

THE REACTION OF AMYLASES WITH STARCH GRANULES

by

YEE SIK KIM

B. S., Kansas State University
of Agriculture and Applied Science, 1957

A THESIS

submitted in partial fulfillment of the

requirements for the degree

MASTER OF SCIENCE

Department of Chemistry

KANSAS STATE UNIVERSITY
OF AGRICULTURE AND APPLIED SCIENCE

1960

L.D
2668
T4
1960
K55
c.2
Documents

TABLE OF CONTENTS

	Page
INTRODUCTION.....	1
REVIEW OF THE LITERATURE.....	2
The Structure of Starch Granules... ..	2
Degradation of Raw Starch.....	5
MATERIALS AND METHODS.....	7
Preparation of Starch.....	7
Preparation of Enzyme.....	8
Determination of Enzyme Activity.....	9
Enzyme Treatment of Starch Granules.....	10
Determination of Total Carbohydrate.....	11
Photomicrography.....	12
Fractionation.....	12
Characterization of Starch and Starch Fractions.....	15
RESULT AND DISCUSSION.....	21
ACKNOWLEDGMENT.....	45
LITERATURE CITED.....	46

INTRODUCTION

Starches from various sources have been studied by many workers for more than a century. Especially during the past 50 years, many points of detail have been clarified by the use of physical, organic and biological methods.

Starch was fractionated into its components, and their molecular weights estimated by the use of the high speed centrifuge. Application of a number of chemical methods have not only defined the chemical structure and properties of each fraction but have opened up the vast field of the industrial utilization of starch. The important physiological roles of starch in nature have been well studied by the use of biochemical methods.

However, our present knowledge on the subject is still far from complete. Although the presence of two basic components of starch, namely amylose and amylopectin has been established, their distribution and the pattern of the organization in the granules are unclear. While a precise knowledge of carbohydrase action with gelatinized starch and with the fractionated components are known in some cases, the mode of action of enzymes with raw starch granules is not well established. Nevertheless, in plants the starch is metabolized from the granule state.

The purpose of this study was to observe the mode of enzyme action on raw starch granules and to formulate theories of the structure of granules with the help of chemical and physical methods of characterization.

REVIEW OF THE LITERATURE

The Structure of Starch Granules

As early as 1716, Leewenhok (37) investigated starch granules microscopically, and thought that the granule consisted of a nucleus, which was soluble in water, and an insoluble outer hull (or shell). This idea of a granule having a special hull substance still persists (3).

When a starch granule is examined under the microscope, a shell like portion can be observed along the periphery. Furthermore, it is very difficult to disrupt starch granules completely with acid or with hot water, i.e. there are always observed hard-to-dissolve particles. These hard-to-dissolve particles are thought to be the shell material, which is different from the rest of the starch material in its structure.

Sandstedt and other workers (6) reported amylase action on raw starch granules was very slow. However, the same authors observed that the enzyme action proceeded easily when the granule was damaged, i.e. the inside of the granule was more easily hydrolyzed than the surface.

Contrary to the above evidences, Ulman (44) proposed that the hull is an artifact. The starch granule was swollen with a sodium salicylate solution and the hull substance was separated by means of an aluminum oxide column. On the column, there were no other components but amylose and amylopectin. Hence, he concluded that the native starch granule possessed no hull, but the latter was

formed during swelling by tangential motion of the starch layers and by consequent compression of the amylopectin fraction.

Earlier than that, the presence of two distinguishable groups of components was established by Meyer (26) and Samec (38). Today, the concept of the two components, amylose (a linear chain polyglycoside) and amylopectin (a branched chain polyglycoside) is widely accepted. The principal linkage in both components is α -1,4-, but at the site of branching in amylopectin the linkage is α -1,6-. Application of methods such as methylation (17) or periodate oxidation (14) enabled the characterization of each component, and physical method such as ultracentrifugation (5) or osmotic pressure (10) determined the approximate size or mass of each fraction.

Meyer in his review (27), related microscopic observations to the concept of the above chemical structure. According to him, amylopectin was primarily responsible for the frame work throughout the granule. Further, he visualized concentric layers in each of which highly ramified amylopectin molecules constituted a reticulated net work. He thought the linear portion of the branches and linear chain fraction, amylose, were generally directed in a radial direction. Meyer believed that this reticulated net work structure of amylopectin molecules provided an explanation for the swelling of starch without dissolving, and for the leachability of amylose with hot water.

In 1934, Hanson and Katz (13) microscopically observed starch granules after they were swollen with hot water and with other

chemicals, and suggested a block like structure for the granule. According to this theory, a granule consists of cubes of about 1μ which are oriented both radially and circumferentially.

More recently Nikuni and Hizukuri (30, 31) proposed a new microgranular structure of starch granules, which was similar to the theory proposed by Hanson and Katz. They observed starch granules and granule sections of 0.05μ in thickness by means of the electron microscope. According to them, the fine granular structure (microgranule) was clearly seen in graminaceae starch, especially in the granules treated with acid or amylase. For potato starch, they claimed that the microgranular structure was observed in the raw starch granule. It was distinctly observed on amyloextrin prepared by soaking potato starch in a dilute acid for several months. They suggested spheric microgranule of $200 - 300 \text{ \AA}$ in diameter which consisted of amylopectin. They thought outer branches of the amylopectin molecules formed net work between the amylopectin sphere, and amylose was present in an amorphous state and filled the spaces around the microgranules.

Pacsu (33), Dumarzert (9), and other earlier workers (11) advanced a theory that a starch granule having a homogeneous chemical structure, i.e. the amylose and the amylopectin are merely artifacts. However, today the concept of starch having two distinct fractions is so firmly established by many lines of experimental evidence that the theory of homogeneous chemical structure is almost entirely abandoned.

Degradation of Raw Starch

Enzyme degradation. In comparison with the great amount of work done on enzymic degradation of gelatinized starch, comparatively little is known about the enzyme degradation of raw starch granules.

For gelatinized starch, it is well established that α -amylase splits α -1,4- linkage between branches, whereas β -amylase splits off a maltose unit at a time from the non-reducing end of the chain, and the action is inhibited near the branching points (12).

The principal mode of action of these enzymes on raw starch granules seem to be the same as for gelatinized starch, but the action pattern appears to be somewhat modified due to the structure of the granule and the availability of the substrate.

Aspinall et al. (2) investigated starch isolated from malted barley. By potentiometric iodine titration, they found that malted barley starch contained 26 percent amylose which was higher than the amount present in unmalted barley. The starch from malted barley was fractionated into amylose and amylopectin components. Methylation end-group assay in combination with amylolysis and periodate oxidation studies indicated that the amylopectin contained the normal type of branched structure but with one non-reducing end-group per 18 glucose residues instead of 26 as in the starch from the unmalted barley. Therefore, it was concluded that during the malting of barley the amylopectin underwent enzymic attack causing shortening of the outer chains but

with retention of the branched structure, while the amylose component was relatively little degraded.

A photomicrographic study of starch granules in connection with the enzyme digestion was carried out by Sandstedt (39). He demonstrated that damaged granules were digested by amylases faster than undamaged granules. Also, within a granule its susceptibility to the enzyme action was not uniform, i.e. amorphous regions were digested faster than crystalline portions of the granule. From these observations, he suggested that the block structure proposed by Hanson and Katz (13) might be correct.

Acid degradation. Degradation of raw starch by dilute acid was tried by some workers in order to study the structure.

