

# EFFECT OF ESTERIFIED PHOSPHORUS IN POTATO STARCH ON THE ACTION PATTERN OF SALIVARY ALPHA-AMYLASE<sup>1</sup>

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## ABSTRACT

Potato starch and potato amylopectin were exhaustively hydrolyzed with salivary alpha-amylase for various lengths of time. The phosphorus-containing limit dextrans were isolated by anion exchange and examined by paper electrophoresis. Chemical analyses showed a decrease in the molar ratio of glucose to phosphorus as time of hydrolysis increased. Treatment with alkaline phosphatase (calf mucosa) resulted in 75% dephosphorylation of the phosphodextrans. The remaining 25% proved resistant to further phosphatase action. The dephosphorylated dextrans were identified by paper chromatography. Three members of the homologous maltodextrin series were shown to be present. These were: maltotriose, maltotetraose, and maltopentaose. This range of dextrans represents a degree of amylolysis one stage beyond that reported by other workers. A theoretical treatment, based on the known action pattern of salivary alpha-amylase, is offered to explain the formation of these limit dextrans.

It is now well established that tuber starches, and particularly potato starch, contain an appreciable amount of organically bound phosphorus (1,2), and that this phosphorus is esterified onto the carbon-6-hydroxyl of glucose (3). Most is found in the amylopectin, or branched fraction, and little with the straight-chain amylose (1,4).

Treatment of potato starch with alpha- or beta-amylase in all cases leads to a concentration of the phosphorus in the limit dextrans (4,5,6,7). These limit dextrans also contain all the alpha-(1 → 6)-, or branch point linkages (8). The only phosphorus-containing compounds which have been identified in enzyme hydrolysates of potato starch so far are linear in structure. These are: phosphomaltohexaose and phosphomaltotetraose. The former was shown to yield phosphomaltotetraose on further alpha-amylolysis (2). This latter molecule was believed to be the smallest phosphorus-bearing limit dextrin formed during alpha-amylolysis, and was fully characterized in 1961 by Parrish and Whelan (9).

The primary aim of this work was to determine more specifically the effect of esterified phosphorus on the action of salivary alpha-amylase at high concentration and for extended periods of time. This was approached by separating the phosphorus-containing alpha-limit dextrans from the nonphosphorylated dextrans. Once isolated, the

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phosphodextrins were identified by physicochemical and enzymatic procedures.

### Materials and Methods

*Materials.* Potato starch and potato amylopectin which had been prepared in a previous study in our laboratory were used (7). All samples had been exhaustively defatted, and the phosphorus contents were 0.075 and 0.168% for the whole starch and amylopectin respectively.

Salivary alpha-amylase solution was prepared by filtering human saliva through Celite and sintered glass. The resulting filtrate was free of mucin and displayed no detectable maltase or phosphatase activity when incubated with maltose or sugar phosphate solution.

*Preparative Methods.* Solutions (3%) of starch and amylopectin were digested with alpha-amylase for different lengths of time varying from 100 to 360 hr. The alpha-amylase was added initially in small aliquots to minimize precipitation of the starch in solution. When liquefaction was observable, more was added until the ratio of enzyme solution to substrate on a volume:weight basis was between 1:1 and 1.5:1.

The phosphorylated limit dextrins were separated from the neutral end products of alpha-amylolysis by anion exchange. The technique used was essentially that of Harris and MacWilliams (10). The phosphorus-containing carbohydrates were adsorbed onto a Dowex 1-X4 anion exchange resin column (13 × 1.7 cm.) and the neutral carbohydrates washed through the column with water. The phosphodextrin fraction was subsequently recovered by elution with 0.01N hydrochloric acid.

Dephosphorylation of the phosphodextrin was carried out with an alkaline phosphatase (Sigma Chemical Co. alkaline phosphatase from calf mucosa). Equal amounts of substrate and enzyme (about 1% solution) were incubated at 37°C. for 14 hr. at pH 8.66. The end products were isolated by means of a combination of anion exchange and charcoal column adsorption-elution techniques. By this means the dephosphorylated carbohydrate was separated from any remaining phosphorylated dextrin and was free of buffer salts and inorganic phosphate.

An attempt at dephosphorylation with an acid phosphatase from potato was made. The results were unsatisfactory, since this enzyme failed to dephosphorylate the phosphodextrin to any appreciable extent. Other workers have reported a similar result with acid phosphatase (10).

*Analytical Methods.* Total carbohydrate was determined by the anthrone method as modified by Dimler and co-workers (11). A modified Fiske-Subbarow technique was employed for phosphorus assay (7).

