



The isolation, identification, and structural study of Ymer barley pectin  
by William A Curran

A THESIS Submitted to the Graduate Faculty in partial fulfillment of the requirements for the degree  
of Doctor of Philosophy in Chemistry

Montana State University

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Abstract:

A pectin has been isolated from Ymer barley flour. It was found to be composed of galacturonic acids linked  $\alpha$ -1 $\rightarrow$ 4 with an apparent minimum chain length of 150 units as determined by periodate oxidation. This polymer does not appear to be branched since methylation data failed to show any branching points. Associated with it, is a glucan which, on the basis of periodate oxidation, appears to have a chain length of 38 units and is linked  $\alpha$ -1 $\rightarrow$ 3. This is the first time such a polymer has been reported and the name, "nigeran", is suggested for this substance. This would replace the use of nigeran for the alternating 1 $\rightarrow$ 3, 1 $\rightarrow$ 4 polymer.

Alpha and beta amylase were both found to be effective in hydrolyzing the glucan. This was an unusual observation and warrants further study.

THE ISOLATION, IDENTIFICATION, AND STRUCTURAL STUDY  
OF YMER BARLEY PECTIN

by

WM. A. CURRAN JR.

A THESIS

Submitted to the Graduate Faculty

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partial fulfillment of the requirements

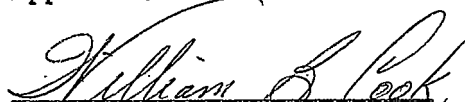
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# ABSTRACT

A pectin has been isolated from Ymer barley flour. It was found to be composed of galacturonic acids linked  $\alpha$ -1 $\rightarrow$ 4 with an apparent minimum chain length of 150 units as determined by periodate oxidation. This polymer does not appear to be branched since methylation data failed to show any branching points. Associated with it, is a glucan which, on the basis of periodate oxidation, appears to have a chain length of 38 units and is linked  $\alpha$ -1 $\rightarrow$ 3. This is the first time such a polymer has been reported and the name, "nigeran", is suggested for this substance. This would replace the use of nigeran for the alternating 1 $\rightarrow$ 3, 1 $\rightarrow$ 4 polymer.

Alpha and beta amylase were both found to be effective in hydrolyzing the glucan. This was an unusual observation and warrants further study.

## INTRODUCTION

The cereal grain which is most common to the Pacific Northwest is wheat. This is due to the relatively short growing period required and the high net returns per acre obtained by the farmer.

Now, however, due to the enormous increase in the production of wheat that was brought about by World War II, the supply has overtaken the demand and a large surplus has resulted. Because of this surplus, governmental restrictions have been made on wheat acreage and it is very likely that many more acres will be removed from wheat production as a means of bolstering wheat prices.

Barley is the cereal grain best adapted to acreage removed from wheat production. Because much of the acreage that the farmer once planted in wheat has now been planted in barley, there is a growing surplus of barley in certain areas. This surplus will increase as more and more acreage is diverted to barley.

In view of the impending barley surplus, a study was made to exploit the commercial possibilities of barley (1). During the course of the investigation, Imsande and Goering (2) found approximately 1.5% of barley flour was made up of a pectin polymer. Commercial pectin is a valuable compound selling at present for \$1.60 per pound and which is in short supply. The present investigation was initiated to determine the composition and structure of the pectin isolated from Ymer barley flour. The development of an economical process for the extraction of this pectin from barley would constitute a substantial contribution to the utilization of this grain for chemical purposes.

There exists in nature several groups of polysaccharides which are more complex than starch or cellulose. They consist of more than one type of sugar residue and the elucidation of their fine structure is dependent on the techniques developed for the investigation of starch and cellulose. Some examples of the more complex polysaccharides are pectins, gums and mucilages. There are, however, further complications which arise from the colloidal character of these polysaccharides and the close association, in nature, with other carbohydrate materials. This results in isolation and purification problems. Pectin was one of the first of this type of polysaccharide to be examined because of its relative ease of isolation, its commercial value and its importance in plant physiology (3).

Pectin occurs in practically all plant materials, especially in fruit and young plant tissues. It also has been reported to occur in wood (4), but it must be understood that the general term "pectin" is used to describe products which vary greatly in composition and possibly also in chemical constitution (3).

All pectins isolated to date give, on hydrolysis, in various proportions L-arabinose, D-galactose, and D-galacturonic acid (5). In addition, D-xylose and L-fucose have been reported in certain pectins, but the evidence is still somewhat conflicting (6), (7), (8), (9), (10).

Pectins occur naturally in both soluble and insoluble forms. Soluble pectin occurs in plant juices and insoluble pectins tend to occur in the green part of plants, in fruit and in root crops (11). This insolubility is apparently due either to the presence of calcium or magnesium or because it is combined with cellulose or some other insoluble polysaccharide (12).

The soluble form of pectin consists, in varying percentages, of the methyl ester of pectic acid, together with an associated galactan and araban. The function of the ester group is uncertain but it may be to prevent undue acidity in the plant.

The role of pectin in plant physiology is still far from clear. It has been postulated to be a cementing agent to bind cells together. In instances where these substances act as the principal binding agents, their removal naturally leads to the separation of the cells. This is the case when fruits or vegetables become overripe and also when they are decomposed by microorganisms.

Because of the apparent contradictions in the early literature, Whistler and Smart (13), (14) have listed some definitions for the clarification of the nomenclature dealing with pectic substances. In brief, the definitions are:

Pectin Substances. This is a group designation for those complex, colloidal carbohydrate derivatives which occur in or are prepared from plants and contain a large proportion of anhydrogalacturonic acid units which are thought to exist in a chainlike combination. The carboxyl groups of polygalacturonic acids may be partly esterified by methyl groups and partly or completely neutralized by one or more bases.

Pectin. The general term "pectin" (or pectins) designates those water soluble polygalacturonic acids of varying methyl ester content which are capable of forming gels with sugar and acid under suitable conditions. They may also have other polysaccharides either chemically or physically bonded to them.

Pectic Acids. The term "pectic acids" is applied to pectic substances mostly composed of colloidal polygalacturonic acids and essentially free from methyl ester groups.

## EXPERIMENTAL

### 1. Isolation of Ymer Barley Pectin.

Ymer barley flour was stirred in an aqueous solution of ammonium oxalate-oxalic acid (0.25% of each) at 75°C for one and one-half hours (15). A solid:liquid ratio of 1:10 was used. The extracts were removed by filtration through folded cheesecloth followed by centrifugation to remove the smaller solids. The addition of two volumes of ethanol, acidified with 5 ml. of concentrated hydrochloric acid per liter, to the filtrate brought about the precipitation of the gelatinous pectin material. The precipitation procedure was repeated three times or until no more ethanol insoluble material could be obtained. The pH of the extracts ranged from 3.6 to 4.0. The precipitated pectin was filtered, with suction, on Whatman No. 1 paper, washed several times with ethanol and then with ether, and dried under vacuum. The dried powder, approximately 1.3% of the original by weight, was dialyzed against running tap water for 24 hours. The non-dialyzable product was spotted on Whatman No. 1 paper and using two separate solvent systems, i.e., n-butanol:pyridine:water (3:2:1.5) and isopropanol:pyridine:water:acetic acid (8:8:4:1), showed no free sugars or uronic acids.

Ymer barley flour was also extracted in an aqueous solution of 0.4% hydrochloric acid for 1.5 hours at 80°C (16). The pH range for the extraction was between 2.3 and 2.6. A solid:liquid ratio of 1:10 was used. The extracts were filtered through folded cheesecloth followed by centrifugation to remove the smaller solids. The addition of ethanol brought about the precipitation of the gelatinous material. The precipitation procedure

was carried out three times or until no more ethanol insoluble material could be obtained. The precipitated material was treated as in the ammonium oxalate extraction and was chromatographed using the same solvents and techniques. Again no free sugars or uronic acids could be determined.

## 2. Preliminary Examination.

All of the preliminary examinations were made using the precipitated material from both the ammonium oxalate extraction and the 0.4% hydrochloric acid extraction.

The dialyzed residues were checked several times for sulfur, nitrogen, bromine, iodine, and chlorine using the sodium fusion method (17). The results in all trials were negative.