Kerr (21) prepared a sample of commercial acid modified thin boiling starch and fractionated it by the method of Lansky, Kool and Schoch (24). The fractions were examined for molecular weight, intrinsic viscosity, iodine affinity, alkali number and reducing value. During the acid treatment the degree of polymerization of the amylopectin fraction dropped, then slowly declined with increasing fluidity. The degree of polymerization of amylose fraction remained nearly constant at 400 - 500 until high fluidities were reached. In the initial stages of acid hydrolysis the amorphous region in the starch granule was attacked with break down of amylopectin. Degraded amylopectin formed a complex with amylose which could not be separated by the method employed and which resisted hydrolysis.

Cowie and Greenwood (8) studied the effect of 0.2 M hydrochloric acid at 45°C on the granular structure and the molecular

size of the components of potato starch. Fractionated starch components were characterized by iodine binding power, osmotic pressure, viscosity and sedimentation before and after the acid treatment. Under the condition used, the granular structure was apparently unchanged, but both the amylose and the amylopectin were partially degraded. The rate of degradation indicated that the amylopectin underwent preferential hydrolysis. It was suggested that the surface membrane of the starch granule was the primary site of attack and that the amorphous regions were more readily degraded, since there was no evidence of swelling in the acid media.

MATERIALS AND METHODS

Preparation of Starch

For the purpose of this study, a quantitative recovery of starch from sorghum grain was not necessary. However, it was desirable not to damage the starch granules during the operation. A wet milling pilot plant process devised by Johnston (20) was followed closely, except that the method was modified to the laboratory scale as follows:

About 100 g. of grain was thoroughly washed and soaked in distilled water for 24 hours. After the soaking, the grain was ground in water with mortar and pestle. When most of the starch had separated from the bran, the starch was washed through a 40 mesh cloth. The grinding and the washing were repeated three times, then finally strained through 400 mesh sieves. The crude

starch was suspended in water and allowed to settle overnight. The water was siphoned out, and the starch again suspended in water for centrifugation. After each centrifugation, the darker layer on top of the good starch layer was manually removed as cleanly as possible. After five such washings, fatty acid was extracted from the starch by Schoch's exhaustive hot methanol process (41). The starch obtained in this manner contained approximately 0.05 percent of nitrogen by the Kjeldahl method, and 0.1 - 0.2 percent of fatty acid by the standard AOAC method (1).

Preparation of Enzyme

Sorghum enzyme extract. Sorghum grain was germinated by the rag doll method used by Kneen et al. (23). It was known that germinated sorghum grain was high in α -amylase and contained a trace of β -amylase (22).

About 100 g. of grain was rolled in an unbleached satin cloth and placed in an incubator at 30°C and 85 percent relative humidity. Water was sprinkled over the dolls every 12 hours. Under these conditions, it was found that the enzyme strength per weight of meal in terms of Sandstedt's α -amylase activity units (40) was maximum at the end of three days germination. After the three day germination period, the grain was dried in the vacuum oven for 24 hours. Then the whole batch was ground in a Wiley Mill through 20 mesh sieves and stored at a low temperature until used.

For an extraction of the enzyme, 100 g. of the meal was stirred with 150 ml. of 0.1 percent calcium acetate at 30°C for five hours. The extract was centrifuged, and was dialyzed against tap water for 24 hours to remove all low molecular weight sugars. After the dialysis the extract was pervaporated to the volume desired.

β -amylase and Salivary Amylase. Throughout this work a commercial preparation of β -amylase was used. Salivary amylase from human saliva (freshly collected and filtered) was adjusted to the strength desired by diluting with distilled water.

Determination of Enzyme Activity

Throughout this study two methods were used to determine the activity of enzymes. Whenever specifically α -amylase activity was desired, the method by Sandstedt et al. (40) was used. For a comparison of activity of two different species of enzyme, i.e.

α -amylase and β -amylase, the ability to produce reducing sugar was compared by the use of Somogyi's reducing sugar analysis method (43).

The original Sandstedt et al. method expressed the activity unit as the number of grams of soluble starch which, under the influence of an excess of β -amylase, were dextrinized by one gram of malt in one hour at 30°C.

$$\text{Unit} = \frac{\text{g. of soluble starch} \times 60 \text{ (min.)}}{\text{g. of malt equiv.} \times \text{dext. time}}$$

For this study, the method of enzyme extraction was different from that of the original method, and it was considered to be

more convenient to express the activity per volume of enzyme extract rather than the weight of the meal used for extraction. However, since the rest of the procedure and the reagents were the same, the original formula for the activity could be modified simply as follows:

$$\text{Unit} = \frac{\text{g. of soluble starch} \times 60 \text{ (min.)}}{\text{ml. of enzyme extract} \times \text{dext. time}}$$

In order to determine the ability to produce reducing sugars, a given amount of enzyme was allowed to digest a 1 per cent soluble starch solution for 30 min., and the reducing sugar was estimated by Somogyi's method described in the section of β -amylase assay.

Enzyme Treatment of Starch Granules

Enzymic digestion was carried out as follows: 10 g. of starch was suspended in 20 ml. of sorghum enzyme extract prepared in the manner previously described. The enzyme activity was determined in terms of the ability to produce reducing sugars. To the starch-enzyme solution mixture was added 0.2 ml. of 1 M acetate buffer, and the mixture was covered with toluene to inhibit bacterial growth. The flask was placed in a 30°C water bath with occasional shaking. After three days of incubation, the starch was centrifuged out at 2,000 r.p.m. for 20 min. The starch was washed with water in like manner five times. Fatty acids were extracted and the starch was dried in a vacuum oven for 24 hours. The prepared starch was stored over calcium chloride for future study.

For β -amylase and salivary amylase treatments, each enzyme preparation was made identical to that of the sorghum enzyme extract in terms of reducing sugar production. The procedure thereafter was the same.

A comparison of the activity of two different species of enzyme is not readily made, but the above measures were taken to at least make the condition of digestions similar.

In order to encourage the shifting of iodine titration end points, and to depress the percent hydrolysis by β -amylase, various methods and strength of the sorghum enzyme treatments were tried. Under the following conditions the maximum shifting and the maximum depression was obtained, and this method was used for the subsequent fractionation and characterization purpose.

Fifty grams of starch were treated with 100 ml. of sorghum enzyme solution extracted from 70 g. of sorghum meal at 30°C. After 5 days treatment, again 100 ml. of fresh enzyme solution of the same strength was added to the reaction mixture, and kept for an additional 5 days under the identical condition. Thus treated starch had characteristics of 37.6 percent amylose (20 ml. of iodine solution) by iodine titration, and 40 percent hydrolysis by β -amylase assay.

Determination of Total Carbohydrate

Throughout this study two methods were used to determine the amount of carbohydrate. Whenever it was possible, the sample was weighed out directly on a dry basis. When a direct weighing was

impossible, the amount was determined by hydrolysis (28). The hydrolysis was carried out by dispersing starch in a given amount of water and to this was added concentrated hydrochloric acid to make 0.2 M acid solution. This was refluxed on a hot plate for 4 hours. The resulting hydrolyzate was adjusted to pH 7 with sodium hydroxide, and its reducing power was determined by Somogyi's method (43) with a glucose standard.

Photomicrography

Microscopic observation of starch granules often rendered some explanation of the physical structure of the starch granule. Conclusions were sometimes difficult however, because of optical effects. In the present study, observations were used as a means of determining the course of progress of the enzyme action.

A Baush and Lomb microscope was used with photographic camera and spherical lamp made by the same company. Magnifications of 210 X and 970 X with oil immersion were used for the photography. By making an exposure strip, the following conditions were found to be optimum: The distance between the light source and the sub-stage mirror was 12 in., the sub-stage diaphragm opening $3/4$, and the exposure time $1/25$ sec.

