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Enzyme and Flavor Components of Fruits Isolated

ENZYMES

Course of Action of Polygalacturonase on Polygalacturonic Acids

ROLLAND M. McCREADY and ELIZABETH A. McCOMB
Western Regional Research Laboratory, Albany, Calif.

In studying the biochemical processes that take place during ripening of fruits, further information on pectic enzymes acting on pectic substances was desired. The course of action of a purified fungal polygalacturonase on polygalacturonic acid was followed by qualitative and quantitative paper chromatography. Crystalline brucine salts of galacturonic di- and trigalacturonic acids were isolated and used as standards to prove the reaction course. These and other oligogalacturonic acids appeared in the very early stage of hydrolysis and ultimately all were converted to galacturonic acid. The rates and apparent course of hydrolysis of polygalacturonic acids were independent of the molecular weights of substrates within the limits of 1900 to 35,000.

THAT PECTIC ENZYMES produce a series of oligouronides from pectic substances is shown by recently published chromatographic studies by Jermyn and Tomkins (*9*), Altermatt and Deuel (*1*), Ayers *et al.* (*3*), Dingle *et al.* (*4*), and Roelofsen (*21*). While much preliminary information can be gained from paper chromatographic studies, isolation and characterization of materials are required as final proof. Phaff and Luh (*19*) and Altermatt and Deuel (*1*) obtained preparations of galacturonic, di-, and trigalacturonic acids that analyzed as monohydrates. The latter workers (*2*) used a combination of ion exchange and lead precipitation to obtain tetragalacturonic acid. None of these oligouronic acids were distinctly crystalline.

In studies of the action of purified polygalacturonase (PG) on polygalacturonic acids the hydrolysis products were separated by paper chromatography on heavy filter paper and crystallized as their brucine salts (*12*). These salts and the free acids regenerated from them were characterized by chemical analyses and used as final proof of the reaction course of polygalacturonase on polygalacturonic acids.

A purified fungal polygalacturonase prepared by the method of Jansen and MacDonnell (*8*) was shown to hydrolyze the glycosidic linkage of pectic acid in such a manner that the rate of hydrolysis was 17 times faster up to 50% hydrolysis than from 50% on. It was suggested that the rapid stage of the reaction might be concerned with the formation of digalacturonic acid, and the second slow stage with the hydrolysis of digalacturonic acid to galacturonic acid. It was found that galacturonic, di- and trigalacturonic, and probably tetragalacturonic acids appeared in the very early stages of hydrolysis. Tetragalacturonic acid was present up to about 72% hydrolysis, but traces of trigalacturonic acid were demonstrated when 95% of the bonds were hydrolyzed. Traces of digalacturonic acid were still present at 97% hydrolysis. These results suggest a somewhat random attack of polygalacturonic acid by polygalacturonase. No differences in initial rates and apparent course of hydrolysis of polygalacturonic acid substrates with polygalacturonase were observed within the limits of molecular weights of 1900 to 35,000.

Methods and Materials

Polygalacturonic acids of several molecular weights were prepared from commercial citrus pectin (Pectinum N.F. VII, manufactured by Sunkist Growers, Ontario, Calif.).

Enzyme-de-esterified polygalacturonic acid was made with orange pectinesterase (PE) by the method of MacDonnell, Jansen, and Lineweaver (*14*). Pectin in 1% solution plus orange pectinesterase was maintained at pH 6.0 by adding dilute sodium hydroxide over a period of an hour. No further additions of alkali were then necessary to maintain pH 6.0 and hydrochloric acid was added to pH 1.5. The precipitated polygalacturonic acid was pressed free of most of the solution, washed once in an equal volume of water, pressed, and washed in 60% ethyl alcohol to remove hydrochloric acid. After the washings were essentially free of chloride ion, the polygalacturonic acid was further dehydrated with 95% ethyl alcohol, air-

Table I. Analyses of Pectin and Polygalacturonic Acids[a]

Substance	Anhydrouronic Acid, %	Methoxyl, %	Specific Rotation	Molecular Weight
Commercial citrus pectin	82	9.8	+230	35,000
Enzymic polygalacturonic acid	90	0.5	265	35,000
Acid-insoluble polygalacturonic acid	99	..	270	3,400
Acid-soluble polygalacturonic acid	98	..	270	1,900

[a] Analyses expressed on water-free and ash-free basis.

dried, and ground for analysis.

Hot dilute acid was used to prepare two polygalacturonic acids of high anhydrouronic acid content and low molecular weight as follows. To 2 liters of boiling 1*N* sulfuric acid was added 100 grams of pectin. Boiling while stirring was continued for 1 hour. The mixture was cooled and the acid-insoluble polygalacturonic acid was removed by filtration, washed free of sulfuric acid with 60% ethyl alcohol, further dehydrated with 95% ethyl alcohol, air-dried, and ground for analysis. A yield of 35 grams of acid-insoluble polygalacturonic acid was obtained.