The pectin gave the characteristic uronic acid color reaction, a deep blue color, with sulfuric acid and carbazole (18), (19). This test can also be used to distinguish between glucuronic and galacturonic acid, however, in this work it was used only as a qualitative test. This was due to the fact that the original polymer was very unstable to heat and acid so that checks were made periodically to be sure that the polymer had not decarboxylated.

The polymer, suspected of being a true pectin, was treated with an aqueous, 1% hydrochloric acid solution at 90°C for three hours to bring about hydrolysis of the araban portion of the pectin (20). The material was filtered because of the insolubility of the Ymer pectin and the filtrate was subjected to further hydrolysis with 6 N hydrochloric acid at 90°C for two hours. The solution was then evaporated to dryness under vacuum and chromatographed using the same solvents as before. Only

glucose was found on the chromatogram.

Another experiment using acid hydrolysis was tried to see if the "pectic triad", which supposedly consists of galactan, araban, and a polygalacturonic acid, could be detected (21). The material was treated with 3 N sulfuric acid at 80°C for two hours. During the initial phase of the treatment a large amount of gas was liberated. At the end of the two hours the solution was cooled and the sulfate ions were precipitated as the barium salt. The solution was then filtered and the filtrate was evaporated to dryness under vacuum. The residue was chromatographed as before and only one spot was found to be present. With the use of known sugars and the color spray reagent, CD-1 (22) the spot was identified as glucose. Since a milder hydrolysis might give something other than glucose, the 3 N sulfuric acid hydrolysis was repeated this time keeping it at room temperature for two hours. With the same procedure as before two spots were detected, one being glucose and the other, not immediately identified, having a Rf of 0.52.

Since the evolution of gas and the positive carbazole reaction indicated the presence of an uronic acid, an enzymatic hydrolysis using the enzyme pectinase was attempted. The pectinase enzyme is supposedly specific for the  $\alpha$ -1 $\rightarrow$ 4 link in a linear polymer of galacturonic acid units. (23) Samples were withdrawn at various time intervals and checked for free galacturonic acid. No free acid was obtained even when the enzyme was allowed to act for 48 hours.

In the structural classification of a polymer one of the simplest and most reliable tests is that of film formation. One spread an aqueous

solution of the material on a glass plate and allows it to air dry. If the film is brittle, a highly branched polymer is indicated. If a strong pliable film is formed, a linear one is indicated. All film tests made on the pectin gave very strong films.

During the preliminary examination for elements it was noticed that a flame test gave a reddish-yellow color, indicating the presence of calcium. A quantitative determination of calcium was run using a Perkin-Elmer flame photometer. A small amount, (0.2 gm), of the polymer was "wet ashed" using concentrated nitric acid and perchloric acid (3:2 v/v). The material was taken to incipient dryness and then diluted to 10 ml. The diluted material showed that the original polymer had a calcium percentage of 0.75%. This could account for the rather high degree of insolubility that was encountered when working with the polymer.

### 3. Determination of a One Component System.

Glucose is not normally found as a hydrolysis product of pectins. Therefore, since glucose was recovered from this polymer after hydrolyzing with sulfuric acid, it was assumed that the glucose may have been bound, either chemically or physically, to the main chain or that a glucan may have been associated with the main chain which would make it appear as if it were a one component system.

Using a Spinco Electrophoresis Cell, Model R-Series B, Durrum Type, with a Spinco Duostat regulated D.C. power supply at 350 volts and 10 ma., the pectin was checked for migration in a pH 3.5 0.02 M citric acid-trisodium citrate buffer. Different strips were removed every hour up to six hours when maximum migration was reached. The paper was air dried and

sprayed with CD-1 developer. Only one band was detected indicating the presence of only one polymer. If the glucose were present as a polymer it must be either chemically bonded to the main chain or strongly physically bonded to the main chain. It is possible, of course, that two polymers with acid groups could migrate at the same rate.

In line with the second assumption, i.e., that the glucose was either chemically or physically bonded to the main chain, an attempt to separate the glucose was made using a Narda Series 600 Sonblaster (24). The polymer itself is highly insoluble in water, however, glucose is soluble and it was felt that any glucose separated could be isolated in the water. The sample was left in the bath from 0 to 360 minutes, varying the bath temperature from 4°C to 40°C. The samples were centrifuged to remove the polymer and the supernatant liquid was evaporated under vacuum. The evaporation gave no visible residue and chromatography showed no free sugars or uronic acids.

#### 4. Quantitative Determination of Carbon Dioxide.

McCready et.al. (25) have developed a technique for the determination of carbon dioxide from uronic acid polymers. The method and a brief description of their apparatus is as follows:

Air, the carrier gas, passes through an Ascarite or soda lime column which removes traces of carbon dioxide. A mercury valve allows the gas to pass in one direction through the apparatus. This valve is connected through a side tube to a reaction flask by means of a rubber connector. The reaction flask is immersed in an oil bath which is kept at 145°C. From the reaction flask the carrier gas passes upward through a reflux

condenser, through a trap containing 25 grams of granulated zinc or tin, and finally into the absorption flask. An absorption tower is connected to the flask and has a bulb of approximately 100 ml. capacity to serve as a trap to prevent the possible loss of alkali by foaming. The carrier gas passes from the tower assembly to a soda lime tower. This is connected to a water pump which causes the air to be swept through the assembly during the heating period.

A 250 mg. sample of pectin was placed in the reaction flask. Thirty milliliters of 19% hydrochloric acid and a small boiling tube were added. A stream of carbon dioxide-free air was drawn through the reaction flask and reflux condenser to remove traces of carbon dioxide before the absorption tower was attached. The flask and tower were swept free of carbon dioxide and 25 ml. of 0.25 N sodium hydroxide and 5 drops of butanol-1 were added to the absorption tower. The oil bath, previously brought to 145°C was put into position so that the level of the oil bath was approximately 1 to 2 mm. below the level of the liquid in the reaction flask. After the initial evolution of gas had ceased the system was swept by the use of an aspirator and the heating of the reaction was continued for 1.5 hours. The absorption flask and tower were then disconnected from the apparatus and the alkali was washed down from the tower into the absorption flask.

Ten milliliters of 10% barium chloride dihydrate solution and two drops of phenolphthalein indicator were added to the absorption flask and the excess alkali present was titrated with 0.100 N hydrochloric acid. A control standardization was run with each different trial.

McCready found a carbon dioxide percentage of 20.8 using a purified citrus pectin as his source of carbon dioxide. The Ymer barley pectin had a carbon dioxide content of 18.8%. This figure is an average of six runs. Maximum variance in the trials was 0.4%.

#### 5. Beta Amylase Action on Ymer Barley Pectin.

During the preliminary investigation, it was observed that a large amount of glucose was liberated by acid hydrolysis. The original material was tested with an iodine solution to see if it would give the characteristic starch color. The color produced was a very pale blue which is not that obtained from starch. (26)

Beta amylase hydrolyzes starch from the non-reducing end and is unable to break the  $\alpha$ -1 $\rightarrow$ 6 linkages thus a fairly large polymer remains which is known as a "beta limit dextrin". The dextrin is large enough to produce the deep purple color with iodine. When Ymer barley pectin is hydrolyzed with beta amylase the residue fails to give any color with iodine, indicating that the chain length is less than 4 - 6 units. (27).

The pectin was incubated with beta amylase at 30°C at a pH of 4.4 for 8 hours. At the end of this time the enzyme was destroyed and the solution was centrifuged to remove the "beta limit dextrin". The supernatant liquid was evaporated to dryness under vacuum and chromatographed as before. Only one spot could be detected on the chromatogram. This had an Rf of 0.52, which corresponded to the spot observed in the preliminary work. The Rg was 0.80 using the Rf of glucose as 0.65. The Rg is defined as the Rf of any sugar divided by the Rf of glucose. Known samples of maltose and isomaltose were then spotted along with the unknown and failed

to give either the correct Rf or color when developed. The Rf values and colors were: maltose 0.49, brown; isomaltose 0.34, green-brown; unknown 0.52, grey-brown. A reproduction of this chromatogram is given in Figure 1.

Since the only known product of beta amylase hydrolysis is maltose there was some doubt that these results represented beta amylase action. Therefore, a control was run on amylose and amylopectin using the same conditions. The isolation and identification of the hydrolysis products was conducted as before. Amylopectin gave only maltose, amylose gave maltose and longer chain polysaccharides.