Fractionation

Ultracentrifugation. It was expected to find some changes in chemical structure of the starch components after the enzyme treatment of sorghum starch granules. In order to trace the change in the chemical structure of starch, each fraction, i.e.

amylose and amylopectin had to be characterized separately. Among various separation methods (24, 29, 41) available, the method of Montgomery and Senti (29) was thought to be the best for the purpose. Due to the nature of the method, it was not expected to yield absolutely quantitative results, but the fractionation of the raw starch standard and of the modified starch should provide a relative comparison.

This method consisted of two parts. The first part involved a pretreatment with an organic solvent, 85 percent glycerol being recommended. In doing so, the organic solvent extracted fat from the starch, and weakened the bonding between amylose and amylopectin. This was followed, in the second part, by a hot water extraction of amylose. The above authors found that about three-fourth of the total amylose could be leached out in a pure form by the first single extraction. The residue was relatively pure amylopectin after two or more additional extractions.

The glycerol used was thoroughly sparged with nitrogen gas. Five percent by weight of starch was suspended in 85 percent glycerol-water (w/w). The sparging with nitrogen gas and slow stirring were continued while the suspension was kept in a water bath, and the temperature was slowly raised from 30°C to 89°C during 1 - 1.5 hours. The suspension was kept at this temperature for an additional hour. The flask was then placed in a water pan at a temperature of 0 - 2°C to cool the contents quickly to 25°C and diluted with several volumes of ethanol. The starch was filtered on a sintered glass funnel and washed with

ethanol until free of the pretreatment solvent. The starch was dried in the oven at 35°C and 30 in. of mercury for 24 hours.

For hot water extraction, the dried starch was weighed out exactly so that the concentration of starch in the final volume was 2 percent. The suspension was adjusted to pH 6.0 - 6.3 by the use of phosphate buffer and water. The slurry was then transferred into a three-necked distillation flask of a volume three times larger than that of the starch solution and was placed in a 98°C water bath. After 15 min. of heating with constant stirring (200 r.p.m. with a finger type stirrer), the flask was placed in 0 - 2°C ice-water bath to cool it to room temperature. The solution was centrifuged in the No. 20 rotor of the Spinco ultracentrifuge at 20,000 r.p.m. (c.a. 42,000 g) for 1 hour. The upper clear liquid layer was collected and the residue was redispersed in a 98°C water bath with stirring 15 min. Then the solution was centrifuged again in the same manner. The extraction was repeated twice. All the amylose and the amylopectin were pooled into separate batches.

Butanol treatment. Crude amylose fraction obtained directly from the ultracentrifugation was also further fractionated to pure amylose, fraction A, and amylopectin, fraction B by use of butanol as follows:

The crude amylose was saturated with butanol at room temperature and kept for 2 - 3 hours. When complexing was complete and most of the amylose-butanol complex had settled on the bottom of the flask, the mixture was centrifuged at 2,000 r.p.m. for 20 min.

The sediment (fraction A) was again dissolved in hot water. These two separate fractions were placed on a hot plate and sparged with nitrogen gas until all the butanol was eliminated from the solutions.

The starch fractions thus obtained were analyzed for their concentrations by the hydrolysis method, and were immediately used for the characterization studies.

Characterization of Starch and Starch Fractions

Potentiometric iodine titration. Bates, French and Rundle (4) studied the difference in the ability of amylose and of amylopectin to bind iodine to form a complex. Solutions of amylose and of amylopectin were titrated potentiometrically with iodine, and the iodine bound in the complex was determined by measuring the potential due to free iodine in the solution after equilibrium was reached. They failed to establish a stoichiometric formula for the starch-iodine complex, but the end point of the reaction between amylose and iodine was determined experimentally. They found that amylose, which was the best amylose yet prepared, took up 18.7 percent iodine at the end point in 0.05 M potassium iodide solution. They titrated a mixture of amylose and amylopectin with 0.001 M iodine solution and found an exactly linear relationship between the number of ml. of iodine solution required to reach the end points and the amylose content.

For the studies reported here, a Leed and Northrup No. 7551 potentiometer with No. 2420 galvanometer was used. Four-hundredth of a gram of an amylopectin rich sample was dispersed in 10 ml. of

0.5 M potassium hydroxide solution. To this was added 20 ml. of water and adjusted to pH 4 with hydriodic acid. Then the sample was made up to 100 ml. The final solution was 0.001 M in iodine, 0.05 M in potassium iodide and contained 0.04 percent of starch.

The amylose content was calculated from the titration by using the following equation:

$$\% \text{ amylose} = \frac{\text{ml. of } I_2 \text{ solution for sample} \times 100}{64 (\text{ml. of } I_2 \text{ solution needed for } 0.04\% \text{ amylose})}$$

β -amylase assay. β -amylase degrades starch stepwise into maltose from the non-reducing end of the chain, and its action is inhibited by the branching point. The specificity of this enzyme action pattern has been thoroughly established, and at the present no chemical means can imitate this reaction. Therefore the β -amylase assay provides definite information on the length of the outer chains of amylopectin.

There are still some disagreements on the extent of the hydrolysis of amylose. Some workers (15) claimed complete hydrolysis of amylose into maltose, but other workers (34) working with crystalline β -amylase, reported only 70 percent hydrolysis of amylose. Today, it is generally acknowledged that β -amylase may consist of two parts and when the two parts (of which one part is called Z-enzyme) together act on amylose, it goes to 100 percent hydrolysis (35).

A commercial preparation of β -amylase (non-crystalline) was used. When the starch sample was a whole starch and was easy to bring into complete dispersion, the starch was dispersed in water

by heating as follows: Five-tenths gram of starch was suspended in about 15 ml. of water and gelatinized on an open flame with constant shaking. To this was then added 2 ml. of 1 M acetate buffer of pH 4.7 and the sample made up to 50 ml. with water. An excess of β -amylase was then added, and the solution shaken vigorously for 30 sec. Immediately 2 ml. of the mixture was taken for the zero time standard. The remainder was incubated in a 37°C water bath and 2 ml. of samples taken at the scheduled time intervals. The reaction was carried out for 4 hours, but usually the curve leveled off within 2 hours. Each sample of hydrolyzate was then analyzed for reducing sugar by Somogyi's method (43) as below: One-tenth ml. of the hydrolyzate was transferred into a Folin-Wu tube by means of a 0.1 ml. pipette. One ml. of Somogyi's reagent was added and the tube heated in a boiling water bath. At the end of 20 min. heating time, the tube was immediately placed in a cold water pan to cool the contents to room temperature. One ml. of Nelson's arsenomolybdate reagent was added and mixed thoroughly. The tube was filled to the 25 ml. mark with water, and the absorbance measured at a wave length of 520 m μ .

When the starch was already in solution, the total carbohydrate of the solution was determined by using the hydrolysis method already described. The rest of the procedure was the same as the above.

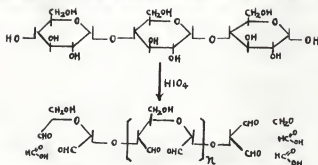
Electrophoresis. Electrophoretic techniques have been most useful for the separation of similar types of compounds with differences in the electrical charges. Northcote (32) carried out

electrophoresis studies of neutral polysaccharides by complexing them with borate and other complexing agents. He found that the mobility of borate complex was greater than any other complexing agent tried.

In order to characterize the crude amylose fraction obtained from the ultracentrifugation, electrophoresis was tried by the following method devised by the above author:

Twenty ml. of crude amylose, i.e. the supernatant of the ultracentrifugation, was placed in a dialysis bag, and dialyzed against 1000 ml. of 0.05 M borate buffer for 24 hours with constant shaking. The electrophoresis was carried out with an Aminco Electrophoresis Apparatus, at 15 m.amp. for 45 min.

Sodium metaperiodate oxidation. The first experimental oxidation of corn starch with periodic acid was investigated by Jackson and Hudson (18). Caldwell and Hixon (7), and Jackson and Hudson (19) separately carried out further studies on the oxidation process, and elucidated the mechanism of the oxidation.