Qualitative analysis of hydrolysates was carried out by single-descent paper chromatography for 40 hr. on Whatman No. 1 paper. The irrigation solvent was n-butanol:pyridine:water, 6:4:3 (12). The position of reducing sugars was shown by reaction to alkaline 3,5-dinitrosalicylate (11).

Electrophoresis of the starch hydrolysates and the phosphodextrin fractions thereof was carried out on Whatman No. 3MM paper. The procedure followed was essentially that of Runeckles and Krotkov (13,14) for the separation of phosphate esters. The phosphate areas were located by a modified Wade-Morgan dip reagent which permits the detection of as little as 2  $\gamma$  phosphorus (13,15).

### Results and Discussion

In this study all the hydrolysates had reached the second stage of salivary alpha-amylolysis, as was shown by chromatographic examination at the termination of hydrolysis. (See Fig. 1, alpha-limit dextrans.) No maltotriose could be detected in any of the hydrolysates, this trisaccharide having been hydrolyzed to maltose and glucose. This hydrolysis constitutes the second stage of alpha-amylolysis, and studies by other workers have shown that it is a much slower, more difficult hydrolysis than the original dextrinizing process, requiring considerably more enzyme (8). The presence of isomaltotetraose (IM4) was a further indication of the extent of amylolysis. This is the smallest dextrin containing an alpha-(1  $\rightarrow$  6)- linkage known to be formed during the second stage of salivary amylolysis.

Electrophoretic examination of the hydrolysates showed that all contained a phosphodextrin component which appeared on the papergram as an elongated zone, traveling more slowly than either glucose-1- or glucose-6-phosphate. This relative rate of travel indicated that all the phosphate was bound to dextrin molecules larger than one glucose unit.

The technique of anion exchange to separate the phosphorylated carbohydrate from the neutral end products of hydrolysis was found to be quite efficient. No free sugars were detected in the phosphodextrin fraction on chromatographic examination.

The results of chemical analyses made on the phosphodextrin fractions of the hydrolysates are shown in Table I. As the length of hydrolysis was increased, there was a corresponding decrease in the molar ratio of glucose to phosphorus (from 4.7 to 4.0). This decrease was not

TABLE I  
GLUCOSE AND PHOSPHORUS CONTENTS OF PHOSPHODEXTRIN

PHOSPHODEXTRIN	ALPHA-AMYLOLYSIS	RATIO OF GLUCOSE UNITS TO PHOSPHORUS
	<i>hr.</i>	
From amylopectin	100	4.7 : 1
From whole starch	215	4.3 : 1
From amylopectin	360	4.0 : 1

great, amounting to only 0.7 glucose unit over a period of 260 hr. However, the results would seem to indicate that the phosphodextrin continues to be hydrolyzed, but only with difficulty in the latter stages of amyolysis.

Treatment of the isolated phosphodextrin with alkaline phosphatase did not result in complete dephosphorylation. By chemical analysis it was found that 25% of the original organic phosphate was still bound to a portion of the carbohydrate. This phosphodextrin was isolated and reincubated with phosphatase. It remained resistant to the action of the enzyme. This observed resistance suggests the possibility of steric hindrance such as might be provided by the presence of a branch linkage near the phosphate group. The fact that other workers have obtained small amounts of isomaltose on acid hydrolysis of their phosphodextrin would support such a conclusion (10).

The dephosphorylated carbohydrate, representing 75% of the original phosphodextrin, was examined chromatographically and the sugars present were identified by their relative mobilities on paper (Fig. 1). Three saccharides were present in quantity and readily identifiable: maltotriose, maltotetraose, and maltopentaose. There were traces of sugars of higher molecular weight, probably six and seven glucose units in size, although amounts were so small that positive identification could not be made. No glucose or maltose was detected.

The identification of a series of phosphorus-bearing alpha-limit dextrans is consistent with the observed action pattern of salivary alpha-amylase (8). Since alpha-amylase does not hydrolyze terminal bonds, it could be anticipated that in the course of random hydrolysis there would be formed not just a single phosphorus-containing limit dextrin, but several. The smallest dextrin observed in this study is a trisaccharide, and this represents a degree of amyolysis one stage beyond that reported by other investigators (2,3,9). Parrish and Whelan (9) had isolated phosphomaltotetraose from their hydrolysates and could not hydrolyze it further with alpha-amylase. They used a sensitive periodate erosion technique to characterize this limit dextrin and found the phosphate group esterified onto the third glucose unit