Inactivation of the beta amylase was tried using temperature and pH variance (28), (29). For the pH inactivation the beta amylase was incubated in a series ranging from pH 3.4 to pH 6.1 for 1 hour at room temperature. The pectin and the starch control solutions were held at pH 4.4 and 0.5 ml. aliquot of the acid treated beta amylase was added. Incubation was for 3 minutes at 20°C and was stopped by the addition of the 3,5 dinitrosalicylic acid reagent (30). All determinations were made on a Beckman Model B Spectrophotometer at 540 mμ. The results of this trial are shown in Figure 2. Although the total amount of maltose released from starch was approximately 100 mg. more than the unknown released from the Ymer barley polymer, the shape of the curve for both substrates was the same and both had a maximum activity at pH 4.4.

The second study was made by increasing the temperature while maintaining the pH at 4.4. The temperature was varied from 20°C to 70°C and the time of incubation was held constant for 10 minutes. The results of this study are shown in Figure 3. The shape of the curve again followed

Starting line

Isomaltose

Maltose

Unknown

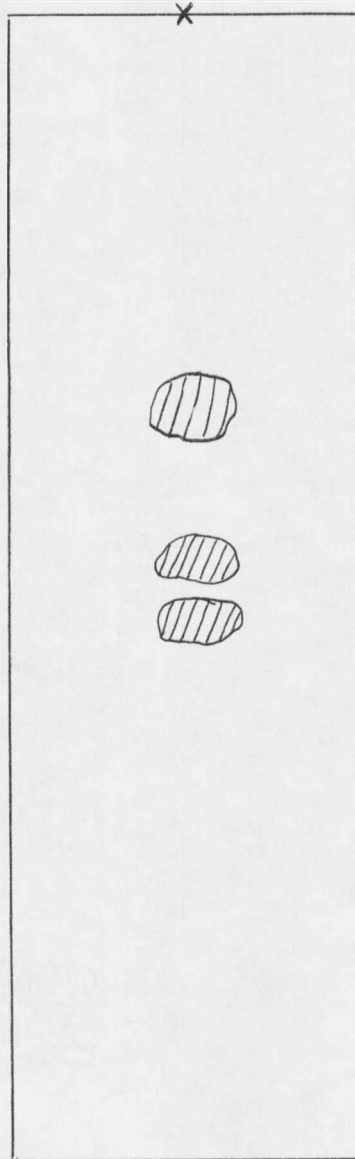


Figure 1. A comparison of the unknown sugar, recovered from beta amylase action, with maltose and isomaltose.

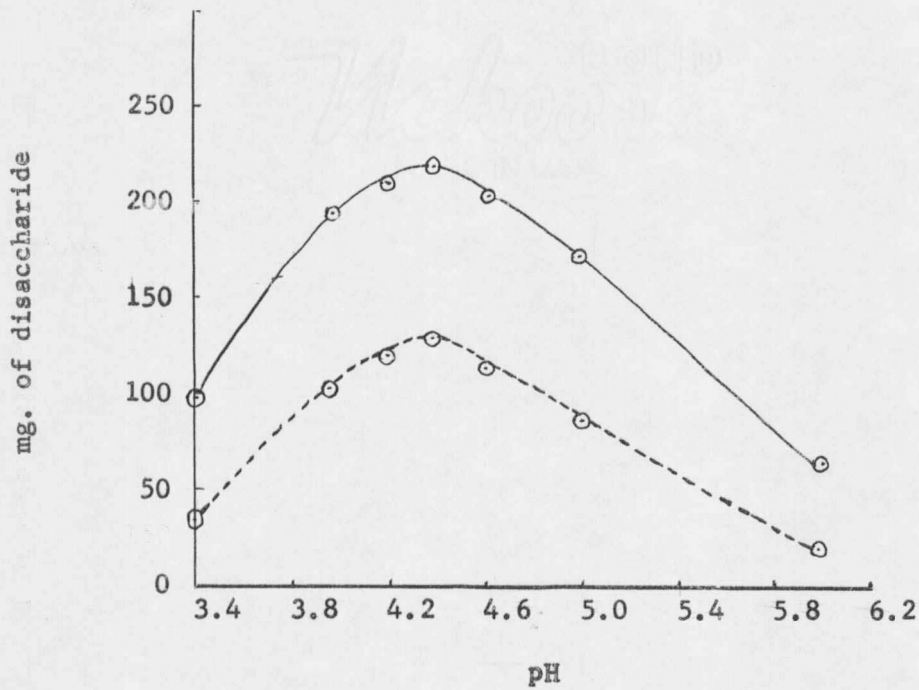


Figure 2. Inactivation of beta amylase by pH  
Legend: ----- Ymer barley pectin  
          \_\_\_\_\_ Starch

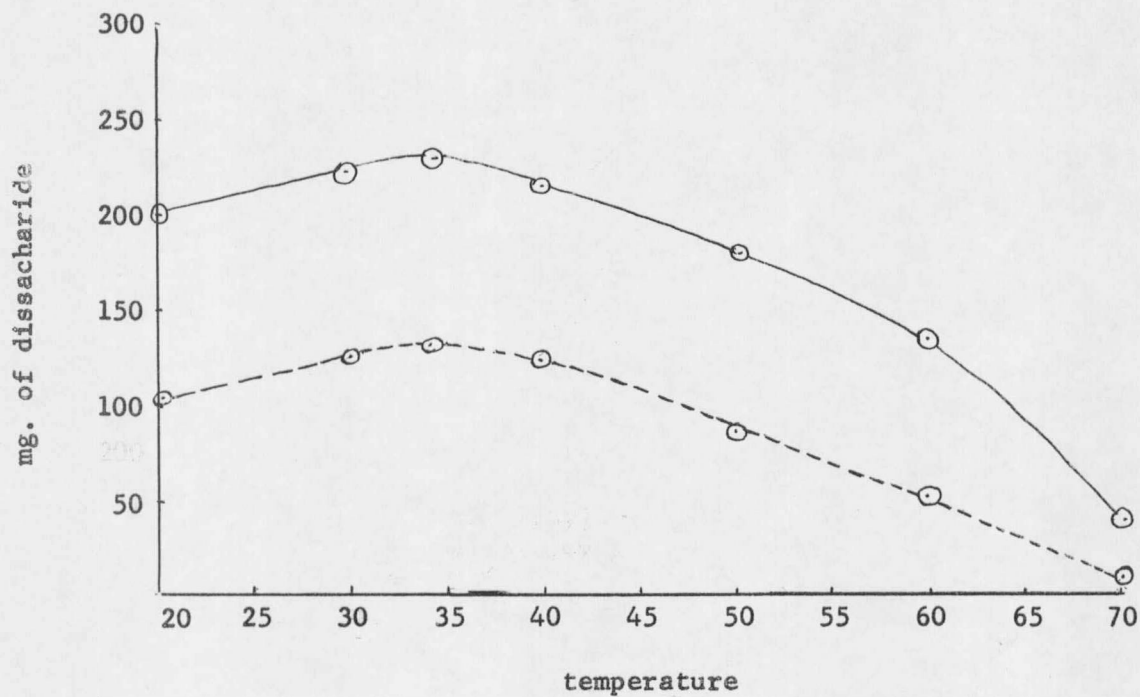


Figure 3. Inactivation of beta amylase by temperature  
Legend: ----- Ymer barley pectin  
————— Starch

that of the starch control. It was observed that the activity on both substrates increased from 20°C to 34°C where it reached a maximum and then rapidly fell off. This suggested that the Ymer barley pectin was hydrolyzed by beta amylase and that the hydrolysis is not due to a contaminating enzyme system.

The results shown in Figures 2 and 3 were reproducible.

#### 6. Identification of the Unknown Disaccharide as Nigerose.

From the data presented in Figure 1 the assumption was made that the unknown sugar was a disaccharide. The unknown sugar was hydrolyzed with acid and chromatographed as before. Acid hydrolysis indicated the unknown disaccharide to be composed of glucose since this was the only sugar obtained from the hydrolysate.

