LOCATION AND POSSIBLE ROLE OF ESTERIFIED PHOSPHORUS IN STARCH FRACTIONS[1]

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ABSTRACT

Phosphorus determinations were made on various amelose and amylopectin fractions by a modified Fiske-Subbarow technique. Results were reproducible to ±2.5%. A positive correlation was found between the beta-amylase limits and the phosphorus content of seven potato amylose samples. Hydrolysis of amylose with beta-amylase resulted in a concentration of phosphorus in the beta-limit dextrin. The phosphate groups appear to be responsible for the incomplete hydrolysis of amylose by enzymes. A decreasing gradient of phosphorus content existed between the amylopectin (0.165%), the intermediate (0.083%), and the amylose (0.008%) fractions of potato starch. Beta- or alpha-amylolysis of waxy corn starch resulted in a concentration of its phosphorus in the limit dextrins, indicating the presence of chemically bound phosphorus. The results also suggest an association between the esterified phosphorus and the branch points which would explain the distribution of phosphorus in starch fractions.

A number of questions related to the presence of phosphorus in starch are as yet unsolved. Attempts to explain the origin of the ester-phosphate groups located at the carbon-6 hydroxyls of tuber amylopectins (15), and in smaller proportions in tuber amyloses, have been unsatisfactory. Why is most of the starch phosphorus located in the branched fraction (8,20) and why do only root, tuber, and stem starches contain chemically bound phosphorus (7)? Phosphoric acid groups in a chain can act as barriers to enzymes such as beta- and alpha-amylases (16), but it is debatable whether the small amount (0.001%) of phosphorus found in amylose is the barrier. Peat and co-workers (14) and Banks and Greenwood (2) feel that these groups are not responsible for the incomplete beta-amylolysis of amyloses, since phosphatases had no effect on the beta-amylase limits. Apparently, complete dephosphorylation was assumed. However, there is evidence that phosphatases do not effect a complete dephosphorylation of starch (11,23). Therefore the possibility still exists that phosphate groups are responsible for the incomplete hydrolysis of amylose.

The work presented here is part of an endeavor to clarify some of the questions concerning starch phosphorus. This involved an examination of the phosphorus content of various starch fractions and

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their enzymically modified dextrins by a refined assay procedure. The results throw some light on the function and location of phosphorus in starch and on the type of barrier to beta-amylase in amylose.

Materials and Methods

Starches. Potato starch was prepared from No. 1 New Brunswick potatoes by conventional procedures. It was not defatted prior to fractionation. Unmodified waxy corn starch was furnished by the American Maize-Products Co., Roby, Indiana. This was defatted with boiling 85% methanol under reflux (10). The potato amyloses had been prepared by several fractionation procedures in a previous study (18). Potato amylopectin and the corresponding intermediate fraction were prepared by the method of Lansky and co-workers (10).

The alpha-limit dextrins were isolated from a salivary alpha-amylolysate of waxy corn starch (19) by elution from a Darco G60-Celite column with 50% ethanol. The procedure for the preparation of beta-amylase-modified waxy corn starch dextrins was essentially that of Hodge and co-workers (7). Hydrolysis was stopped after 21.0, 32.0, and the limit 54.47" conversion to maltose by inactivation of the enzyme with heat. The beta-limit dextrin of potato amylose was prepared according to Neufeld and Hassid (12).

Analysis. Beta-amylase was prepared from untreated whole-wheat flour by a modification of the procedure of Kneen and co-workers (9). To ensure complete inactivation of alpha-amylase, the acid treatment was extended to a further 24 hours at 2°C. The convertibility of the amyloses by beta-amylase was measured under the same conditions employed by Peat and co-workers (14), with the exception that the amyloses were dissolved in 1*N* sodium hydroxide at room temperature. Hydrolyses were followed by the ferricyanide-ceric sulfate method of Hassid (6). The average degrees of polymerization ($\overline{DP}$) of the amyloses were determined from intrinsic viscosity (η) measurements in 1*N* potassium hydroxide at 25°C., using the viscosity-molecular weight relationship of Cowie and Greenwood (4): $(\eta) \times 740 = \overline{DP}$. The chain unit and the degree of branching of the amylopectin dextiins were determined by the periodate oxidation method of Potter and Hassid (17).