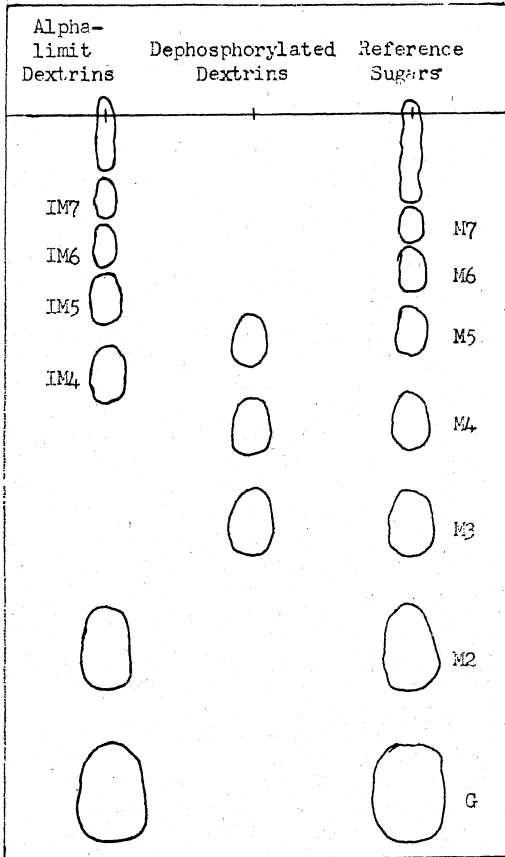


Fig. 1. Paper chromatographic fractionation of the dephosphorylated dextrin from potato starch hydrolysates. Reference sugars obtained from acid hydrolysis of potato amylose.

from the reducing end. From this evidence they concluded that it protects three adjacent alpha-(1 → 4)-bonds as depicted in Fig. 2, (a).

However, it is the interpretation of the writers, based on the observed action of alpha-amylase, that, in addition to the tetraose, there should be formed two configurations of a phosphopentaose, Fig. 2, (b) and (c), and a phosphohexaose, Fig. 2, (d). The additional alpha-(1 → 4)-glucosidic linkages in the last three structures are resistant to further enzyme action by virtue of their terminal positions.

The smallest phosphorus-bearing dextrin found was a trisaccharide, indicating that only two bonds, not three, are resistant to alpha-amylolysis, owing to the presence of an esterified group.

From Fig. 2, two sets of hypothetical limit phosphodextrins have

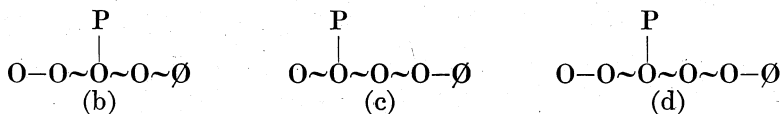
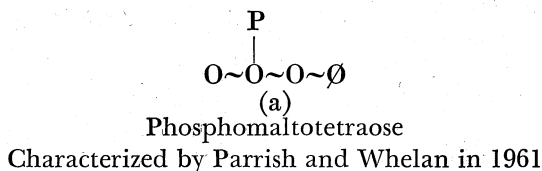


Fig. 2. Alpha-limit phosphodextrins postulated to be present after salivary alpha-amylolysis if phosphate group protects three alpha-(1 → 4)-glucosidic linkages. Key: P, phosphate group; O, glucose unit;  $\emptyset$ , reducing glucose unit;  $\sim$ , resistant alpha-(1 → 4)-bond.

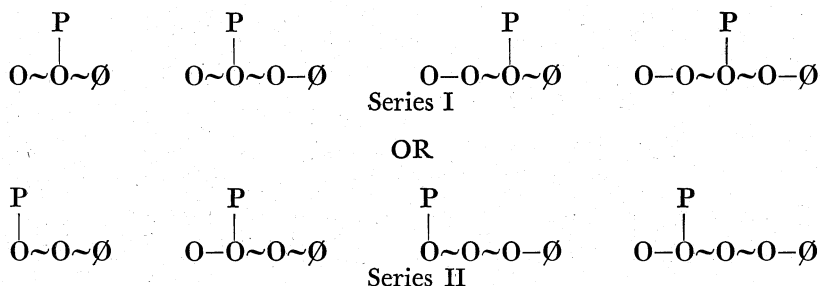


Fig. 3. Two hypothetical series of limit phosphodextrins (two alpha-(1 → 4)-glucosidic bonds resistant to alpha-amylolysis).

been constructed as shown in Fig. 3. These are the only series containing two resistant bonds per molecule which can be derived from the structures depicted in Fig. 2. All the bonds shown are resistant to further enzyme action, either by virtue of their proximity to the phosphate group or by their terminal placement. In series I the bonds on either side of the phosphorylated glucose residue are resistant to alpha-amylolysis owing to the phosphate. In series II the phosphate group protects the reducing end linkage and that next to it from enzyme action. Either of these sequences would explain the formation of the three limit dextrins in this study.

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