In 1914 Dox and Niedig (31) reported isolating a polyglucosan, "mycodextran", from Penicillium expansum. A similar polysaccharide was isolated by Dox (32) from Aspergillus niger. Barker, Bourne, and Stacy (33) reported isolating a polyglucosan, probably identical to those mentioned by Dox, which had been synthesized from sucrose by Aspergillus niger. The structure of the polysaccharide has been shown to be made up of glucose units in which the links are alternating  $\alpha$ -1 $\rightarrow$ 3 and  $\alpha$ -1 $\rightarrow$ 4. Hydrolysis of the polysaccharide yielded, as one of the products, an unknown disaccharide which was classified as two glucose units joined  $\alpha$ -1 $\rightarrow$ 3. Barker suggested the name "nigerose" for the disaccharide and all references to the Ymer disaccharide will be as nigerose.

After the disaccharide has been classified by Barker to be an  $\alpha$ -1 $\rightarrow$ 3 linkage many workers reported on this sugar. Pazur et. al. (34) reported

that the nigerose phenylosazone gave a melting point of 204°C-205°C. The phenylosazone of the Ymer barley disaccharide was made following the work of Pazur and gave a melting point of 203°C-205°C.

Dr. Wolfrom and Dr. Thompson were kind enough to send a sample of  $\beta$ -nigerose octaacetate. The Ymer disaccharide was acetylated to form the acetate derivative for a mixed melting point determination. The following procedure was followed in order to obtain the beta derivative (35). One gram of the disaccharide, 0.5 gram of sodium acetate, and 10 ml. of acetic anhydride were refluxed for 10 minutes and evaporated to dryness to remove most of the acetic acid and the acetic anhydride. The dark colored residue was purified by removing the sodium acetate by repeated extraction with warm water and finally taking up the residue in absolute alcohol and heating with charcoal. After recrystallization from hot alcohol the melting point was 151°C-152°C. Mixed melting point was 151°C-153°C.

Charlson and Perlin (36) have shown that oxidation with lead tetraacetate, of a reducing disaccharide having a 1 $\rightarrow$ 3 linkage between the two residues in which the reducing moiety is glucose, produced D-arabinose. From the data observed in Figure 1 it was apparent that the unknown disaccharide from Ymer barley was not an  $\alpha$ -1 $\rightarrow$ 4 or an  $\alpha$ -1 $\rightarrow$ 6 linkage, however, it was not known if it contained an  $\alpha$ -1 $\rightarrow$ 2 or an  $\alpha$ -1 $\rightarrow$ 3 linkage. Feingold, Neufeld, and Hassid (37) working with the  $\beta$ -1 $\rightarrow$ 3 linked glucan extracted from Phaseolus aureus also determined the presence of the 1 $\rightarrow$ 3 link. Their method was used for this determination, however, the concentrations were increased 100 fold so that the products could be detected on chromatograms.

One hundred mg. of the disaccharide was added to 1 ml. of water and

the mixture diluted to 10 ml. with glacial acetic acid. The solution containing the disaccharide material was oxidized with 500 mg. of lead tetraacetate dissolved in 40 ml. of glacial acetic acid. The reaction was allowed to proceed for 10 minutes at 28°C and then stopped by the addition of 20 ml. of glacial acetic acid containing 200 mg. of oxalic acid. The precipitate which formed was discarded, the supernatant liquid diluted with 150 ml. of water and shaken with 20 grams of Dowex 50 (acid form) to remove residual lead ions. The reaction mixture was concentrated to dryness in a vacuum desiccator, taken up in 50 ml. of 1 N hydrochloric acid, held at 100°C for 30 minutes, and again concentrated to dryness. The residue was chromatographed as before and two spots, glucose and arabinose were detected.

#### 7. Alpha Amylase Action on Ymer Barley Pectin.

The normal products of alpha amylase action on starch are glucose, maltose and some longer branched fragments. Ymer barley pectin was incubated with alpha amylase, pH 4.4, for 8 hours at 30°C. The residue remaining, for want of a better name, was called an "alpha limit dextrin". The enzyme was removed by precipitation with 20% trichloroacetic acid and the supernatant liquid was evaporated to dryness under vacuum. The material was then chromatographed as before and identified by using known sugars as controls. The only sugar found to be present was glucose. According to current concepts of alpha amylase action these results were not expected. Therefore, a control was run using amylose and amylopectin with the alpha amylase under the same conditions. Only glucose, maltose and longer chain polysaccharides were obtained from both amylose and

amylopectin.

The fact that only glucose was obtained from the action of alpha amylase on the pectin polymer suggested that it might be of interest to try the hydrolysis of nigerose with this enzyme, since this sugar was formed by beta amylase action on the original material and therefore this  $\alpha$ -1 $\rightarrow$ 3 link must be hydrolyzed by alpha amylase. Glucose was the only sugar obtained from this experiment. This also was a surprising result because maltose, which has an  $\alpha$ -1 $\rightarrow$ 4 link is not hydrolyzed by alpha amylase as shown in Table I. A study was made as to the effect of pH, temperature and mercuric chloride on the ability of alpha amylase to hydrolyze nigerose. Beta treated starch (38) was used as a control. The temperature and pH experiments were carried out as in section 6 except that the time of incubation in the temperature experiment was for 4 hours instead of 10 minutes. The results of these experiments are reported in Figures 4 and 5. The inactivation of alpha amylase by the use of mercuric chloride was determined using the method of Underkofler and Roy (39). They have shown that a concentration of 1 mg. of mercuric chloride/ml. completely inactivates alpha amylase. The enzyme was incubated with increasing concentrations of mercuric chloride for 4 hours at room temperature. The results are reported in Figure 6.

With the use of an alpha amylase concentrate purchased from National Biochemical Corporation a purification experiment (39) was carried out using nigerose and beta treated starch as substrates. The results of this experiment are presented in Figure 7.

Maltose and nigerose were incubated with the alpha amylase, pH 4.4,

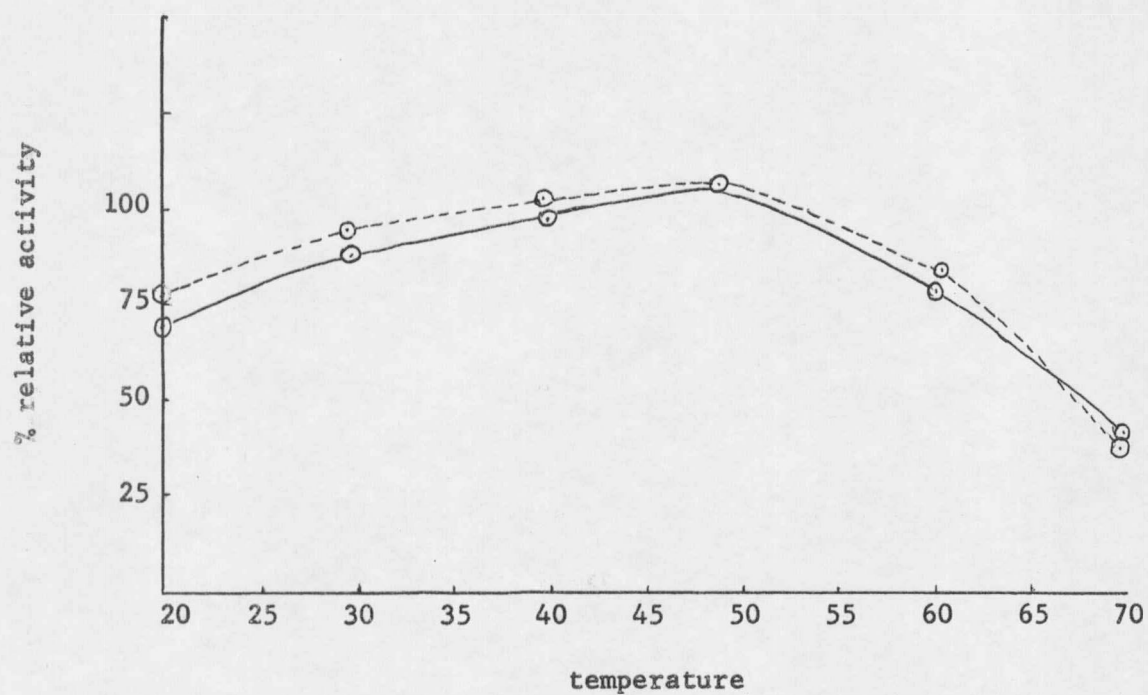


Figure 4. The inactivation of alpha amylase by temperature.

