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

Abstract:
A pectin has been isolated from Ymer barley flour. It was found to be composed of galacturonic acids linked α-1→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 α-1→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→3, 1→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.

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ABSTRACT

A pectin has been isolated from Ymer barley flour. It was found to be composed of galacturonic acids linked α-1⇒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 α-1⇒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⇒3, 1⇒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 developement 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, expecially 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 somthing 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 α-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 form, 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 α-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 know 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 mu. 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

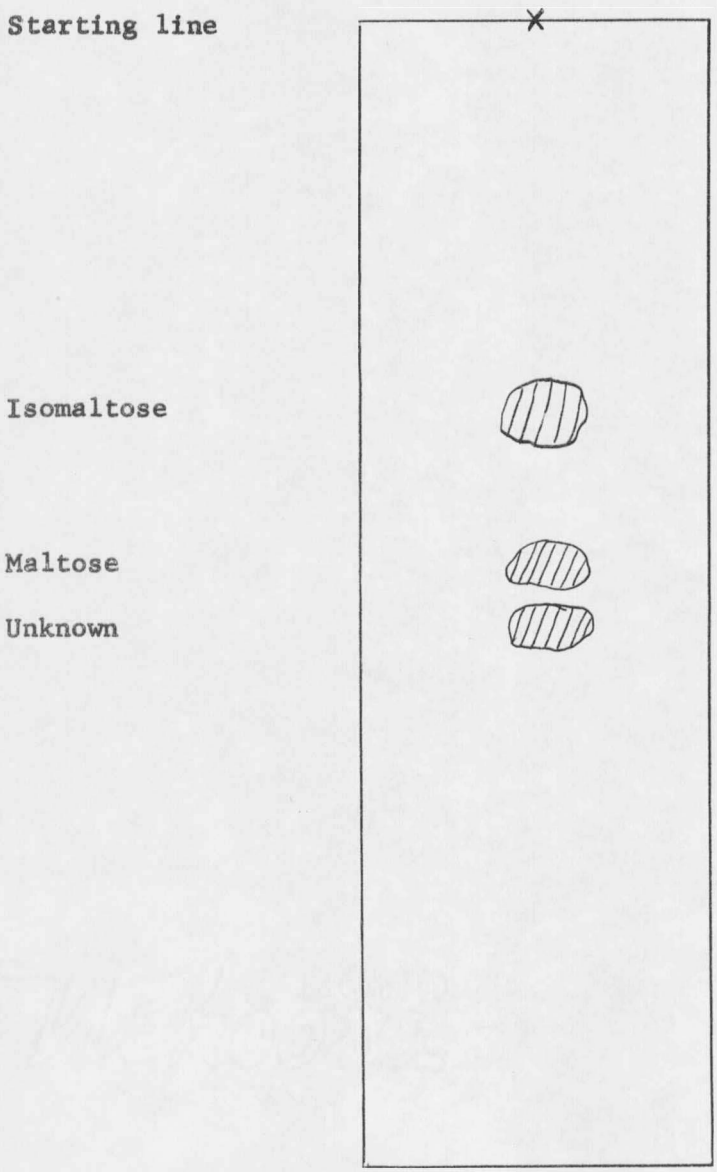

Figure 1. A comparison of the unknown sugar, recovered from beta amylase action, with maltose and isomaltose.

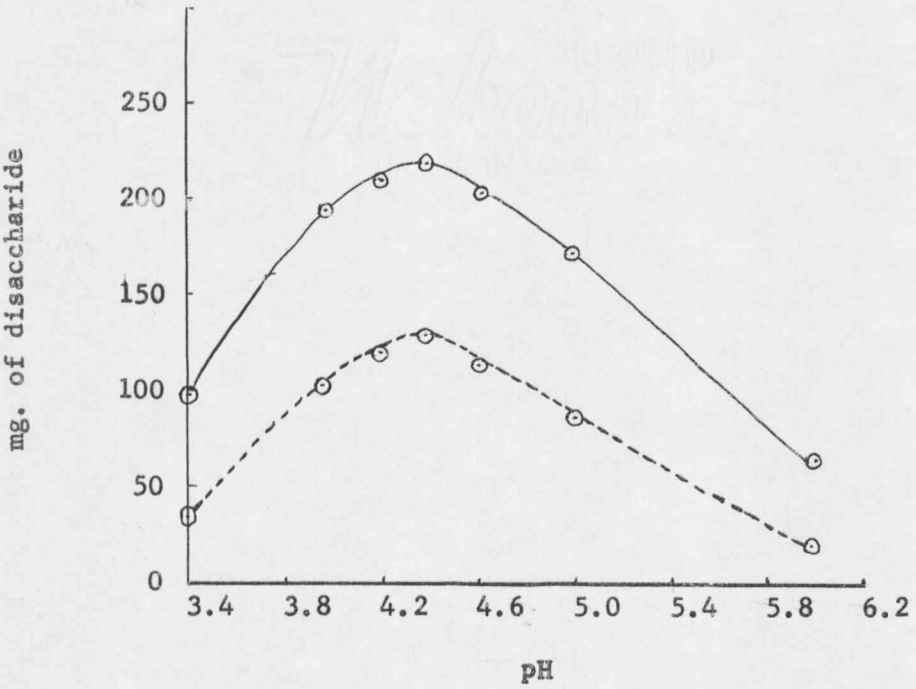

Figure 2. Inactivation of beta amylase by pH
Legend: -------- Ymer barley pectin
_____ Starch