Phosphorus was assayed by the modified Fiske-Subbarow technique of Bartlett **(3).** This method shows increased sensitivity and reliability over other procedures. To make it applicable to starch, the acid digestion step was altered. Amylose (50–80 mg.), amylopectin (10–20 mg.), or starch (10–20 mg.) was weighed directly into carefully

washed (acid bath) borosilicate test tubes (2.3 × 10 cm.). Sulfuric acid (0.5 ml., 10*N*) and 0.5 ml. of distilled water were added and the tubes heated in an oven at 150°–160°C. for 1–1.5 hours until charring was complete. Approximately 10–20 drops of 30% hydrogen peroxide were then added to the cooled tubes and these were replaced in the oven for 0.5 hours. This addition of hydrogen peroxide and the reheating period were repeated until the final solution was clear.

The determination of phosphorus in starch has been difficult, owing to the low amounts of phosphorus and the high carbon-to-phosphorus ratio. Banks and Greenwood (1) reported a variation in phosphorus results of ±5% for starch and amylopectin, ancl ±15% for amylose. The present results were reproducible to ±2.7% for all samples.

Results and Discussion

In this study, the first series of phosphorus determinations were carried out on fractions prepared from nondefatted potato starch (column 1, Table I). According to other workers (10), potato starch does not contain any fatty acids or phospholipids. The results show that

TABLE I

PHOSPHORUS CONTENT OF POTATO STARCH FRACTION

SAMPLE	PHOSPHORUS	
	Untreated	Defatted
	%	%
Amylopectin	0.160	0.165
Intermediate	0.081	0.083
Amyloses		
D	0.087	0.008
F	0.073	0.015
	0.097	0.039

the phosphorus content of the amylopectin was in approximate agreement with literature values but the amyloses exhibited abnormally high phosphorus contents. Therefore, all these samples were exhaustively defatted and the phosphorus assay repeated (column 2, Table I). Although little change occurred in the phosphorus content of the amylopectin and intermediate fractions, the phosphorus values of all the amylose samples were lowered by the defatting procedure. This indicates the presence of phospholipids in potato starch which are mainly associated with the linear fraction. Furthermore, this amylose-phospholipid association is not destroyed by fractionation. Schoch and Williams (21) have presented evidence that fatty acids in corn

starch are preferentially adsorbed on the linear-chain component. As a precautionary measure, all samples were defatted after fractionation.

Since published studies on the effect of phosphate in linear chains on the course of enzyme action have been carried out on only one to three amylose samples (1,2,14), any conclusions are difficult to make. Table II shows seven amylose samples of various degrees of polymerization and different limits of beta-amylolysis, together with their respective phosphorus contents. Three samples from the literature have been included. A definite relationship was found between the percent

TABLE II

DEGREES OF BETA-AMYLOLYSIS AND PHOSPHORUS CONTENT OF POTATO AMYLOSES

SAMPLE	$\overline{DP}$	BETA LIMIT	P
		%	%
Ref. 1	2300	94.0	0.001
A	1150	78.0	0.003
Ref. 1	3200	83.0	0.004
B	1250	80.1	0.004
C	1200	74.9	0.005
D	1800	74.4	0.008
Ref. 14	....	70.0	0.009
E	800	63.2	0.014
F	2620	67.7	0.015
G	2000	53.0	0.040

phosphorus and degree of convertibility by beta-amylase. It was deemed significant that amyloses with the highest conversion into maltose by beta-amylase shoulcl have the lowest phosphorus content. The beta-limit dextrin of one of the amylose samples (F) was isolated. It represented approximately 33% of the original molecule. Phosphorus assay showed:

Original amylose (F)	0.0150% P
Beta-limit dextrin	0.0408% P

Degradation of the amylose resulted in a concentration of the phosphorus in the limit dextrin. Within experimental error, all the phosphorus was found in the beta-limit dextrin. Peat and co-workers (14) have reported a similar finding, but appear to have overlooked the significance of their results. Their potato amylose contained 0.0086% phosphorus whereas the beta-limit clextrin contained 0.0340%.

An esterified-phosphate group will apparently protect two to three glucosidic linkages in its vicinity from alpha-amylase attack (13,16). In this study, potato amylose (F) was hydrolyzed by the technique of Dimler and co-workers (5) to the achroic point with malt alpha-

amylase. The achroic dextrins consisting of four to eight unit dextrins were isolated and assayed for phosphorus:

Original amylose	0.0150% P
Achroic dextrins	0.0510% P

All the phosphorus was concentrated in the achroic dextrins, indicating that phosphate groups do impart immunity to a few glucosidic linkages in amylose from alpha-amylase.