Legend: ----- Nigerose  
————— Beta treated starch

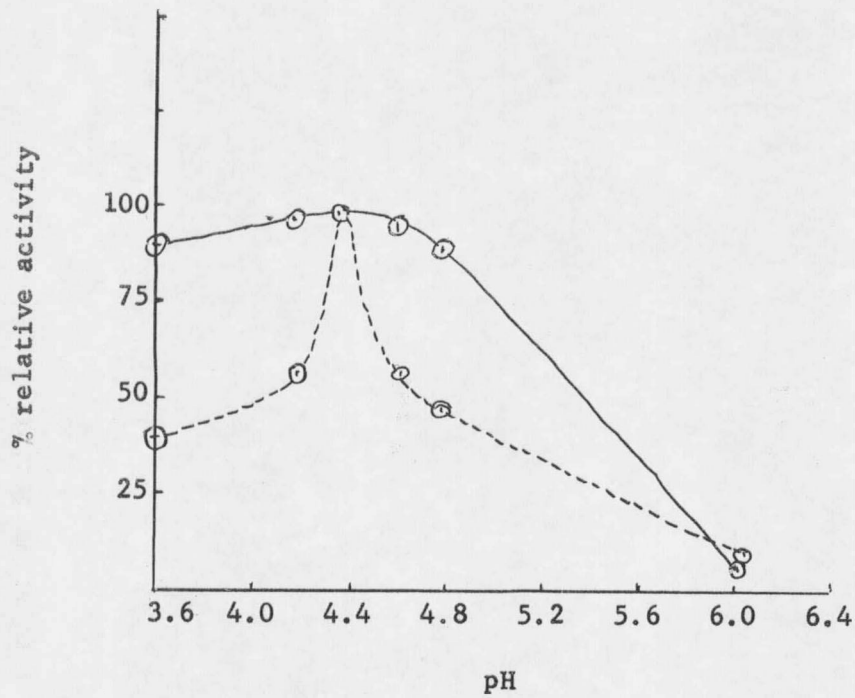


Figure 5. Inactivation of alpha amylase by pH  
Legend:  
----- Nigerose  
————— Beta treated starch

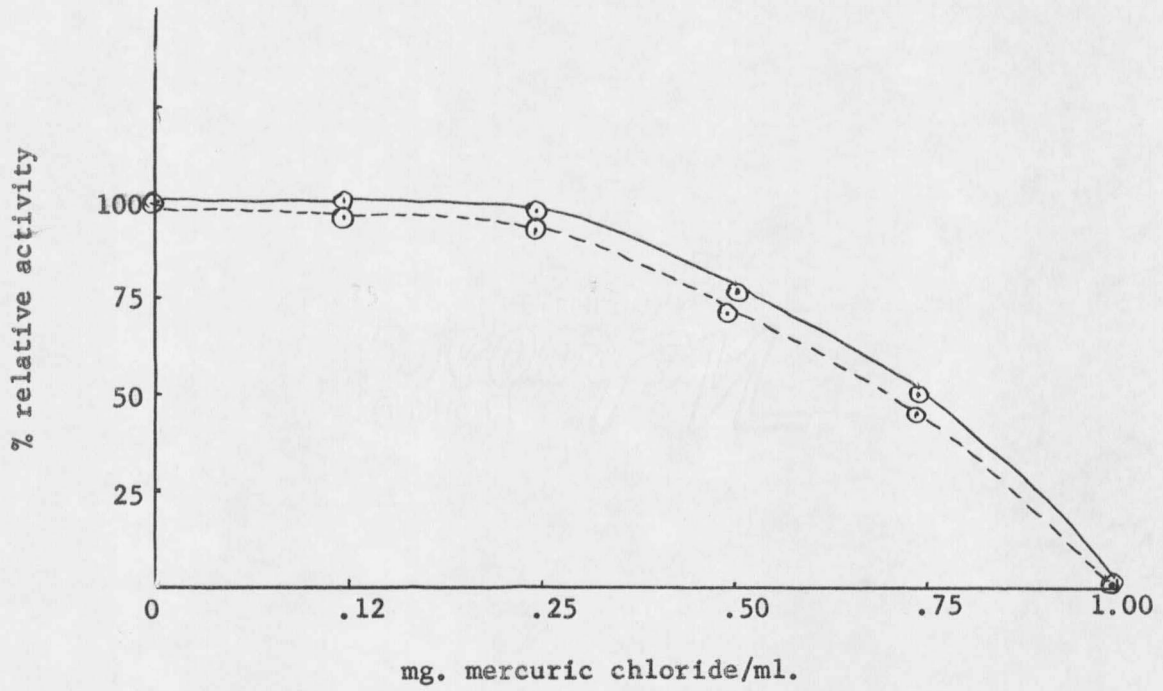


Figure 6. Inactivation of alpha amylase by mercuric chloride  
Legend: ----- Nigerose  
————— Beta treated starch

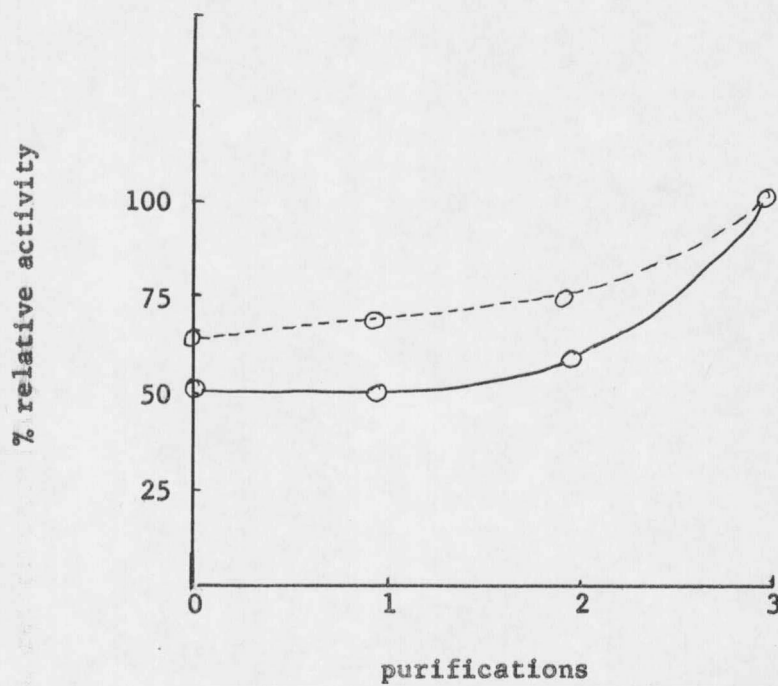


Figure 7. Increase in activity of alpha amylase due to purifications  
Legend: ----- Nigerose  
————— Beta treated starch

for 8 hours at 30°C (30). From this data which is reported in Table I, it is apparent that there is a definite difference in the action of alpha amylase on these two substrates.

Nigerose was also incubated with salivary amylase in a phosphate buffer at pH 7. Pazur, French, and Knapp (40) have shown that there is little or no maltase activity at this pH so that any glucose produced would not be the result of the enzyme maltase. After 8 hours, complete hydrolysis to glucose was obtained.

In order to check for other enzymes which may have been present as impurities in the alpha amylase, maltose, isomaltose,  $\alpha$ -methyl-O-glucoside, gentiobiose, melezitose, turanose and nigerose were incubated with alpha amylase for 8 hours. Only in the case of nigerose could any hydrolysis be measured. This was of interest because turanose and melezitose both have an  $\alpha$ -1 $\rightarrow$ 3 linkage although the hexose moieties are different from that found in nigerose. Turanose is 3-O- $\alpha$ -D-Glucopyranosyl-D-fructose while melezitose is O- $\alpha$ -D-Glucopyranosyl-(1 $\rightarrow$ 3)-O- $\beta$ -D-fructofuranosyl-(2 $\rightarrow$ 1)- $\alpha$ -D-glucopyranoside. This result should be more thoroughly investigated as it might shed some light on this mechanism. The sample of  $\beta$ -nigerose octa-acetate received from Drs. Wolfrom and Thompson was deacetylated using the method of Schultz and Wagner (41). The residue was put with alpha amylase and again complete hydrolysis to glucose was observed.

From these results it can be concluded that either crystalline alpha amylase can hydrolyze the  $\alpha$ -1 $\rightarrow$ 3 linkage present in nigerose or that a new enzyme, "nigerose", is present as an impurity in the alpha amylase. However, it is more probable that alpha amylase hydrolyzes the  $\alpha$ -1 $\rightarrow$ 3 linkage.

