THE ISOLATION AND CHARACTERIZATION OF A STARCH POLYSACCHARIDE FROM THE LEAF TISSUE OF THE APPLE TREE (MALUS MALUS)*

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In a previous investigation conducted in this laboratory (1) a starch polysaccharide, apparently identical with the water-soluble component of the common cereal and tuber starches, was isolated from the woody tissue of the apple tree (Malus malus). Thus it appears that starches of widely different origin may contain a common water-soluble polysaccharide.

Through an application of the method (2) used above a crude starch polysaccharide was obtained from the leaf tissue of the apple tree (Malus malus), which was separated into a water-soluble and a water-insoluble fraction. The water-soluble fraction was an amorphous white powder, \([\alpha]_D^{30} = +172^\circ\), which reacted with aqueous iodine-potassium iodide to give a blue color that disappeared on heating and reappeared on cooling. As this material contained minor quantities of erythrodextrin and pentosan, it was further purified by forming the iodine-iodide complex, which after precipitation with half saturated ammonium sulfate (3) was decomposed with silver nitrate. This procedure yielded an amorphous white polysaccharide which was soluble in water and formamide and possessed a specific rotation of \([\alpha]_D^{30} = +185^\circ\). An aqueous solution of the purified starch polysaccharide produced a pure blue color with the iodine-iodide reagent. Elementary

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analysis indicated a compound of type formula \((\text{C}_\text{H}_{\text{O}_6})_\text{n}\), and

as pentosans were shown to be absent, it was evident that the

polysaccharide was a polyhexosan.

Evidence as to the structural similarity of the starch polysaccharide of the apple leaf to that of the apple wood was obtained from hydrolytic studies. Dilute acid hydrolysis of the leaf starch polysaccharide yielded a solution whose reducing power, calculated as glucose, was 98 per cent of that required for a theoretical yield. The equilibrium rotation of the hydrolysate was \([\alpha]_b^{30} = +62^\circ\). The absence of \(d\)-fructose, \(d\)-mannose, and \(d\)-galactose and the exclusive presence of \(d\)-glucose in the acid hydrolysate were established by the usual methods (4). The kinetics of the hydrolysis of the leaf starch polysaccharide in 5 N sulfuric acid were studied at 80° and 90°. The constants found were \(K_{50} = 2.2 \times 10^{-2}\text{min.}^{-1}\) and \(K_{90} = 7.2 \times 10^{-3}\text{min.}^{-1}\).

Enzymatic hydrolysis of the leaf starch polysaccharide with barley malt diastase yielded a digest from which maltose was isolated in the form of its phenylosazone. The kinetics of the enzymatic hydrolysis of the leaf starch polysaccharide and a soluble potato starch polysaccharide (e.g., the \(\beta\)-amylose fraction) were studied under comparable conditions. The velocity constant for the leaf polysaccharide was \(K_{37} = 1.2 \times 10^{-3}\text{min.}^{-1}\), and for the potato polysaccharide \(K_{37} = 1.5 \times 10^{-3}\text{min.}^{-1}\). Further, it was demonstrated that the leaf starch polysaccharide and the potato starch polysaccharide were hydrolyzed at approximately identical rates, even though different diastatic enzymes were employed.

From the above it is apparent that the starch polysaccharide isolated from the leaf tissue of the apple tree is a polyglucosan, similar, if not identical, in structure to the \(\beta\)-amylose component of common cereal and tuber starches and to the starch polysaccharide previously isolated from apple wood (1).

Since the experimental portion of this study was completed, Campbell (5) and Spoehr and Milner (6) have reported the isolation of various wood and leaf "starches."1 A critical comparison

1 It is of interest to note that in 1926 Dr. R. G. L. Beazeley isolated the so-called transitory starch present in potato leaves. This work was mentioned in a lecture by Professor A. R. Ling of the Biochemistry Department of the University of Birmingham, England (7). The starch was character-
of the "starches" obtained by these investigators with our preparations is not possible, since the same criteria of characterization have not been employed. However, on the basis of existing data it appears quite probable that the leaf "starches" isolated by Spoehr and Milner are similar to the polysaccharides described in this and a previous communication (1).

