Carbohydrate Research, 67 (1978) 189–195

ACTION OF SWEET-POTATO BETA-AMYLASE ON PHOSPHODEXTRIN OF POTATO STARCH*

SHIRO TABATA, SUSUMU HIZUKURI, AND KAZUSHI NAGATA

Department of Agricultural Chemistry, Faculty of Agriculture, Kagoshima University, Korimoto, Kagoshima (Japan)

(Received June 21st, 1977, accepted for publication, November 17th, 1977)

ABSTRACT

The specificity of sweet-potato beta-amylase in the vicinity of the phosphate ester groups was studied by determining the structures of the phosphorylated oligosaccharides (alpha-phosphodextrin and beta-limit-alpha-phosphodextrin) formed by its action on potato starch. The beta-limit-alpha-phosphodextrin was separated by chromatography on Dowex-1 ($HCOO^-$) resin into three fractions that were distinguishable by the d.p. and by the ratio of D-glucose 6-phosphate residues to total organic phosphate. Each fraction contained linear molecules having one phosphate ester group that was not located at the reducing or non-reducing terminals. The smallest phosphodextrin was 6^2-phosphorylmaltotriose. It was deduced that beta-amylase hydrolysed (1→4)-α-D linkages from the non-reducing end until one or two D-glucosyl residues remained attached to the phosphorylated residue, depending on whether there was originally an odd or even number of glucosyl residues on the non-reducing side of the phosphorylated residue.

INTRODUCTION

It was established by Meyer *et al.* [1] and confirmed by Schoch[2] that the phosphate ester groups present in potato starch are mainly attached to the amylopectin component. The phosphate groups of amylopectin protect (1→4)-α-D-glucosidic linkages in their vicinities from the action of various amylases, in a manner similar to that of (1→6)-α branch-linkages. Therefore, phosphorylated oligosaccharides can be prepared by the hydrolysis of potato starch with alpha-amylase. Posternak[3] isolated phosphorylated maltotetraose after exhaustive hydrolysis of potato starch with pancreatic alpha-amylase. The same product was obtained when salivary alpha-amylase was used[4], and it was suggested that the phosphate groups were attached to the third D-glucosyl residue from the reducing end of the tetraose. McBurney *et al.* [5] isolated a series of phosphomalto-oligosaccharides, from tri- to penta-saccharide, by

*Studies on Starch Phosphate. Part 4.

exhaustive hydrolysis of potato amylopectin with salivary alpha-amylase We have shown[6] that the phospho-oligosaccharides obtained by exhaustive hydrolysis of potato starch with glucoamylase had d p 2–7, and that each contained one phosphate group The major portion of the phosphate in the smaller phospho-oligosaccharides was located at C-6 of a D-glucosyl residue (*i e* , P-6), whereas that in the larger molecules was located[7] at C-3 (*i e* , P-3) Both for research and industrial purposes, it is important to determine the specificities of action of various amylases in the vicinity of the phosphorylated residues in starch and the structure of the resulting phospho-oligosaccharides

We now report on the specificity of action of sweet-potato beta-amylase in the vicinity of the phosphate groups in a phosphodextrin prepared from potato starch by a near-limit hydrolysis with *B subtilis* alpha-amylase

MATERIALS AND METHODS

Preparation of alpha-phosphodextrin[8] — A suspension of commercial potato starch (200 g) in 0 1M $CaCl_2$ (2 l, pH 6 5) containing crystalline *B subtilis* alpha-amylase (4 mg) was heated to 80° and then cooled to 45° alpha-Amylase (16 mg) was added, and the solution was incubated at 45° for 24 h until the increase of reducing power had almost ceased The hydrolysis was terminated by elevation of the temperature to 100° for 15 min and the resulting precipitate was removed by centrifugation The supernatant, from which the lipids were extracted with diethyl ether (0 5 vol), was applied to a column (0 7 × 7 cm) of DEAE-Sephadex (Cl^- form) Neutral sugars were eluted with distilled water and phosphodextrins with 0 01M HCl containing 0 2M NaCl The latter eluate was neutralised to pH 5–7 with dilute sodium hydroxide and then lyophilised to give the phosphodextrin (α-PD) which is a near-limit dextrin The reducing power of α-PD was increased by 90% on incubation for 4 h with large amounts of crystalline alpha-amylase (2 5% of α-PD) and yielded the alpha-limit phosphodextrin that was not further attacked by beta-amylase