TABLE I

Action of Alpha Amylase on Maltose and Nigerose

| <u>pH</u> | <u>uM of Glucose Produced</u> |                  | <u>% Conversion of Nigerose<br/>to Glucose</u> |
|-----------|-------------------------------|------------------|------------------------------------------------|
|           | <u>Maltose</u>                | <u>Nigerose*</u> |                                                |
| 3.6       | 0                             | 3.32             | 35.5                                           |
| 4.2       | 0                             | 6.10             | 65.2                                           |
| 4.4       | 0                             | 8.90             | 95.1                                           |
| 4.6       | 0                             | 6.10             | 65.2                                           |
| 4.8       | 0                             | 4.70             | 50.2                                           |
| 6.1       | 0                             | 1.39             | 14.9                                           |

---

\*100% conversion of nigerose used would yield 9.36 uM of glucose

An equimolar mixture of maltose and nigerose was incubated with alpha amylase for 8 hours at 30°C. The resulting solution was chromatographed as before and only maltose and glucose were found. This would indicate that maltose does not inhibit the hydrolysis of nigerose. A mixture of nigerose and beta treated starch was also incubated with alpha amylase. Using a starch control, the dextrinizing time was 15 minutes. When the nigerose was added the time was still 15 minutes. This was done to find out whether the nigerose would inhibit the hydrolysis of starch. Further work should be done in this area to find out if starch inhibits the hydrolysis of nigerose.

#### 8. Identification of the Glucan.

Acid hydrolysis of the original Ymer barley pectin, followed by chromatographic separation and elution, gave a total glucose content of 20%.

During the course of the work with alpha amylase it was noticed that limited action, 10-15 minutes, on the Ymer barley pectin gave rather large fragments which were observed when the chromatogram was descended 3-4 times. Therefore, it was decided to elute these fragments to see if a determination of linkages could be made. Since beta amylase gave nigerose as the only product and because the only known polymer (33) containing nigerose was made up of alternating alpha 1→3 and 1→4 links, it was assumed that the glucan from Ymer barley pectin was made up in a similar manner. If this was true, after treatment with periodic acid, the residue should contain approximately 50% of the original glucose. However, if the polymer was all 1→3 linkages, all of the glucose would be recovered, except the molecule on each end of the chain which would be oxidized under

these conditions.

The original Ymer barley pectin was incubated with alpha amylase, pH 4.4, at 50°C for 3 minutes. The incubation time was kept as short as possible so that only a small amount of glucose would be produced and so that the largest possible fragment of the glucan could be isolated. Twenty percent trichloroacetic acid was then added to precipitate the enzyme and the supernatant liquid was dried under vacuum. The polymer was separated by paper chromatography using 3 descents. Only one detectable band appeared along with a trace of glucose. The polymer was eluted with water and the resulting solution divided into three parts. All three were subjected to periodic oxidation using the method of Baddiley, Buchanan, and Szabo' (42). Two of the fractions were titrated with 0.01 N sodium hydroxide. According to Potter and Hassis (43), a good approximation of the chain length of a linear polymer is possible if 3 moles of formic acid are produced per chain. The two fractions gave a chain length of 19 units. The third sample was dialyzed, hydrolyzed and the hydrolyzate chromatographed as before. The glucose was eluted, dried under vacuum and weighed. Assuming the 19 unit figure to be correct, the total amount of glucose produced after hydrolysis would be 17 units, if it was a 1→3 polymer. If it was an alternating 1→3, 1→4 polymer then 8-9 units of glucose should be recovered depending upon which link came first at the non-reducing end.

Since 95.68% of the theoretical yield for 17 glucose units was recovered, it was apparent that the glucan was composed of 1→3 linkages. The action of alpha amylase confirmed this observation. If the glucan contained alternating 1→3, 1→4 links maltose should have been detected in

the final products.

The infrared spectra of this alpha fragment is presented in Figure 8.

9. The Identification of the "Alpha Limit Dextrin".

After the action of alpha amylase on the original unknown Ymer barley pectin it was noticed that a definite residue remained.

A very strong pliable film was formed from this residue indicating a linear molecule.

The residue gave a positive carbazole-sulfuric acid reaction indicating uronic acids, therefore, a carbon dioxide analysis was run on this fraction. The percent carbon dioxide found was 19.2. According to McCready the theoretical yield for pectic acid is 19.8%. Pure galacturonic acid would give 25% carbon dioxide. McCready's value is based on the number of methoxy groups present in the polymer, the assumption that the acids are all in the monohydrate form, and that the presence of inorganic ions caused the deviation from the percentage found in pure galacturonic acid. The "alpha residue" was found to have 0.94% calcium, 2.2% methoxy groups (44), 2% glucose, which was obtained by mild acid hydrolysis, and also water. Assuming that the acids are in the monohydrate form, the percentage carbon dioxide was found to vary only 3.6% from theory.

The residue, after the removal of the residual glucose, was analyzed on a Beckman-IR4 Infrared Spectrophotometer. The spectra gave a definite indication of carboxyl groups by the absorbance at  $1710\text{ cm}^{-1}$ . A known pectic acid was analysed in the same manner and gave a spectra that was very similar to that of the alpha residue. The result of these analyses is presented in Figures 9 and 10.

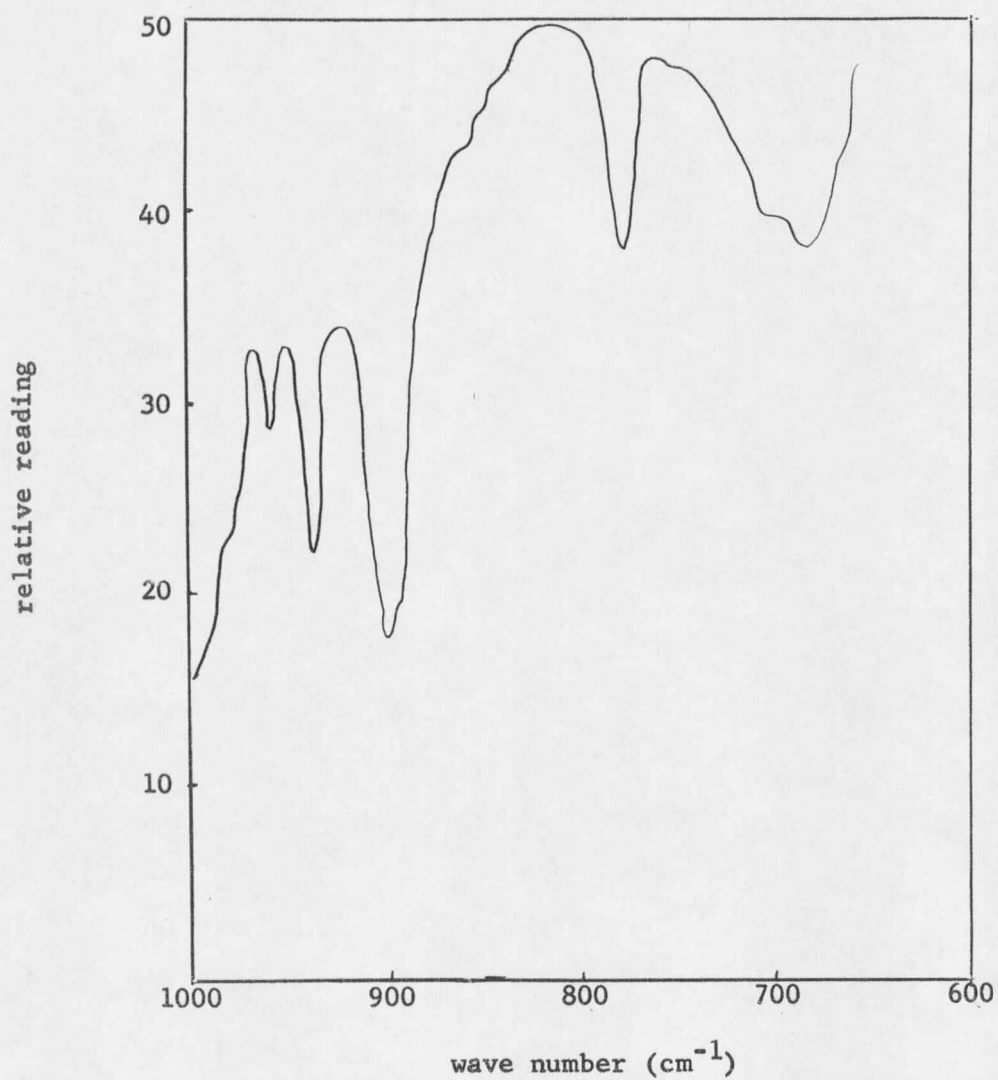


Figure 8. Infrared spectrum of acetylated nigran (KBr pellet)

