetraose, respectively. Similar cleavages of the non-reducing terminal linkage with alpha-amylases have been reported for oligosaccharides[32] and phosphorylated oligosaccharides[4]. No hydrolysis of the C linkages of 3^4-phosphorylmaltohexaose and 3^4-phosphorylmaltopentaose was observed (Table II), although the enzyme hydrolysed the C linkage of P3-glucan (Fig. 6) since it produced 3^3-phosphorylmaltopentaose. These findings suggest that the enzyme hydrolysed the C linkages of 3-phosphorylmalto-oligosaccharides having more than four D-glucosyl residues on the reducing-side chain. The enzyme hydrolysed the $\bar{B}$ and C, and $\bar{A}$ and C, linkages of 6-phosphorylated (1→4)-α-D-glucan (P6-glucan) (Fig. 6), yielding 6^3-phosphorylmaltotetraose and 6^3-phosphorylmaltotriose (Table I), respectively, in the molar ratio ~1:4. These products were the same as those with porcine-pancreatic alpha-amylase[4], and the yields of these products suggested that *A. oryzae* alpha-amylase hydrolysed the $\bar{A}$ linkage more rapidly than the $\bar{B}$ linkage of P6-glucan.

The smallest molecules formed from P3- and P6-glucans were 3^3-phosphorylmaltotetraose and 6^3-phosphorylmaltotriose, respectively, indicating that the limiting actions of the enzyme cleaved the $\bar{B}$ and C linkages of P3-glucan and the $\bar{A}$ and

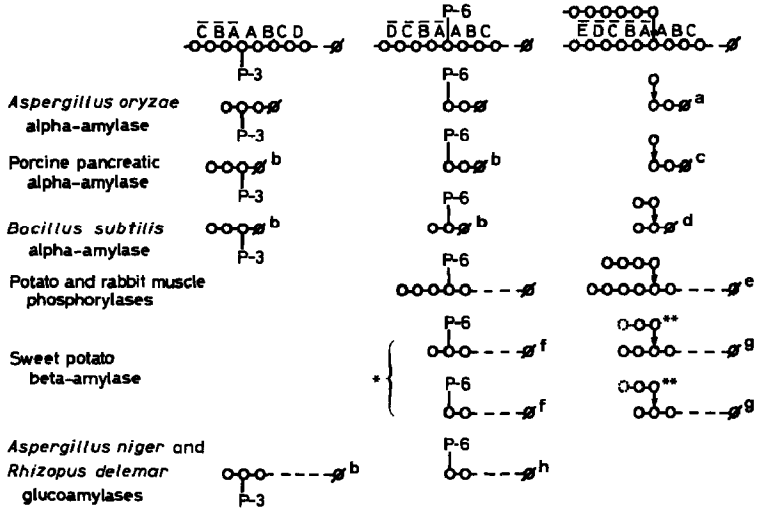

Fig. 6. Phosphorylated and branched malto-oligosaccharides a^5, $b^{4,15}$, c^5, $d^{6,7}$, e^9, f^2, g^8, and $h^{3,15}$ produced from (1→4)-α-D-glucans phosphorylated at positions 3 and 6 and from amylopectin by exhaustive degradation with various amylases and phosphorylases; *, odd and even numbers of D-glucosyl residues on the non-reducing-side chain; **, maltosyl or maltotriosyl branch; ↓, α-(1→6) linkage; see Table I for other symbols.

C linkages of P6-glucan (Fig. 6). These actions on the non-reducing-side chain coincide with those of *A. niger*[3,4] and *Rhizopus delemer*[15] glucoamylases. The limiting action of *A. oryzae* alpha-amylase on P6-glucan coincided with that of porcine-pancreatic alpha-amylase and differed from that of *B. subtilis* alpha-amylase, whereas *A. oryzae* alpha-amylase had an action on P3-glucan different from those of porcine-pancreatic and *B. subtilis* alpha-amylases[4], implying structural differences in the active sites of these alpha-amylases. All these alpha-amylases produced 6- and 3-phosphorylated malto-oligosaccharides of the same sizes, but the 6-phosphoryl products were smaller than the 3-phosphorylated products, and glucoamylase hydrolysed the $\bar{A}$ linkage of P6-glucan but not that of P3-glucan. These observations implied that the 6-phosphate is less obstructive than the 3-phosphate. This may be due to the 6-phosphate indirectly binding to the pyranose ring through a methylene group, whereas the 3-phosphate binds directly.

Rabbit muscle phosphorylase *a* and *b* degraded the 6-phosphorylglucan at a reduced rate compared to potato phosphorylase (Fig. 5), because of the lower affinity of muscle phosphorylase for amylose[33]. Potato and rabbit muscle phosphorylases were unable to by-pass the phosphorylated residue and their actions ceased at the same site on the substrate, that is, three D-glucosyl residues remained attached on the non-reducing side of the 6-phosphorylated residue. This limiting extent was larger than that of rabbit muscle phosphorylase for the main chains of branched molecules such as glycogen and amylopectin, since Walker and Whelan[9] suggested that the enzyme degrades until four D-glucosyl residues remain attached to the D-glucosyl residue carrying the branch. The limiting action of potato phosphorylase on the main chain is uncertain but may be the same as muscle phosphorylase, judging from the similarity of their limiting actions on P6-glucan. However, the action of potato phosphorylase is slow because of the lower affinity of potato phosphorylase for glycogen and amylopectin[33]. The action of phosphorylases on P6-glucan indicates that the 6-phosphate is the most obstructive for phosphorylases among (1→4)-α-D-glucan-degrading enzymes, since amylases leave one or no D-glucosyl residue attached to the 6-phosphorylated residue on the non-reducing side (Fig. 6). These differences in the action specificities of exo-enzymes such as beta-amylase[2] and glucoamylase[3,4,15] are helpful in structural analyses of phosphorylated (1→4)-α-D-glucans.

The limiting action of *A. oryzae* alpha-amylase on P6-glucan coincides with that on the main chain of a branched molecule[5] (Fig. 6), as reported for porcine-pancreatic and *B. subtilis* alpha-amylases[4], but is different from that of muscle phosphorylase in that the enzyme cleaves the $\bar{E}$ linkages of the main chains of amylopectin and glycogen but cannot cleave the $\bar{D}$ linkages. These observations suggest that the 6-phosphate is less obstructive for phosphorylase than the maltotetraosyl side-chain. Similar obstructive behaviour was observed for sweet-potato beta-amylase (Fig. 6), since the enzyme is unable to cleave the $\bar{A}$ linkage of the main chain of amylopectin with an even number of D-glucosyl residues on the non-reducing-side chain[8], whereas the enzyme can hydrolyse the $\bar{A}$ linkage of a branched

(1→4)-α-D-glucan having a D-glucosyl stub[34], as in a (1→4)-α-D-glucan having a 6-phosphate[2]. By analogy, a side chain shorter than maltotetraose, probably a D-glucosyl group, may allow phosphorylases to cleave the $\bar{D}$ linkage of the main chain, but this requires clarification. The inhibitory behavior of the 6-phosphate for amylases and phosphorylases seems to be a bulk rather than an electronegative effect as reported for porcine-pancreatic and *B. subtilis* alpha-amylases[4], and that of the 3-phosphate seems also to be a bulk effect since the hydroxyethyl group bound at C-3 of the D-glucosyl residue has a similar obstructive effect on the action of porcine-pancreatic alpha-amylase[14].

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