EXPERIMENTAL

All analytical constants and rotations are reported on the ash- and moisture-free basis.

Preparation of Crude Starch Polysaccharide—The leaves from 8 year-old apple trees (same variety and vegetative condition) were collected on a sunny afternoon during the latter part of August, 1933. They were first subjected to a temperature of 115° under 15 pounds pressure for 10 minutes to inactivate the enzymes, and then dried rapidly in a strong current of air at 65° (8, 9). The dried tissue was ground to pass a 120 mesh sieve, and exhaustively extracted first with acetone and then with a mixture of benzene and ethanol (ratio 2:1). 6 kilos of the air-dried extracted tissue were refluxed for 45 minutes with 24 liters of 85 per cent ethanol containing 0.75 per cent nitric acid. The solids were recovered by centrifugation and again treated with the acid-alcohol reagent. This operation was repeated until two successive extractions produced solutions of the same color. The residue was then suspended in 20 liters of cold water, thoroughly stirred for 15 minutes, and centrifuged. The insoluble portion was washed with water until the washings were neutral to litmus. After spinning to partial dryness, the mass was extracted for 20 minutes with 30 liters of hot water in a steam-jacketed vessel to remove the starch polysaccharide. The insoluble leaf residue was separated from the hot solution by centrifugation and again extracted for 20 minutes with 30 liters of boiling water. The first and second aqueous extracts were combined and concentrated in vacuo (15 to 20 mm.) at 30° to 2 liters. The concentrate was filtered through

ized as an amorphous white substance, soluble in cold water, and yielded (like β-amylase) the theoretical quantity of maltose with barley diastase. Dr. Beazeley has informed us in a private communication that a complete report of this work has so far not been published.
an asbestos mat and the light yellow filtrate poured into 4 volumes of 95 per cent ethanol. The precipitated material was allowed to settle and the major portion of the supernatant liquid was removed. The remaining gelatinous suspension was made up to 5 liters with a 1:1 acetone-ethanol mixture, and allowed to stand for several days. After removing most of the acetone-ethanol solution by decantation, the starch polysaccharide slurry was diluted with acetone, collected on a suction filter, pressed dry under a rubber dam, and finally washed with dry acetone. After the cake was disintegrated, the product was dried in vacuo over calcium chloride for 4 days. After it was ground to a fine powder, it contained 11 per cent moisture. Yield, 100 gm.

Preliminary Purification of Starch Polysaccharide—80 gm. of the crude polysaccharide were shaken with 2 liters of cold water for 2 hours. The resulting dispersion was centrifuged, yielding a cloudy supernatant liquid and a mucilaginous residue. The latter was suspended in 2 liters of water, centrifuged, and the resulting aqueous extracts combined. This turbid solution was passed through a Berkefeld type N filter, and the polysaccharide recovered from the clear but slightly yellow filtrate by precipitation with ethanol and acetone. Yield, 25 gm. 20 gm. of the above product were dispersed in 1 liter of cold water, the resulting solution filtered through a type N Berkefeld filter, the polysaccharide recovered from the filtrate as above, and dried in vacuo over calcium chloride. Yield, 15 gm. The polysaccharide thus obtained contained 9 per cent moisture and 2.7 per cent ash. It was soluble in water, yielding a slightly opalescent solution. This aqueous solution on treatment with the iodine-potassium iodide reagent produced a blue color which was discharged on heating and which reappeared on cooling. The blue starch iodide color was also discharged by the addition of aqueous silver nitrate (10). The application of Small’s test for erythrodextrin (3) and Bial’s reaction for pentosan (4) indicated that these substances were still present in the polysaccharide which possessed the following constants.