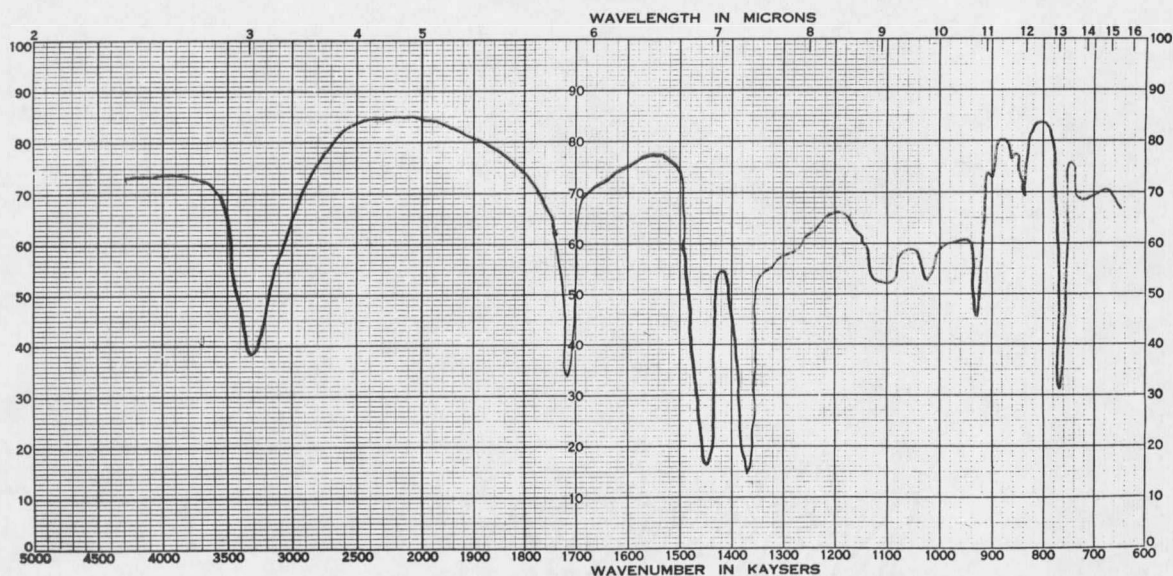


Figure 9. Infrared spectrum of alpha amylase residue

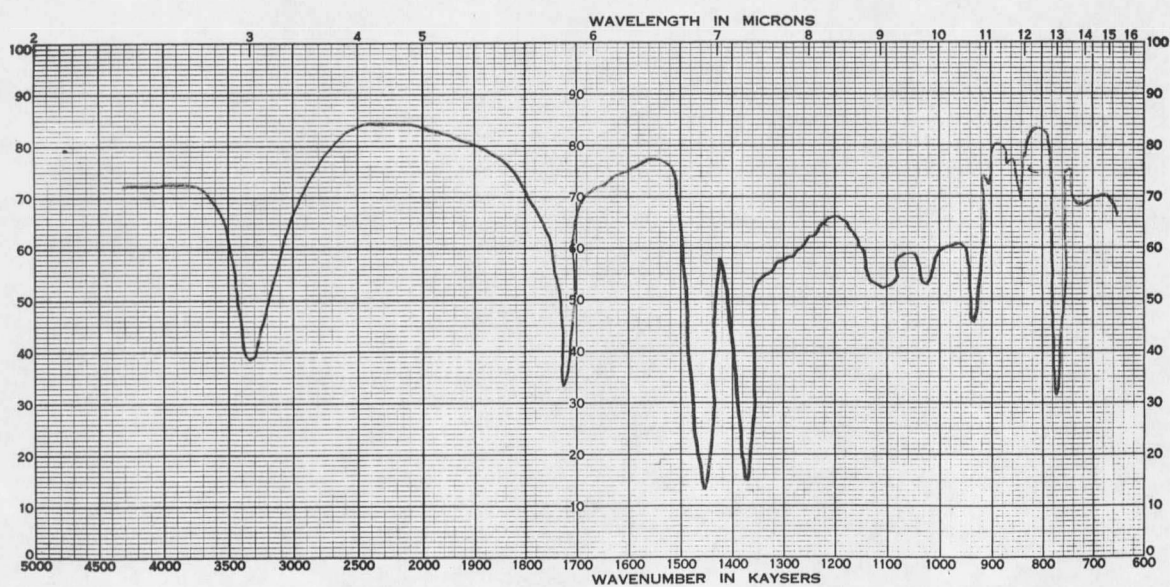


Figure 10. Infrared spectrum of commercial pectic acid

Because of the similarity of the infrared spectra the residue was then incubated with pectinase, which had failed to hydrolyze the original pectin in the preliminary investigation. The hydrolysis product was identified by paper chromatography and the only product present was galacturonic acid. This indicated that the residue is made up of  $\alpha$ -1 $\rightarrow$ 4 linkages. This would indicate that the glucan, present in the original Ymer barley polymer, inhibited the hydrolysis of the galacturonic acid polymer.

The residue, without glucose, was subjected to periodic acid oxidation (42). A 0.5 gram sample was thoroughly dispersed in a phosphate buffer at pH 7 and 0.1 N sodium periodate was added. The samples were allowed to react for 5 days at room temperature in the dark. At the end of this time the excess periodate was destroyed by the addition of ethylene glycol. Blank determinations were made on all trials. Using the method of Potter and Hassid (43) an attempt was made to estimate the chain length. The results of this were very confusing giving values of 200, 407, and 606 units respectively.

The decarboxylated residue after the removal of glucose was again subjected to an end group determination using sodium periodate. The results of three trials gave chain lengths of 149, 149 and 151. If this can be considered a minimum chain length then with the 20% glucose added it would give a minimum length of 187 units if the glucan and the uronic acid polymer are chemically linked.

When the decarboxylated residue was dried under vacuum all attempts to get it back into solution failed. All available solvents were tried with this residue. When galacturonic acid is decarboxylated the product is

arabinose. A hydrolysis of the araban was attempted by the use of concentrated sulfuric acid for 6 hours at 80°C. The only sugar found was arabinose and this was present only in very small amounts. The difficulty of hydrolysis and solubility of this araban should be investigated further in view of the ease of hydrolysis of the arabans reported in the literature. This is probably because it exists in the pyranose form. The alpha residue was subjected to a methylation study (45) to determine the linkage present.

Three grams of the alpha residue were methylated with dimethyl sulfate and 40% sodium hydroxide. This procedure was repeated 4 times and each methylated product was extracted with methanol to remove the neutral polysaccharides. After the fourth methylation the material was dissolved in water and refluxed with methyl iodide containing 5% methanol. Silver oxide (7.5 grams) was added over a 4 hour period and the refluxing was continued another 4 hours. The product was passed through a column of Dowex 50 (acid form) to remove any cations and the resulting acid was extracted with acetone and ethanol and hydrolyzed with 1 N hydrochloric acid for 5 hours. The hydrolyzate was evaporated under vacuum which yielded a syrupy mixture of sugars. The resulting chromatograph showed 3 distinct spots which were identified by their R<sub>f</sub> and R<sub>g</sub> values. The third spot was placed on the chromatogram purposely for the identification of the methylated uronic acids. The three components were the 2,3,4 trimethyl galacturonic acid (46), the 2,3 dimethyl galacturonic acid (46) and the 2,3,4,6 tetramethyl glucose (47), which was used for identification of the other two. The results of this experiment are presented in Figure 11 and would indicate that the uronic acid polymer is a chain of

Starting line

Dimethyl galacturonic acid

Tetramethyl galacturonic acid

Tetramethyl glucose

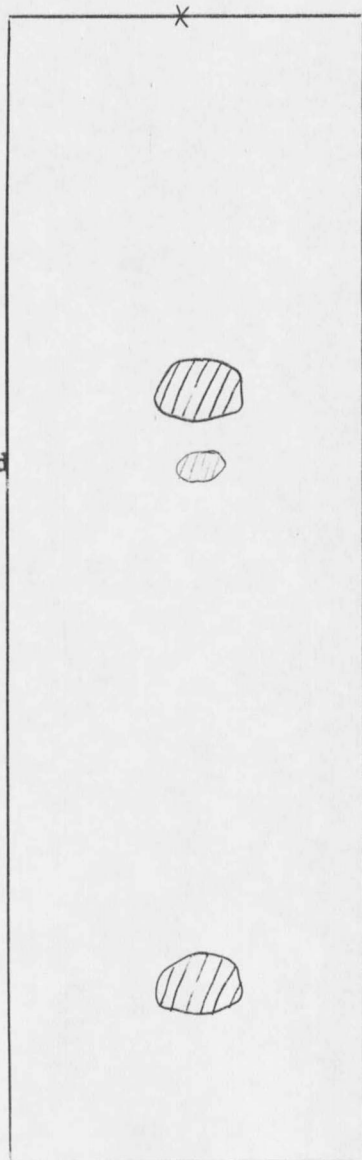


Figure 11. Chromatogram of the methylated uronic acids in the alpha residue.