The intermediate glucose units would be oxidized to dialdehyde compounds as shown, while the terminal units would give rise to three molecules of formic acids, and one molecule of formaldehyde. Also, it was found that the oxidation of starch by periodic

acid gave the identical results whether the oxidation was carried out on raw starch granules or on gelatinized starch.

Measurement of the formic acid production from a given amount of starch could be utilized for determination of the number of glucose residues per non-reducing end group. It was clear from the reaction mechanism that any linear chain starch fraction could give rise to three moles of formic acid per mole of amylose; one from the non-reducing end, and two from the reducing end. However, in case of amylopectin which has a great number of branches, the number of reducing ends are negligible in comparison with the number of non-reducing ends. Theoretically there should be only one reducing end per amylopectin molecule. Thus the amount of formic acid produced from the reducing end could be ignored.

The number of glucose residues per non-reducing end can be calculated as follows:

$$\begin{array}{l} \text{For amylose:} \\ \text{D.P. per non-reducing end} = \frac{\text{Weight of sample} \times 3}{\text{N of acid} \times 162} \end{array}$$

$$\begin{array}{l} \text{For amylopectin:} \\ \text{D.P. per non-reducing end} = \frac{\text{Weight of sample} \times 1}{\text{N of acid} \times 162} \end{array}$$

The procedure devised by Potter and Hassid (36) was closely followed. When a starch fraction was in solution, the total carbohydrate was determined by the hydrolysis method already described.

Five-tenths gram of amylose or 0.2 g. of amylopectin sample was placed in a 125 ml. erlenmeyer flask which was painted black to exclude light. Ten ml. of 3 percent sodium chloride solution was added and cooled to 0 - 2°C, after which 0.37 M of sodium metaperiodate was added. The flask was kept in dark at 0 - 2°C for

25 hours with constant shaking.

Colorimetric study of starch-iodine complex. It has been known for a long time that starch forms a blue complex with iodine. Also it has been known that pure amylose forms a much more intensely colored complex than does amylopectin. Mixtures give intensities intermediate between the two.

McCready and Hassid (25) used this property for differentiation of the two components of starch. The absorbance under a given set of conditions, i.e. $25 \pm 0.03^{\circ}\text{C}$, 1.0 mg. of starch, 2.0 mg. of iodine, and 20 mg. of potassium iodide per 100 ml at 6800 \AA with Bausch and Lomb Spectronic 20 Colorimeter, has been called Blue Value. Today it is known that the intensity of the blue color does not exactly correlate with the length of chain, but it can be used for qualitative means for differentiation of the two components (16).

In this study, instead of taking the blue value at the given wave length, the absorbance of the complex was taken for the range of $400 \text{ m}\mu$ to $675 \text{ m}\mu$ by using a Bausch and Lomb Spectronic 20 Colorimeter. The preparation of the complex was identical to the procedures originally devised for the blue value determination.

Alkali number. The alkali-lability is an empirical test for characterizing polysaccharides. According to Schoch and Jensen (42), terminal reducing groups of glycopyranose chains produced simple acidic compounds such as formic, acetic, and lactic acids upon degradation by alkali. The same authors defined alkali number as the number of mls. of 0.1 N sodium hydroxide consumed by 1 g. of starch during a digestion in the alkali for exactly

one hour at 100°C.

The reaction is a complicated one and the assay thus is quite empirical. However, if the condition of digestion is rigidly kept constant, the alkali number which depends on the number of reducing ends, can be used as a qualitative means for noting changes in molecular weight.

In this experiment every possible effort was made to keep the digestion condition constant. Five-tenths gram of sample was suspended in 10 ml. of water placed in 8 oz. nursing bottle. To this was added 25 ml. of 0.4 N sodium hydroxide with gentle swirling for uniform gelatinization. Then 65 ml. of hot water was added. The bottle was capped and placed in a vigorously steaming sterilizer for exactly 60 min. After the digestion period, the bottle was placed in a cold water pan and 75 ml. of cold water added. Then the excess sodium hydroxide was titrated with 0.2 N sulfuric acid to pH 8 by use of a pH meter. Twenty-five ml. of 0.4 N sodium hydroxide treated identically was used as blank. The alkali number was calculated as follows:

Alkali number =

$$\frac{\text{ml. of acid for blank} - \text{ml. of acid for sample} \times \text{N.F.} \times 10}{\text{Weight of sample on a dry basis}}$$

RESULT AND DISCUSSION

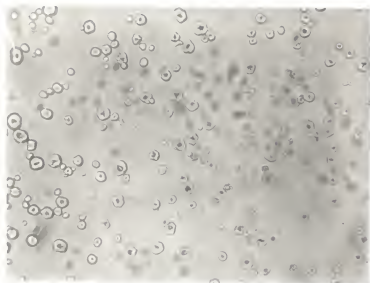
This study was started in an attempt to study the mode of degradation of starch granules during germination of sorghum grain. Sorghum grain was germinated for a week as described, and samples were taken at 24 hours intervals. Starch prepared

from these samples was examined under the microscope, and by the iodine titration method. It was found that by microscopic observations the number of granules affected by the enzyme increased as germination progressed. Also by the iodine titration, it was noticed that the end points were somewhat shifted to the right, i.e. the amylose content was increased as the germination progressed.

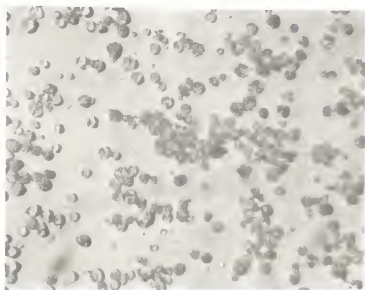
However, the changes were rather slow and barely significant. Furthermore, enzyme activity decreased after three days of germination. Hence the degradation was carried out in vitro with concentrated enzyme solutions under controlled conditions, in order to bring about drastic changes in the starch granules in a short period.

After three enzyme treatments, each of three days duration practically all granules were affected by the enzyme. Fig. 1-a shows starch granules before the treatment, and Fig. 1-b shows the granules after such treatments. Pictures of the same granules with greater magnifications and under polarized light are shown in Fig. 2-a and -b, and Fig. 3-a and -b.

As shown in Fig. 2-a, the surface and peripheries of untreated granules are perfectly smooth, while the treated granules have worm like fissures on the surfaces and saw tooth like peripheries as shown in Fig. 3-a. Under polarized light, the characteristic crosses at the centers were somewhat affected by the enzyme action, although the crosses were clearly seen (Fig. 3-b). If the theory that the crosses are caused by the inside structure of the starch