During the fractionation of amylose sample D, the amylopectin and small amounts of an intermediate fraction were also isolated. Apparently, this intermediate fraction is not as highly branched as amylopectin and possesses longer exterior branches (25). From the present concepts of the dual synthesis of starch, it seems reasonable to assume that the intermediate fractions represent transition stages in the synthesis of one fraction from the other. Since phosphorus plays an important part in the synthesis of starch, its amount in the intermediate fraction was measured and compared to the two major fractions:

Amylopectin	0.165% P
Intermediate	0.083% P
Amylose	0.008% P

A gradient decrease in phosphorus content from amylopectin to the intermediate to amylose was found. The fact that this intermediate fraction is not only intermediate in the degree of branching but also in the phosphorus content suggests some relationship between esterified phosphorus and the branch linkages.

The phosphorus content of waxy corn starch and its beta-amylase-modified dextrins is given in Table III. As more of the outer chains

TABLE III
PHOSPHORUS CONTENT OF WAXY CORN DEXTRINS

SAMPLE	$\overline{CL}$ [a]	BRANCHING	P
		%	%
Whole starch	*20.7*	4.8	0.0025
Dextrin I	*16.1*	6.2	0.0028
Dextrin II	*14.9*	6.7	0.0033
Beta-limit dextrin	*10.9*	9.2	0.0052

[a] Average outer chain length

are removed by the enzyme, the phosphorus content of the dextrins increases, with the limit dextrin containing over double the amount of phosphorus of the parent amylopectin. Although Hodge and co-workers (7) found a similar trend in root and tuber starches, no such con-

centration was found in cereal starches. However, the present results show that waxy corn starch contains chemically bound phosphorus. Further inspection of Table III reveals a positive relationship between the percentage of branch points arid the percent phosphorus.

Why is all the phosphorus concentratecl in the interior of the amylopectin molecule? Workers (13,16) have shown that the phosphate groups are not in the immediate vicinity of the sole reducing group of amylopectin. The concentration of all the phosphorus in the beta-limit dextrin of amylopectin and the gradient of phosphorus concentration from amylopectin to the intermediate to amylose indicate that the phosphoric acicl radicals are situated in the vicinity of, or are associated in some manner with, the points of branching. Further evidence for this was obtained by examining the phosphorus content of the alpha-limit dextrins obtained from the exhaustive alpha-amylolysis of waxy corn starch. These dextrins consist of 20% of the final end-products of the alpha-amylolysis of waxy corn starch and contain all the alpha-(1→6)-linkages (24). They contained 0.0114% phosphorus as compared to 0.0025% in the original undegradecl molecule. The dextrins represent one-fifth of the amylopectin and their phosphorus content is approximately five times greater. Similar concentrations of phosphorus in amylase-limit dextrins of starches were reported in early studies from Myrback's laboratory (11). These results indicate that the phosphate groups in starch are in close proximity to the points of branching, supporting the concept of an association of starch phosphorus with the alpha-(1→6)-linkages.

If this is the explanation for the presence of phosphorus, the small amount found in amylose would signify the presence of an alpha-(1→6)-branch point in the molecule. This would explain why phosphatases could not extend the limit of beta-amylolysis of amylose (2,14). The presence of a branch point near the phosphate group would provide steric hindrance to the enzyme and prevent it from dephosphorylating the sample. The association in some way of phos phoric acid in the 6-position in starch with the formation of the alpha-(1→6)-linkages also might explain some of the results of Schwimmer and Weston (22) who found that, during the synthesis of starch, the presence of phosphate in the medium tended to suppress amylose formation in keeping the chains short. A phosphate group esterified onto the chain at this point, perhaps by a phosphatase or by the incorporation of glucose-1:6-diphosphate, might prevent further phosphorylase action. The branching enzyme, on the other hand, might show activity and insert a branch point onto the end of the chain, providing new end groups for the phosphorylase. If amylo-

pectin is synthesized from amylose in the plant, the above hypothesis would explain the high phosphorus content of the amylopectin fraction of starch.

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