1-4 linked residues.

The decarboxylated residue was subjected to methylation without the removal of the 2% glucose that remained with the chain. This was done in order to obtain the 2,3,4,6 tetramethyl glucose which could be used as the reference spot. The methylated sugars present were the 2,3,4 tri-methyl arabinose, the 2,3 dimethyl arabinose (47) along with the reference spot. The results of this experiment are presented in Figure 12. This would indicate that the decarboxylated residue is also made up of 1-4 linkages.

#### 10. The Structure of Ymer Barley Pectin.

The original pectin polymer was subjected to methylation in order to determine the linkage between the glucan and the uronic acid polymer. The methylated sugars isolated were the 2,3,4 tri-methyl galacturonic acid, the 2,3 dimethyl galacturonic acid, the 2,3,4,6 tetramethyl glucose, and the 2,4,6 trimethyl glucose (48). This data confirms the assumptions made earlier. The uronic acids are linked through the 1-4 position, and the glucan is linked through the 1-3 position. However, there was no evidence of a monomethylated uronic acid. It can be assumed then that the bond existing between the uronic acid polymer and the glucan is not a chemical bond. Separation of the glucan was attempted by refluxing the original pectin with absolute alcohol for 2 hours. The residue was centrifuged and the supernatant liquid evaporated under vacuum. The product of this extraction was hydrolyzed with 4% hydrochloric acid for 2 hours at 70°C. The hydrolyzate was chromatographed as before and the only glucose was isolated. The Ymer pectin was placed in an 8 m urea solution (49) in

Starting line

Dimethyl arabinose

Trimethyl arabinose

Tetramethyl glucose

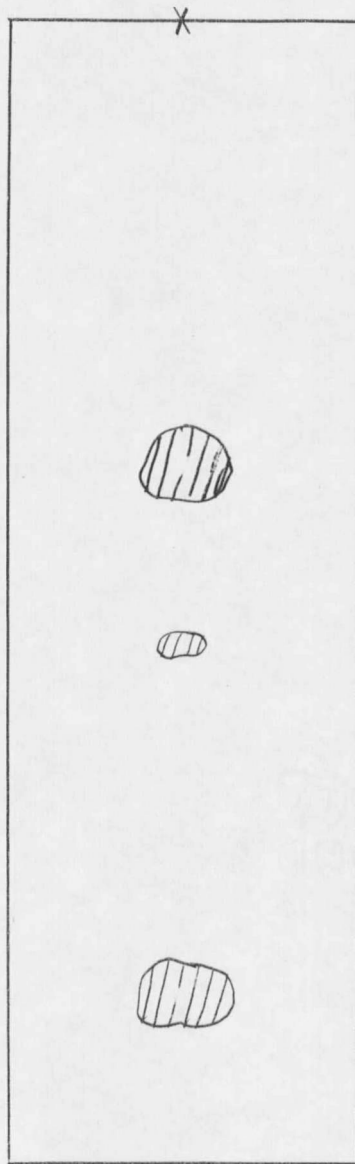


Figure 12. Chromatogram of the methylated arabinose units in the decarboxylated alpha residue.

order to separate the glucan from the uronic acid polymer. The solution was left at room temperature for 5 hours and then dialyzed. The dialyzate was centrifuged and the supernatant liquid dried under vacuum. The residue was chromatographed and no glucose could be detected. The residue was then hydrolyzed with 4% hydrochloric acid for 2 hours at 70°C. The hydrolyzate was chromatographed and only glucose was isolated. This would seem to indicate that the glucan can be extracted in both of these manners, however, the yield is very poor. Further study may give a more practical way to isolate the glucan.

Therefore, from the available data, it would appear that the uronic acids are linked through the 1→4 position and that the glucan is linked through the 1→3 positions. However, the glucan is not chemically bound to the uronic acid polymer. Assuming the uronic acid polymer to be similar to pectic acid, Whistler and Smart (50) have stated that the repeating unit in pectic acid is composed of 3 galacturonic acid residues. This would give a linear length of 50 units if the polymer can be considered to be 150 units. This would correspond very close to the 40 units in the glucan. Two possible structures can account for this data. They are presented in Figure 13. Both structures are feasible, however, (a) would appear to be the one that would account for the action of both alpha and beta amylase.

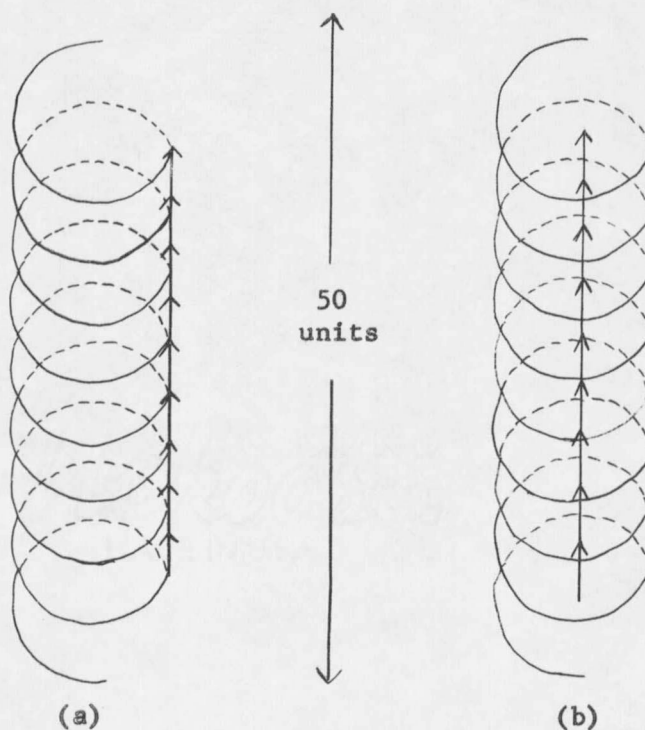


Figure 13. Two possibilities for the Ymer barley polymer. The helical structure would contain 150 galacturonic acid units, while the arrows represent approximately 40 glucose units.

### CONCLUSIONS

1. A pectin has been isolated from barley flour by two different methods.
2. The pectin has associated with it, a glucan, to the amount of about 20%, which is linked through the  $\alpha$ -1 $\rightarrow$ 3 positions. From the data available it would appear that the glucan contains 38 glucose units.
3. The glucan was isolated by the action of alpha amylase on the original material. It proved to be an  $\alpha$ -1 $\rightarrow$ 3 linked polymer as a result of periodate studies. This is the first time that this polymer has been reported as a natural material.
4. The pectin was found to be a polymer of galacturonic acid, with a minimum chain length of 150 units joined  $\alpha$ -1 $\rightarrow$ 4. Methylation and periodate oxidation failed to show any branching points. The infrared resembled that of commercial pectic acid.
5. It has been observed that alpha amylase hydrolyzes the glucan, which shall be named "nigeran", completely to glucose. This is an unusual observation in that alpha amylase has never been observed to hydrolyze any other glucose polymer completely and therefore must alter our concepts in regard to alpha amylase action. Every attempt to prove this unusual activity to be the result of a different enzyme resulted in failure.
6. Beta amylase likewise was proven to hydrolyze the "nigeran" completely to nigerose.

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The isolation, identification,  
and structural study of Ymer

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