Preparation and chromatography of beta-limit-alpha-phosphodextrin — A solution of α-PD (1 76 g) in 0 02M acetate buffer (25 ml, pH 4 8) was incubated at 37° for 5 h with crystalline sweet-potato beta-amylase (2 mg) The reaction was terminated by heating the mixture in a boiling water bath for 10 min The coagulated protein was removed by centrifugation To the supernatant, 99% ethanol (5 vol) was added and the mixture was centrifuged to give beta-limit-α-PD, which was washed twice with 90% ethanol The product was not hydrolysed further on incubation for 4 h with large amounts of beta-amylase

An aqueous solution of beta-limit-α-PD (445 mg) was applied to a column (0 8 × 13 cm) of Dowex-1 ×8 ($HCOO^-$) resin which was washed thoroughly with distilled water to remove neutral sugars Elution with a linear gradient of formic acid up to 0 25M (total volume, 320 ml) then gave the fractions F-1–F-3 shown in Fig 1, based on the carbohydrate content and ratio of 6-phosphate to total organic phosphate

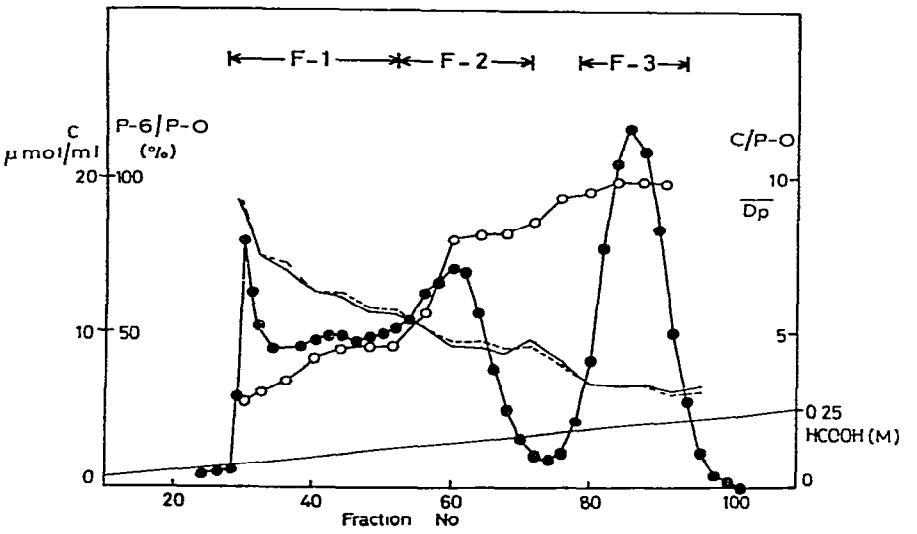

Fig 1 Chromatogram of beta-limit-α-PD on Dowex-1 ×8 ($HCOO^-$) resin —●— carbohydrate as glucose (μmol/ml) —○— 6-phosphate (P-6)/organic phosphate (P-O) (%) ———, d p per reducing end, – – – – –, d p per phosphate

Assays — D-Glucose 6-phosphate, inorganic phosphate (P_i), glucose, reducing sugars, and total carbohydrates were assayed by the methods described previously[6-8]

The non-reducing ends of phosphodextrins were determined by assaying, with a glycerokinase-NAD-dependent glycerophosphate dehydrogenase system the glycerol produced by Smith degradation[9]

Glycerol phosphate dehydrogenase, glycerokinase, D-glucose 6-phosphate dehydrogenase, acid phosphatase grade 1 (potato), and alkaline phosphatase (degree of purity 1) were obtained from Boehringer Crystalline alpha-amylase (*Bacillus subtilis*) was a gift from Nagase Sangyo Crystalline sweet-potato beta-amylase was prepared by the method described previously[10]

RESULTS AND DISCUSSION

The d p of alpha-phosphodextrin (α-PD) was found to be 6 25 by determination of the reducing and non-reducing ends and the phosphate content as shown in Table I, and α-PD was concluded to contain linear molecules each having one phosphate ester The smallest molecule formed on dephosphorylation of α-PD was maltotetraose (Fig 2), although the major products were maltopentaose, maltohexaose, and maltoheptaose α-PD is probably also heterogeneous in the location of the phosphate group The possible structure of the phosphomaltopentaose and the phosphomaltohexaose is discussed below