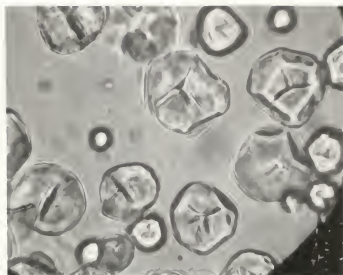


(a) Untreated starch granule 210 X



(b) Partially digested starch granules 210 X

Fig. 1 Photomicrographs of sorghum starch granules

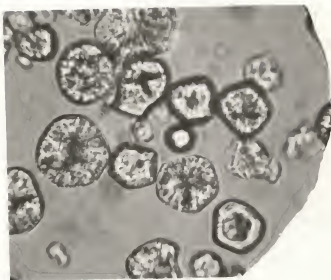


(a) Under ordinary light

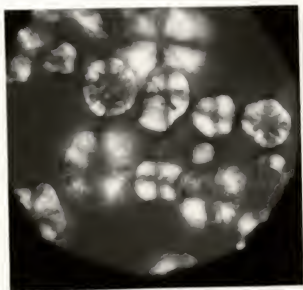


(b) Under polarized light

Fig. 2 Photomicrographs of untreated starch granules 970 X



(a) Under ordinary light



(b) Under polarized light

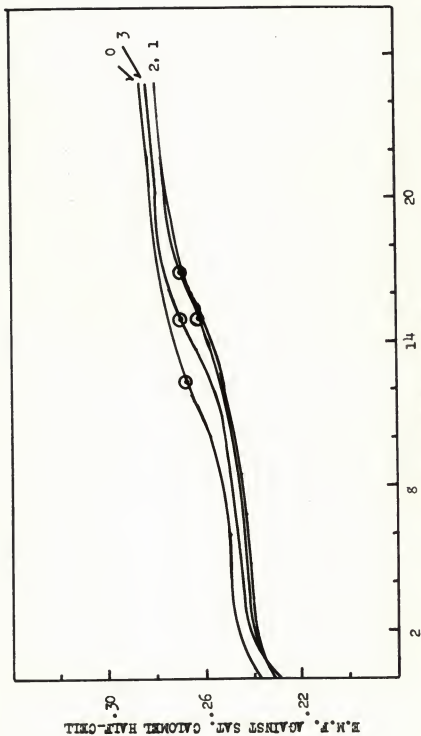
Fig. 3 Photomicrographs of partially digested starch granules 970 X

granule is true, it must mean that the enzyme action did not disrupt the crystalline pattern, or that the enzyme did not penetrate deeply into the internal portion of the granule. From these observations, it was believed that the enzyme action was a surface phenomena, and the worm like erosions were surface etchings rather than deep fissures into the center of the granule.

Degradation patterns of starch granules by other species of enzyme, i.e. β -amylase and salivary amylase were also examined in the same manner. Starch granules degraded by either species of enzyme showed very similar to those degraded by sorghum enzyme extract. One noticeable difference was that during salivary amylase digestion, small starch fragments were produced, although the mother granules kept their shapes and the erosion pattern identical to that produced by the other enzymes.

The individual fractions, namely amylose and amylopectin were also studied to ascertain whether there had been any preferential action by the enzyme. During the enzyme degradation, if any one of the fractions was preferentially digested, the net result should show up as an increase of the other fraction on iodine titration. The iodine titration method was considered to be the most sensitive and accurate means so far devised for the purpose.

In Fig. 4, 5, 6 are shown the titration curves for starches treated with sorghum enzyme extract, commercial β -amylase and salivary amylase respectively. In each Fig., 0 denotes untreated starch standard, 1 for starch treated for the first three days, and 2 for the second three days treatment and so on. The



0.001 N. IODINE SOLUTION, ML.

Fig. 4 Potentiometric iodine titration curves of untreated sorghum starch (0) and sorghum enzyme extracts treated starches (1, 2 and 3).

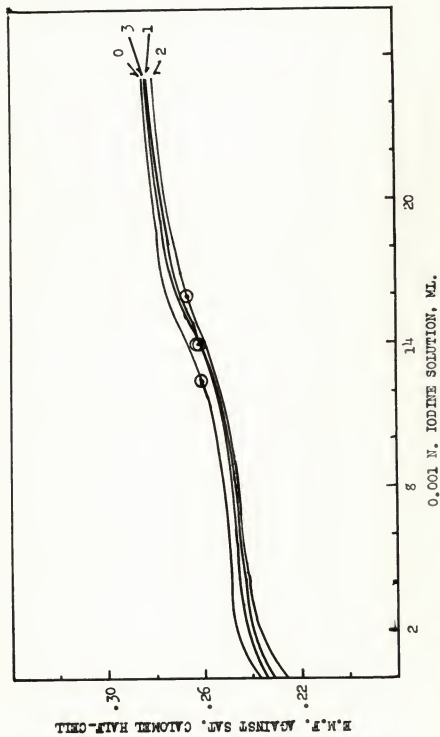


Fig. 5 Potentiometric iodine titration curves of untreated sorghum starch (0) and β -amylase treated starches (1, 2 and 3).

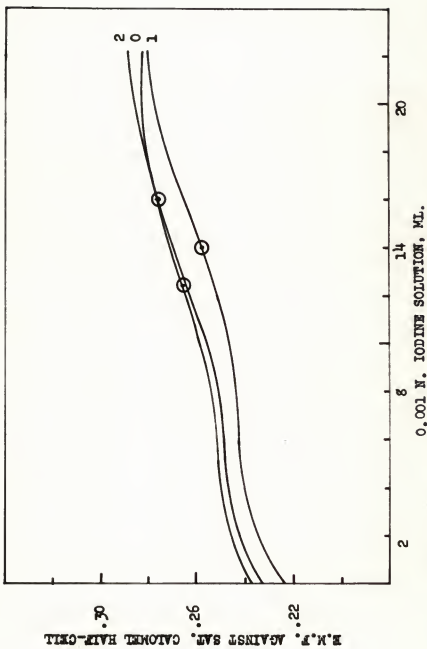


Fig. 6. Potentiometric iodine titration curves of untreated starch (1) and salivary amylase treated starches (2).

corresponding β -amylase assay curves are shown in Fig. 7, 8, and 9.

In case of sorghum enzyme extract and β -amylase, the first three days treatment brought about barely significant changes. However, at the end of the second three days treatment the end points of the titrations were shifted to the right showing about 4 percent increase in amylose content. After the third three days treatment, the end points were shifted back to the original positions. For salivary amylase, a somewhat faster action was observed. The first three days treatment shifted the end point to the right, and at the end of the second three days treatment, the end point shifted back.

β -amylase assay curves showed a similar tendency. Starches from earlier stages of degradation invariably showed high per cent hydrolysis by β -amylase. The values were lowered after the second treatment, then again the values rose as the degradation progressed.

These experimental results suggested that regardless of the enzyme species, during the early stages of the degradation, i.e. after the second three days treatment, mainly the amylopectin fraction was hydrolyzed by these enzymes. The increase of amylose could be accounted for only by a decrease in the amylopectin content. If the iodine titration data are interpreted as the sole result of preferential hydrolysis of amylopectin, the lowering of the per cent hydrolysis by β -amylase should indicate that the outer branches of amylopectin were shortened by the treatment.

In order to investigate the above idea, further, fractionation

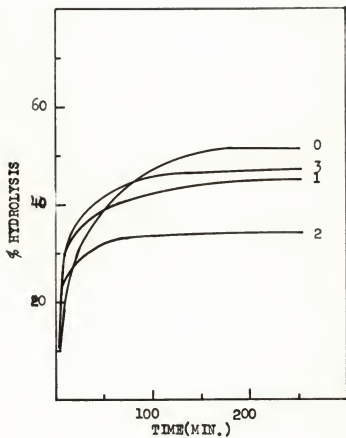


Fig. 7 β -amylase assay curves of untreated sorghum starch (0) and sorghum enzyme extract treated starches (1, 2 and 3)

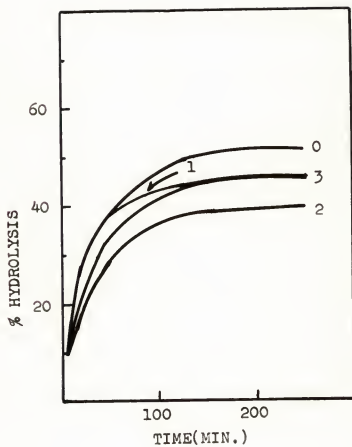


Fig. 8 β -amylase assay curves of untreated sorghum starch (0) and β -amylase treated starches (1, 2 and 3).