Specific Rotation—\([\alpha]_D^\circ = +172^\circ \pm 2^\circ \text{ (in water, } c = 0.9 \text{ per cent).}"

Relative Viscosity—\(t/t_0 = 1.12 \text{ at } 35^\circ \text{ (in water, } c = 1 \text{ per cent).}"

Analysis—\((C_{4}H_{10}O_{5})_z\). Calculated. C 44.4, H 6.1

(Micro-Pregl) Found. " 44.0, " 5.9

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Final Purification of Starch Polysaccharide—3 gm. of the partially purified polysaccharide were dissolved in 200 cc. of warm water, to which 20 cc. of an aqueous solution containing 4 per cent iodine and 6 per cent potassium iodide were added. The starch-iodide complex was then flocculated by the addition of an equal volume of saturated ammonium sulfate (3). The resulting suspension was centrifuged, the supernatant liquid decanted, and the residue taken up in 100 cc. of water. An equal volume of saturated ammonium sulfate was then added, and the process repeated until the supernatant liquid was colorless after centrifugation. After the last washing was decanted, the precipitate was drained and suspended in 100 cc. of water. 0.1 per cent silver nitrate was then added until the blue color was discharged. The solution was filtered (Berkefeld type N) and the filtrate made up to 200 cc. Ammonia was determined on an aliquot portion and to the remainder of the solution sufficient barium acetate was added to react quantitatively with the ammonium sulfate present. The barium sulfate was removed by filtration (Berkefeld type N) and the filtrate poured with stirring into 3 liters of a 1:1 acetone-ethanol solution. The precipitate was collected by centrifugation and taken up in 50 cc. of warm water. This solution was filtered through an asbestos mat and the polysaccharide recovered from the filtrate as above. Yield, 1.6 gm. Aqueous solutions of this polysaccharide on treatment with iodine-potassium iodide produced the pure blue color characteristic of β-amylose. Erythrodextrins and pentosans were definitely absent. The following constants were observed.

Specific Rotation—\([\alpha]_2^\circ = +185^\circ \pm 2^\circ\) (in water, \(c = 0.7\) per cent).

Analysis—\((C_{44}H_{44}O_{54})_2\). Calculated. C 44.4, H 6.1

Found. " 44.6, " 5.95

Acid Hydrolysis of Starch Polysaccharide. Complete Hydrolysis—Samples of the partially purified polysaccharide, the purified polysaccharide, and potato starch were hydrolyzed with 2.5 per cent sulfuric acid by refluxing for 3.5 hours. After cooling, the hydrolysates were neutralized, made to volume, and utilized for the determination of reducing sugars and specific rotation. The values so obtained are as follows:
2.0 gm. of the partially purified polysaccharide were hydrolyzed as above and the acid hydrolysate made up to 100 cc. 50 cc. of this solution were withdrawn, adjusted to pH 4.0, and inoculated with a pure culture of *Saccharomyces cerevisiae* after the addition of an equal volume of double strength yeast water. Upon incubation for 5 days at room temperature, the solution had no perceptible action on boiling Fehling's solution, thereby indicating that the polysaccharide in question was composed of units of fermentable monosaccharides, *e.g.* d-glucose, d-fructose, or d-mannose. The remainder of the acid hydrolysate was neutralized with barium carbonate, the precipitated barium sulfate removed, and the solution concentrated *in vacuo* to a small volume. The application of Seliwanoff's reaction (4), with a small portion of the concentrate, established the absence of d-fructose. On treating the remainder of the concentrate with phenylhydrazine in the presence of acetic acid, the absence of d-mannose was indicated by the non-appearance of its insoluble hydrazone and finally from this reaction mixture d-glucosazone was isolated in good yields. The constants of this preparation are given below.

*Melting Point*—The compound melted at 205-206° with decomposition.

*Analysis*—C₁₃H₂₅O₁₉N₄. Calculated, N 15.64; found, N 15.50 (Pregl).