The data in Fig 1 show that the beta-limit-α-PD contains molecules of various sizes The phosphorus contents of fractions F-1, F-2, and F-3 were 20, 28, and 52%,

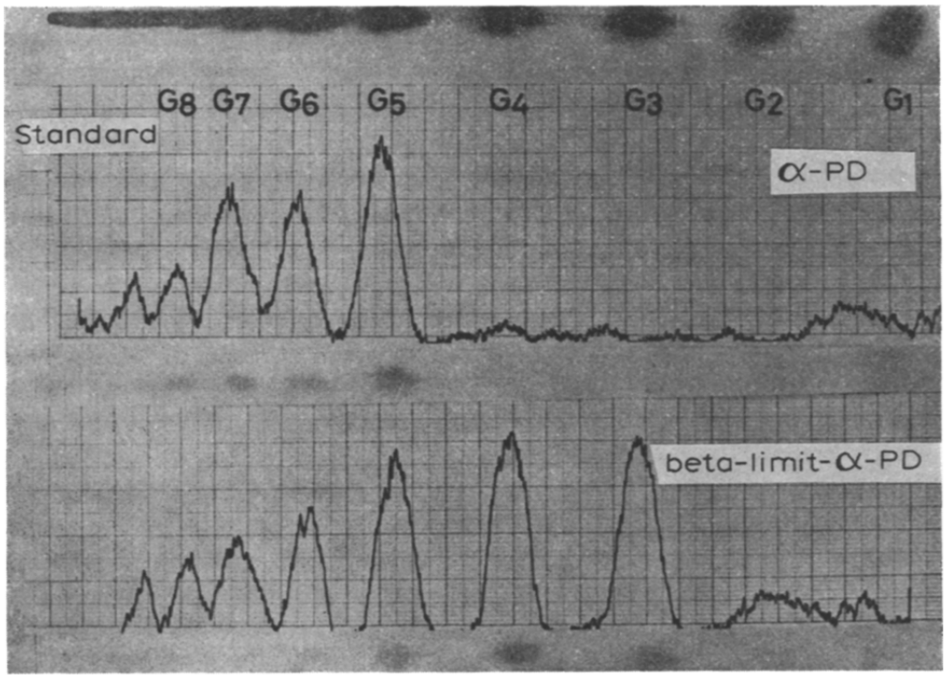

Fig 2 Paper chromatogram of dephosphorylated α-PD and beta-limit-α-PD, phosphodextrin (~1 μmol) was incubated with 1 5 units of alkaline phosphatase at 37° for 15 h in 0 1M ethanolamine–HCl buffer (100 μl, pH 10 0) containing mM $MgCl_2$ and mM $ZnCl_2$ A portion (5 μl, ~50 μg as glucose) of this incubate was subjected to descending p c (3 developments) on Toyoroshi No 51 paper with 1-propanol–water (7 3) at room temperature and detection with alkaline silver nitrate[11] after digestion with glucoamylase[12]

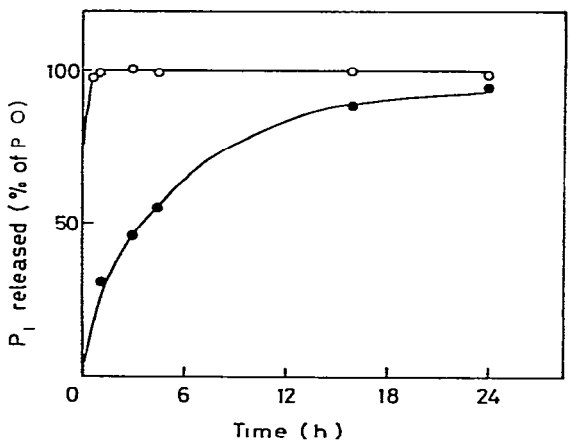

Fig 3 Liberation of inorganic phosphate (P_i) from F-3, phosphodextrin (2 μmol) in 0 05M citrate buffer (pH 5 9) was hydrolysed with 1 2 units of acid phosphatase —●— F-3 —○—, Glc-6-P

respectively, and the corresponding d p values were 6 7, 5 1, and 3 2 Each fraction contained one phosphate group per reducing or non-reducing end (Table I) Fraction F-3 was detected by hydrolysis with acid phosphatase, which almost quantitatively released the phosphate during 24 h, although the rate was much lower than that for D-glucose 6-phosphate (Fig 3), and maltotriose and a trace of maltotetraose were formed