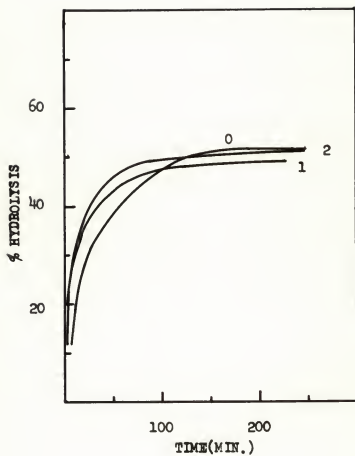


Fig. 9 β -amylase assay curves of untreated sorghum starch (0) and salivary amylase treated starches (1 and 2).

and subsequent characterization of each fraction was tried. At this stage, amylopectin from the treated starch was expected to be different from amylopectin of untreated starch if the preferential hydrolysis of amylopectin was true. However, contrary to the expectation, the enzyme modified amylopectin was found in the crude amylose fraction, i.e. the supernatant of the ultracentrifugation. This led to a new method for the separation of the modified amylopectin (or fraction B) from enzyme treated starch granules.

An untreated starch standard and the treated starch were fractionated into their component fractions by Montgomery and Senti's method (29) under identical conditions. Iodine titration curves of these fractions are shown in Fig. 10. From the curves it was clearly noted that amylopectin fractions from both starches were almost identical in iodine absorption, while the amylose fractions were not. Crude amylose from the treated starch contained about 19 percent more amylopectin than the crude amylose from the untreated starch by the iodine titration. β -amylase assay data for the treated crude amylose indicated about 25 percent lower degree of hydrolysis than those of the untreated crude amylose. Furthermore, total yield of crude amylose fraction from the treated starch was about 33 percent more than that from the untreated starch under identical fractionation conditions. These data indicated that a portion of the amylopectin was mixed into the amylose fraction during fractionation of the treated starch. This portion of amylopectin mixed into the amylose fraction was considered to be the enzyme modified amylopectin.

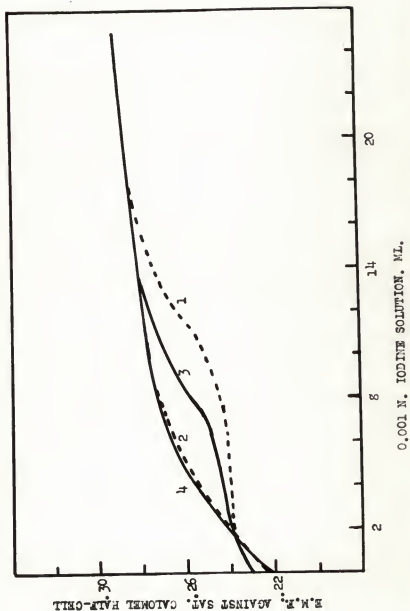
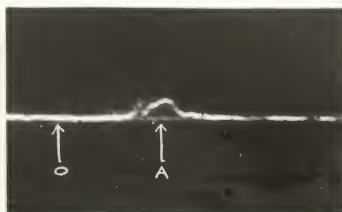


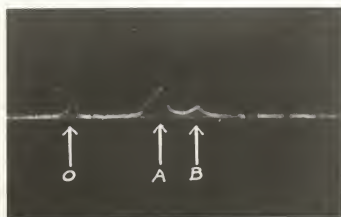
Fig. 10 Potentiometric iodine titration curves of fractions from untreated starch and sorghum enzyme extract treated starch. (1) supernatant of untreated starch (2) sediment of untreated starch (3) supernatant of treated starch (4) sediment of treated starch.

These characteristics of the crude amylose were well supported by electrophoresis studies. In Fig. 11 the electrophoretic patterns of crude amyloses from both starches are shown. The crude amylose from untreated starch did not show any significant separation after 45 min. electrophoresis (Fig. 11-a), while the crude amylose from the treated starch separated into two fractions, A and B, during the same period. Although the original authors of the method showed the fast moving fraction (fraction B in this case) was amylopectin, this was proved again by complexing amylose (fraction A in this case) with butanol as follows: When the crude amylose from the undigested starch was treated with butanol and the electrophoresis was carried out on the supernatant, no peak appeared on the scope. However, when the same was done with the crude amylose from treated starch, fraction A disappeared but fraction B remained and moved exactly the same distance as the fraction B of the mixture did.

In the Table 1 are compared other characteristic properties of the crude amylose fraction from the two starches. From the table it is clear that the amylopectin fraction from both starches have very similar characteristics in all respects. The amylose fraction, however; are very different in their properties. The degree of hydrolysis by β -amylase for the fraction from the treated starch is much lower than that from untreated starch, but the β -amylase assay value for the crude amylose from treated starch is higher than that of pure amylopectin. This can only mean that the amylopectin species (which is lower degree of



(a)



(b)

Fig. 11

- (a) Electrophoretic pattern of a supernatant from untreated starch. The peak moved from "O" to "A" in 45 min., but there was no significant separation. The unsymmetry of the peak merely indicates the inhomogeneity of amylose.
- (b) Electrophoretic pattern of a supernatant from treated starch. Under the identical condition this fraction separated into two subfractions "A" and "B".

Table 1. Comparison of the crude amylose and amylopectin fractions from partially digested and untreated sorghum starch.

Characteristic properties	Untreated starch		Treated starch	
	Crude amylose	Amylo-pectin	Crude amylose	Amylo-pectin
Amylose content (I ₂ titration)	75 %	6.2 %	56 %	6.2 %
Degree of hydrolysis by β -amylase	88 %	40.2 %	63 %	43.2 %
Degree of branching (periodate oxidation)	352	29.8	168	32.5
Alkali No.	22.0	4.8	17.9	5.6

hydrolysis than the amylose) are mixed into the amylose fraction, and depressed the resultant degree of hydrolysis by β -amylase.

The lower value of the degree of branching for the treated crude amylose can be interpreted by the same line of reasoning. When the fraction with lower number of glucose residue per non-reducing end (32 - 29 glucose units for amylopectin) is mixed into the amylose, the net value for the degree of branching has to be lowered.

Alkali number is used as an empirical method for a comparison of amylose to amylopectin. The value for the fraction from the treated starch is intermediate between the two extremes, namely, amylose and amylopectin. This too can be explained by the similar line of reasoning as the above.

The length of straight chain of each starch fraction was compared by the use of absorption spectra of starch-iodine complexes. The method too was used as an empirical means to

characterize each fraction. As is noted in the Fig. 12, a commercial preparation of amylose (0) gave a very high absorption at a relatively higher wave length region. On the other hand, amylopectin indicated a very low intensity and the location of the peak was found at a relatively lower wave length region. It is very interesting to notice that the crude amylose from the digested starch showed lower intensity and the peak at a shorter wave length region compared with those amyloses from the untreated starch. Again the spectra of the amylopectins from both starches are identical. The lower absorbance and the location of the peak indicated that the fraction B is amylopectin which probably was somewhat modified.

Thus far the accumulated evidences indicated that the crude amylose fractionated by means of ultracentrifuge from the untreated starch contained the enzyme modified amylopectin probably different from the mother amylopectin in the length of its outer branch chain.

Direct evidences were sought by characterizing the two fractions, i.e. pure amylose (or fraction A) and the modified amylopectin (or fraction B). It was already shown that these two fractions could be separated by complexing fraction A with butanol. The characteristic properties of these two fractions are shown in the Table 2.

The alkali number and β -amylase assay of the fraction A showed this fraction was identical with the crude amylose obtained from the untreated starch. The number of glucose residue per

Fig. 12 Spectra of iodine complexes with various starch fractions.