*Kinetics of Acid Hydrolysis*—The kinetics of the acid hydrolysis of the partially purified polysaccharide were studied at 80° and 90° in a solution which was 5 N in sulfuric acid and approximately 1 per cent in starch content. The rate of hydrolysis was followed by determining the liberated reducing groups with the volumetric method of Benedict (11). The data so obtained were reduced to a first order equation whose constants are as follows:*

2 In this and the previous communication (1) we have employed this method as a means of obtaining numerical values solely for purposes of characterization. In every case the constants presented are average values. It is not implied that the first order equation describes exactly the course of the acid hydrolysis of starch polysaccharides (12).
Kinetics of 5 x H₂SO₄ Hydrolysis of Apple Leaf Polysaccharide

<table>
<thead>
<tr>
<th>$K_{50}$ min⁻¹</th>
<th>$K_{90}$ min⁻¹</th>
<th>$K_{90}/K_{50}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\times 10^{-2}$</td>
<td>$\times 10^{-2}$</td>
<td></td>
</tr>
<tr>
<td>2.2</td>
<td>7.2</td>
<td>3.27</td>
</tr>
<tr>
<td>1.5</td>
<td>5.1</td>
<td>3.40</td>
</tr>
<tr>
<td>4.5</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Enzymatic Hydrolysis of Starch Polysaccharide. Isolation of Maltose—1 gm. of the partially purified polysaccharide was dissolved in 50 cc. of water. This solution was buffered to pH 4.5 by the addition of 5 cc. of 0.1 M sodium acetate-acetic acid. 10 cc. of a solution of barley malt diastase (1) were added, and the reaction mixture maintained at 37° for 48 hours. The digest was evaporated to dryness at 30°, taken up in hot 70 per cent ethanol, filtered, and the filtrate concentrated under diminished pressure to 10 cc. The concentrate was then treated with phenylhydrazine and maltosazone isolated in the usual manner.

Melting Point—The derivative melted at 205–206° with decomposition.
Analysis—C₂₄H₃₁O₉Na. Calculated, N 10.80; found, N 10.94

Kinetics of the Enzymatic Hydrolysis—The kinetics of the enzymatic hydrolysis of the partially purified leaf starch polysaccharide were studied at 37° in an aqueous solution buffered to pH 4.5. The hydrolysis was conducted as previously described, with barley malt diastase (1). Soluble potato starch was employed as a control. Calculated as before (1), the following constants were obtained: leaf starch polysaccharide $K_{37} = 1.2 \times 10^{-3}\text{min}^{-1}$; potato starch polysaccharide $K_{37} = 1.5 \times 10^{-3}\text{min}^{-1}$.

Determination of "Chromic Period" (13)—2 per cent solutions of the purified leaf starch polysaccharide and a soluble potato starch polysaccharide were prepared. 0.25 cc. of starch solution, 0.05 cc. of 0.1 M sodium acetate-acetic acid, and 0.1 cc. of enzyme solution were introduced into a small test-tube and the volume adjusted to 1 cc. The solution was maintained at 37°, and at intervals a drop was removed and tested against a drop of iodine-potassium iodide reagent. The time required for the disappearance of the last trace of blue color was recorded as the "chromic period." Since this property is greatly dependent upon the
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enzyme concentration as well as the substrate, the values so obtained were reduced to ratios, with the chromic period of the potato starch polysaccharide taken as unity. This procedure offers a comparison of substrates independent of enzyme variation. The values thus found are given in Table I.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Dilution</th>
<th>Relative “Chromic Period” (period for potato starch = 1)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Barley malt diastase</td>
<td>0</td>
<td>1.0</td>
</tr>
<tr>
<td>“</td>
<td>1:10</td>
<td>1.0</td>
</tr>
<tr>
<td>Taka-diastase</td>
<td>0</td>
<td>0.9</td>
</tr>
<tr>
<td></td>
<td>1:10</td>
<td>0.9</td>
</tr>
</tbody>
</table>

**SUMMARY**

A starch polysaccharide has been isolated from the leaf tissue of the apple tree (*Malus malus*). This polysaccharide was found to be a poly-glucosan, similar, if not identical, in structure to the β-amylose component of common cereal and tuber starches and to the starch polysaccharide previously isolated from the woody tissue of the apple tree.

We are indebted to Mr. Robert Harrower and Dr. Eugene Schoeffel for assistance with the analytical determinations and to Professor R. H. Roberts of the Horticulture Department for his cooperation and counsel.

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