TABLE I

STRUCTURAL ANALYSES OF BETA-LIMIT-ALPHA-PHOSPHODEXTRINS

Fractions	*Number of* D-*glucose residues*			*P-6/P-O*[a] (%)
	Reducing end (mol/mol)	*Non-reducing end*	*P-O*[a]	
α-PD	6 25	6 28	6 21	67 0
beta-Limit-α-PD	5 20	5 43	5 27	69 0
F-1	6 70	6 78	6 88	42 2
F-2	5 10	5 26	5 18	65 4
F-3	3 21	3 38	3 26	99 4

[a]P-O organic phosphate, P-6 6-phosphate

TABLE II

YIELDS OF D-GLUCOSE AND D-GLUCOSE 6-PHOSPHATE FROM PHOSPHODEXTRINS BY ACID HYDROLYSIS BEFORE AND AFTER REDUCTION[a]

Phosphodextrin	*Glc*	*Glc-6-P*
	(mol/mol of dextrin)	
α-PD	5 55	0 63
Reduced α-PD	4 58	0 65
F-1	6 08	0 41
Reduced F-1	5 10	0 42
F-2	4 07	0 65
Reduced F-2	3 07	0 65
F-3	2 20	0 98
Reduced F-3	1 18	0 97

[a]Phosphodextrin (1–1 5 μmol) was reduced with 20 μmol of sodium borohydride at room temperature (until no reducing power was detected) and then hydrolysed with 0 7M HCl for 4 h

Acid hydrolysis of beta-limit-α-PD, before and after reduction with sodium borohydride, gave the same amount of D-glucose 6-phosphate (Table II), indicating hat the phosphate group was not located at the reducing terminal

Smith degradation of α-PD and fractions F-1, F-2, and F-3 gave glycerol (0 16, 0 15, 0 19, and 0 30 mol/mol of total carbohydrate as glucose, respectively), but no L-glycerol 3-phosphate Since 70% of L-glycerol 3-phosphate survives the conditions

of acid hydrolysis involved in Smith degradation, the foregoing results imply that no phosphate was located at the non-reducing terminal residues It follows that HO-6 of the middle D-glucosyl residue of the smallest phosphodextrin, F-3, must be esterified, *i e* , F-3 is 6^2-phosphoryl maltotriose

The proportions of P-6 in F-1, F-2, and F-3 were 42 2, 65 4, and 99 4%, respectively The finding that the fraction (F-3) of smallest molecular weight had the lowest proportion of P-3 is consistent with the observations on phospho-oligosaccharide obtained by the hydrolysis of potato starch with glucoamylase[6], and suggests that P-3 has a bigger obstructive effect than does P-6 on the action of alpha- and beta-amylase and glucoamylase

The main components of α-PD are maltopentaose, maltohexaose, and maltoheptaose, and those of beta-limit-α-PD are maltotriose, maltotetraose, and maltopentaose, suggesting that phosphomaltotriose and phosphomaltotetraose of beta-limit-α-PD are derived from phosphomaltopentaose and phosphomaltohexaose of α-PD, respectively

Of the five possible structures of phosphomaltopentaose (V) only V-2, V-3, and V-4 need to be considered (the arabic numeral indicates the D-glucosyl residue that is phosphorylated in relation to the reducing end, which is residue 1), since no phosphate is attached to the terminal residues 6^2-Phosphorylmaltotriose is the only maltotriose present in the beta-limit-α-PD, and no beta-limit-α-PD is further hydrolysed by beta-amylase These facts imply that the beta-amylase cleaves V-2 at linkage $\bar{B}$ in Fig 4, and does not cleave V-3 at linkage $\bar{A}$ or V-4 at linkage A if V-3 and V-4 are present Similarly, beta-amylase attacks phosphohexasaccharide, and possibly produces only phosphomaltotetraoses IV-2 and IV-3, phosphomaltohexaoses VI-2 and VI-3 are possibly present in α-PD, but VI-1 and VI-6 are absent and VI-4 and VI-5 are not hydrolysed if they are present, since no product contains a phosphorylated non-reducing end-group Thus, it is deduced that beta-amylase hydrolyses the (1→4)-α-D linkages from the non-reducing end until one or two D-glucosyl residues remain attached to the P-6 phosphorylated residue, depending on whether there was originally