- 0: Commercial preparation of amylose
- 1: Amylose from untreated starch
- 2: Amylopectin from untreated starch
- 3: Amylose from treated starch
- 4: Amylopectin from treated starch
- 5: Fraction A
- 6: Fraction B

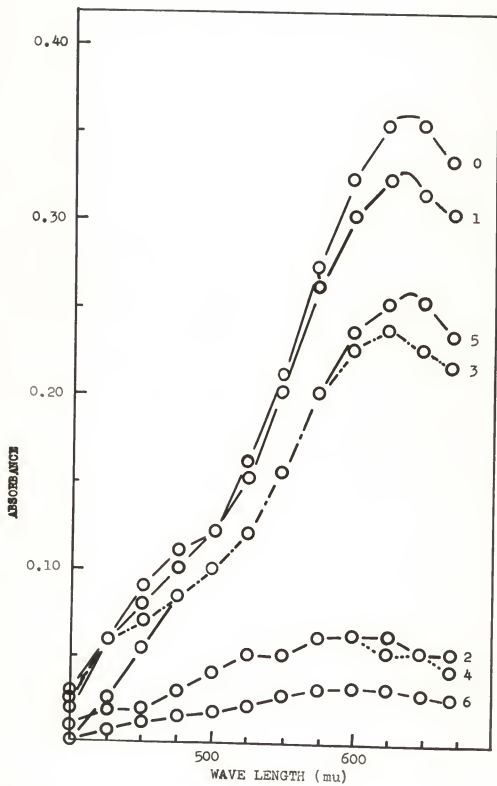


Table 2. Comparison of A and B fractions obtained from partially digested sorghum starch.

Characteristic properties	: : Fraction A	: : Fraction B
Degree of hydrolysis by β -amylase	89.5 %	26.4 %
Degree of branching (periodate oxidation)	289.0	20.6
Alkali number	21.6	7.6

non-reducing end was somewhat lower than that of untreated starch. In fact about 63 glucose residues were split off from the chain under the given condition, i.e. about 18 percent amylose was hydrolyzed by the enzyme treatment, because the amylose from the untreated starch had 352 glucose residues per non-reducing end.

Alkali number of the fraction B was sufficiently low that there were no doubts about this fraction being amylopectin. On the other hand the number of glucose units per non-reducing end of the fraction B was about 10 glucose units less than that of the untreated amylopectin. The lowering of this number of glucose units per chain without modifying the molecular weight greatly could be done only by shortening the outer chain length of the amylopectin molecule. This was further supported by the β -amylase assay of the two amylopectins. Fraction B was about 22 percent lower than the untreated amylopectin in the degree of hydrolysis by β -amylase.

When the data of periodate oxidation were closely examined, the fraction B had about 10 glucose units less than that of

untreated amylopectin per chain. This means about 33 percent glucose residues per chain were split off by the enzyme treatment. This value together with the 18 percent loss in amylose fraction suggested about 15 percent net loss of amylopectin from whole starch. It was interesting to notice that iodine titration indicated about 15 percent increase in amylose after the enzyme treatment.

All the results could be summarized as follows: During the early stages of digestion of starch granules, regardless of the enzyme species used, the surfaces of the granules were extensively degraded. During the digestion period, amylopectin was digested in preference to the amylose. The mode of amylopectin degradation was such that outer branch chains of the molecule were shortened leaving the branching points unaffected.

From these findings together with theories proposed by other workers, the organization pattern of starch granules could be visualized. Since the above findings could not be explained by any known mechanism of α -amylase, which was described earlier, the mode of degradation was sought in the granular structure in terms of the availability of the two fractions. It was believed that amylose and amylopectin were so oriented in the starch granule that outer chains of amylopectin were readily available for the enzyme action.

The microscopic observation described in the earlier section in connection with the preferential digestion of amylopectin well check with the theory proposed by Ulman (44), in which he believed

the outer layer of granules were richer in amylopectin than the center. Furthermore, the amylopectin molecules inside the granules had to be so organized that the outer chains were more readily available than inner chain. In order for starch granules to be grown by the action of phosphorylase in plants, the outer chain ends had to be available on the surface of the growing body.

Finally, it was very interesting to notice that the mode of enzyme degradation of starch granules were very similar to the acid degradation (8 and 21). As was described in the literature survey section, Cowie and Greenwood believed that acid primarily attacked surface membrane of starch granules, and the amylopectin underwent preferential hydrolysis.

The study of the reaction of enzymes with starch granules is a relatively new field. At present, only the degradation aspects of sorghum starch granules were studied. Not only degradation of one type of starch but studies in reactions of other species of carbohydrases including carbohydrate synthesis enzymes, i.e. phosphorylase etc. with starches of various sources should reveal greater insights of granular structures. The use of the electron microscope and of tracer technique in combination with classical characterization method should be very helpful for the future study of this field.

ACKNOWLEDGMENT

With deepest appreciation the author wishes to express his gratitude to his major professor, Dr. P. Nordin. Throughout this work Dr. Nordin's encouragement and guidance have been invaluable.

The author is also indebted to the State of Kansas for providing funds for this project.

LITERATURE CITED

- (1) Association of Official Agriculture Chemists, Methods of Analysis. 8th Ed. Washington, D.C.: Assoc. Offic. Agr. Chem. 1955.
- (2) Aspinall, G. O., E. L. Hirst and W. McArthur.
Constitution of a modified starch from malted barley. Jour. Chem. Soc. (London), 3075-81. 1955.
- (3) Balls, A. K. and S. Schwimmer.
Digestion of raw starch. Jour. Biol. Chem. 156:203-10. 1944.
- (4) Bates, F. L., D. French and R. E. Rundle.
Amylose and amylopectin content of starches determined by their complex formation. Jour. Am. Chem. Soc. 65: 142-8. 1943.
- (5) Beckmann, C. O. and S. Q. Landis.
The molecular weight of β -amylose from corn starch by means of the ultracentrifuge. Jour. Am. Chem. Soc. 61: 1495-593. 1939.
- (6) Blish, M. J., R. M. Sandstedt and D. K. Mecham.
Action of wheat amylases on raw wheat starch. Cereal Chem. 14:605-28. 1937.
- (7) Caldwell, C. G. and R. M. Hixon.
A study of the periodic acid oxidation of starches and dextrin as a means of determining molecular size. Jour. Biol. Chem. 123:595-606. 1938.
- (8) Cowie, J. M.G. and C. T. Greenwood.
Physicochemical Studies on starches. Jour. Chem. Soc. (London), 2658-65. 1957.
- (9) Dumazart, C.
Tosylation of starch and glycogen. Bull. Soc. Chim. 32:983-7. 1950. Chem. Abst. 9904g. 1951.
- (10) Foster, J. F. and R. M. Hixon.
Solution viscosities of the amylose components of starch. Jour. Am. Chem. Soc. 65:618-22. 1943.
- (11) Fouard, E.
Sur les proprietes colloïdales de l'amidon et sur l' unite de sa constitution. Bull. Soc. Chem. Fran. 3: 1170-4. 1908. Chem. Abst. 1146. 1909.