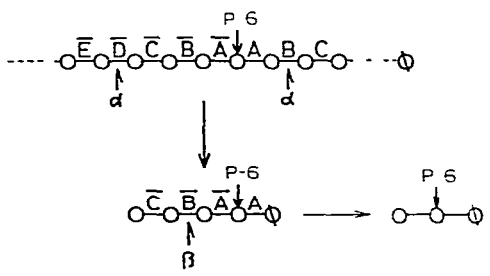

Fig 4 Hydrolysis of (1→4)-α-D-glucosidic bonds of starch in the vicinity of D-glucose 6-phosphate residues (P-6) by successive actions of alpha- and beta-amylase, and the formation of 6^2-phosphoryl-maltotriose ○, D-glucosyl residue, ∅, reducing-end D-glucose, α, first attack with alpha-amylase, β second attack with beta-amylase

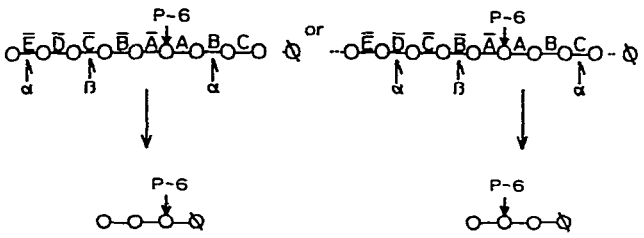

Fig 5 Possible mechanisms for the formation of phosphomaltotetraose by successive actions of alpha-amylase and beta-amylase see Fig 4, for key to symbols

an odd or even number of D-glucosyl residues on the non-reducing side of the phosphorylated residue

The cleavage of the phospho-oligosaccharide by sweet-potato beta-amylase at $\bar{B}$ and $\bar{C}$ in Fig 5 accords with the action on branched oligosaccharides having a maltosyl or maltotriosyl stub[13], but it differs from the action on branched oligosaccharides having a D-glucosyl stub at an adjacent (1→4)-γ-D linkage on the non-reducing side of the branch point[14] This result suggests that, probably due to its ionic nature, the phosphate group of phospho-oligosaccharides has a bigger blocking effect on beta-amylase than does the D-glucosyl stub of the branched oligosaccharides

6^2-Phosphorylmaltotriose is one of the main components of beta-limit-α-PD indicating that the nearest linkage to the location of ester phosphate that is attacked by *B subtilis* alpha-amylase is linkage B in starch (Figs 4 and 5)

REFERENCES

1 K H Meyer, P Bernfeld, and E Wolff, *Helv Chim Acta*, 23 (1940) 854–864
2 T J Schoch, *J Am Chem Soc*, 64 (1942) 2957–2961
3 T Posternak *J Biol Chem* 188 (1951) 317–325
4 F W Parrish and W J Whelan *Stärke* 13 (1961) 231–236
5 L J McBurney and M D Smith *Cereal Chem* 42 (1965) 161–167
6 S Hizukuri, S Tabata, and Z Nikuni *Stärke* 22 (1970) 338–343
7 S Tabata and S Hizukuri *Stärke* 23 (1971) 267–272
8 S Tabata K Nagata and S Hizukuri *Stärke*, 27 (1975) 333–335
9 S Hizukuri and S Osaki *Carbohydr Res* 63 (1978) 261–264
10 Y Takeda and S Hizukuri, *Biochim Biophys Acta*, 185 (1969) 469–471
11 W E Trevelyan D P Procter and J S Harrison *Nature (London)* 166 (1950) 444–445
12 K Kainuma and D French *FEBS Lett* 5 (1969) 257–261
13 R Summer and D French *J Biol Chem* 222 (1956) 469–477
14 K Kainuma and D French *FEBS Lett* 6 (1970) 182–186