- (12) Greenwood, C. T.
Review on carbohydrases action on starch. Advance in Carbohydrate chemistry, Vol. 11. Academic Press, N.Y. 1956.
- (13) Hanson, E. A. and J. R. Katz.
Abhandlung zur physikalischen Chemie der Starke und der Broberereitung. Zeit. Phys. Chem. A168:339-52. 1934.
Inbid. A169:135-42. 1934.
- (14) Halsall, T. G., E. L. Hirst and J. K. N. Jones.
The structure of glycogen. Ratio of non-terminal to terminal glucose residues. Jour. Chem. Soc. (London), 1399-400. 1947. Oxidation of carbohydrates by the periodate ion. Inbid. 1427-32. 1947.
- (15) Hopkins, R. H., B. Jelinek and L. E. Harrison.
The action of β -amylase on potato amylose. Jour. Biochem. 43:32-8. 1948.
- (16) Horan, F. E.
A study of the cold acid treatment of potato starches. Unpublished Ph.D. dissertation. Columbia University, N.Y. 1944.
- (17) Irvins, J. C.
Comprehensive survey of starch chemistry. R. P. Walton Co. N. Y. 1928.
- (18) Jackson, E. L. and C. S. Hudson.
Application of the cleavage type of oxidation by periodic acid to starch and cellulose. Jour. Am. Chem. Soc. 60:989-91. 1931.
- (19) Jackson, E. L. and C. S. Hudson.
The structure of the products of the periodic acid oxidation of starch and cellulose. Jour. Am. Chem. Soc. 60: 989-91. 1931.
- (20) Johnston, R. W.
The production of starch from grain sorghum. Unpublished Master's thesis, Kansas State University, Manhattan, Kansas. 1942.
- (21) Kerr, R. W.
Degradation of corn starch in the granule state by acid. Die Starke. 4:39-41. 1952.
- (22) Kneen, E.
Sorghum amylases. Cereal Chem. 22:112-34. 1945.

- (23) Kneen, E., B. S. Miller and R. M. Sandstedt.
In influence of temperature on the development of amy-
lase in germinating wheat. *Cereal Chem.* 19:11-27. 1942.
- (24) Lansky, S., M. Kooi and T. J. Schoch.
Properties of the fractions and linear subfractions
from various starch. *Jour. Am. Chem. Soc.* 71:4066-75.
1949.
- (25) McCready, R. M. and W. Z. Hassid.
The separation and quantitative estimation of amylose
and amylopectin in potato starch. *Jour. Am. Chem. Soc.*
65:1154-7. 1943.
- (26) Meyer, K. H., M. Wertheim et P. Bernfeld.
Recherches sur l'amidon, IV. *Helv. Chim. Act.* 23:865-
75. 1940.
- (27) Meyer, K. H.
The past and present of starch chemistry. *Experientia.*
8:405-20. 1952.
- (28) Meyer, K. H., G. Noelting and P. Bernfeld.
Recherches sur l' amidon, XXXVII. *Helv. Chim. Act.* 31:
103-10. 1948.
- (29) Montgomery, D. M. and F. R. Senti.
Separation of amylose from amylopectin of starch by an
extraction-sedimentation procedure. *Jour. Polymer Sci.*
28:1-9. 1958.
- (30) Nikuni, Z.
Fine structures of starch granules. Studies with
electron microscope and x-ray diffractometer. *Kagaku,*
(Tokyo). 27:283-8. 1957.
- (31) Nikuni, Z. and S. Hizukuri.
Structure of starch granules. *Mem. Inst. Sci. and Ind.*
Osaka Univ. 14:173-81. 1957.
- (32) Northcote, D. H.
Electrophoresis of some neutral polysaccharides. *Jour.*
Biochem. 58:353-8. 1954.
- (33) Pacsu, E.
Molecular structure of cellulose and starch. *Jour.*
Polymer Sci. 2:565-82. 1947.
- (34) Peat, S., S. J. Pirt and W. J. Whelan.
 β -amylase and the constitution of amylose. *Jour. Chem.*
Soc. (London). 705-13. 1952.

- (35) Peat, S., G. J. Thomas and W. J. Whelan.
Z-enzyme. Jour. Chem. Soc. (London). 722-33. 1952.
- (36) Potter, A. L. and W. Z. Hassid.
End group determination of amylose and amylopectin by
periodate oxidation. Jour. Am. Chem. Soc. 70:3488-
90. 1948.
- (37) Reichert, E. T.
The differentiation and specificity of starches in re-
lation to genera, species, etc. Carnegie Inst. Wash.
Pub. 173:362. 1913. Chem. Abst. 1071. 1915.
- (38) Samec, M.
The structural difference of starch substance which
differ in their colorimetric iodine tests. Ber. Dtsch.
Chem. Ges. 73A:85-92. 1940. Chem. Abst. 646. 1941.
- (39) Sandstedt, R. M.
Photomicrographic studies of wheat starch III, Enzyme
digestion and granule structure. Supplement to Cereal
Chem. 32:17-47. 1955.
- (40) Sandstedt, R. M., E. Kneen and M. J. Elish.
A standardized Wolgemuth procedure for α -amylase
activity. Cereal Chem. 16:712-23. 1939.
- (41) Schoch, T. J.
Fractionation of starch by selective precipitation with
butanol. Jour. Am. Chem. Soc. 64:2957-61. 1942.
- (42) Schoch, T. J. and C. C. Jensen.
A simplified alkali-lability determination for starch
products. Ind. Eng. Chem. Anal. Ed. 12:531-32. 1940.
- (43) Somogyi, M.
A new reagent for the determination of sugar. Jour.
Biol. Chem. 160:61-73. 1954.
- (44) Ulman, M.
Determination of the chemical nature of the hull of a
swollen starch grain. K el. Zeit. 150:128-34. 1957.

THE REACTION OF AMYLASES WITH STARCH GRANULES

by

YEE SIK KIM

B. S., Kansas State University
of Agriculture and Applied Science, 1957

AN ABSTRACT OF A THESIS

submitted in partial fulfillment of the

requirements for the degree

MASTER OF SCIENCE

Department of Chemistry

KANSAS STATE UNIVERSITY
OF AGRICULTURE AND APPLIED SCIENCE

1960

Although starch is metabolized from the granule state in plants, neither the mode of degradation by enzymes nor the organization of the starch molecules within the granules are clearly known. The purpose of this study was to observe the mode of enzyme action on raw starch granules and to formulate theories of the structure of granules with the help of chemical and physical methods of characterization.

Very sound sorghum starch granules were treated with different species of enzymes, i.e. sorghum enzyme extract (which is high in α -amylase and contains a trace of β -amylase), commercial preparation of β -amylase and fresh human salivary amylase. These enzyme treated starches were compared with raw starch granules under the microscope with ordinary light and polarized light. From these observations, it was believed that the action of each enzyme did not disrupt the crystalline pattern of the granules, nor did they penetrate deeply into the granules.

Enzyme treated starches were further examined by means of potentiometric iodine titration and β -amylase assay. The results from these two types of experiments clearly suggested that the outer branches of amylopectin molecules were shortened during the initial stage of the enzyme treatment.

In order to examine the above concept further, fractionation of raw starch and sorghum enzyme treated starch was carried out by use of an ultracentrifuge. The subsequent characterization methods for the supernatant (amylose) and for the sediment (amylopectin) included potentiometric iodine titration, β -amylase assay,

electrophoresis, sodium metaperiodate oxidation, colorimetric studies of the starch-iodine complex and alkali number. All the results from the above methods indicated the amylose fraction from the treated starch contained the enzyme modified amylopectin in addition to amylose. Furthermore, since the amylopectin fractions (sediment) from both starches were identical in every respect, it was believed that any change that took place during the treatment should be sought in the crude amylose fraction (supernatant) of the treated starch.

The crude amylose fraction was treated with butanol and fraction A (amylose) was separated from fraction B (the enzyme modified amylopectin). Properties of these two fractions were as follows: Fraction A: 89.5 percent hydrolysis by β -amylase, 289.0 glucose units per chain, and alkali number of 21.6. Fraction B: 26.4 percent hydrolysis by β -amylase, 20.6 glucose units per chain, and alkali number of 7.6.

All of the data indicated that amylases acting on starch granules first cause shortening of the outer chains of amylopectin with little or no effect on the amylose. These findings are not in accord with the known mechanism of α -amylase acting on gelatinized starch. It is believed that the differences can be ascribed to the organization of the granules. It is postulated that the outer chains are to be found at the surfaces which are accessible to the enzyme.