To the sulfuric acid–containing filtrate was added 3 volumes of 95% ethyl alcohol. A white flocculent precipitate of degraded acid-soluble polygalacturonic acid was separated by filtration, washed free of sulfuric acid with 60% ethyl alcohol, dehydrated with 95% ethyl alcohol, air-dried, and ground for analysis. Twenty grams of acid-soluble polygalacturonic acid was obtained. Molecular weights of pectin and enzymic polygalacturonic acid were estimated by osmotic pressure (*18*) and those for the acid–de-esterified polygalacturonic acids were calculated from reducing groups measured by Jansen and MacDonnell's (*8*) modification of the Willstätter-Schudel method (*24*). Anhydrouronic acid determinations were made with a colorimetric carbazole method (*11*). A summary of the analyses of the original pectin and the polygalacturonic acids is shown in Table I. The molecular weights of the polygalacturonic acids vary considerably. The molecular weight of 35,000 for the enzymic polygalacturonic acid is the same as that for the original pectin, although 10% methoxyl (4.5% methylene) was removed by hydrolysis. One explanation is that the anhydrouronic acid content of the polygalacturonic acid is higher than the original pectin and suggests that about 5% of low-molecular-weight material was lost during the preparation of the enzymic polygalacturonic acid. The acid de-esterification treatment brought about an increase in anhydrouronic acid contents and a decrease in molecular weights of the resulting polygalacturonic acids as compared with the original pectin.

The acid-soluble and insoluble polygalacturonic acids are similar to some made by Ehrlich and Sommerfeld in 1926 (*6*) and by Newbold and Joslyn in 1952 (*16*)

To study the action of polygalacturonide-splitting enzymes a purified polygalacturonase was prepared by Rosie Jang of this laboratory from commercial Pectinol 100 D (Rohm and Haas Co.), using the method of Jansen and MacDonnell (*8*). The activity of this preparation was 0.027 millimole of bonds split, per minute per milligram of enzyme.

Dowex 50 cation exchange resin regenerated with 4% hydrochloric acid, washed free of chloride, and dried in air was used to convert salts of the uronic acids to free acids.

Qualitative and quantitative paper chromatograms were carried out with 45 × 55 cm. sheets of Whatman No. 1 filter papers (not humidified or previously conditioned) by ascending irrigation for 16 to 20 hours at 25° C., with a miscible solvent made with 5 volumes of ethyl acetate, 3 volumes of water, and 2.5 volumes of acetic acid. After irrigation the papers were dried in air for 4 hours or more without heating. They were then sprayed with bromocresol green indicator (0.04% bromocresol green in 95% ethyl alcohol adjusted to pH 6 with sodium hydroxide). The positions of the acidic substances were revealed as yellow bands or spots on a blue background. The colors were further intensified by momentary exposure to ammonia vapor.

The same or a duplicate sheet was then dipped in an ethyl acetate solution containing 2% aniline and 2% trichloroacetic acid, dried in air for a few minutes, and heated to 85° C. for 5 minutes. Flesh to light brown spots revealed the areas occupied by the reducing galacturonides. R_{GA} value is the ratio of distance of migration of a substance compared with galacturonic acid.

Separations of oligogalacturonides by paper chromatography on a macro scale were carried out on Eaton-Dikeman No. 301 filter paper sheets, 50 × 60 cm. and 0.03 inch in thickness. Ascending irrigation was used with the ethyl acetate–acetic acid–water solvent which reaches the top of these papers in about 8 hours or more. After irrigation, the papers were dried in air at room temperature for 8 hours or more to remove the acetic acid. The acidic substances were then revealed as yellow bands on a blue background after spraying with the bromocresol green indicator. As much as 3 grams of mixed oligogalacturonic acids has been separated on one 50 × 60 cm. sheet of the thick filter paper.

Three liters of 1% polygalacturonic acid solution adjusted to pH 5.0 with dilute sodium hydroxide solution was hydrolyzed with 0.01 gram of polygalacturonase for 72 hours at 25° C. Approximately 80% of the polyuronide bonds were split as determined by reducing power and qualitative paper chromatograms of the products of partial hydrolysis revealed the probable presence of galacturonic, di-, and trigalacturonic acids. The solution was heated to destroy the enzyme, cooled, and passed through Dowex 50. The acidic effluent was one third neutralized with sodium hydroxide and then adjusted to pH 7 with strontium hydroxide and concentrated to 100 ml. in vacuo. Sodium strontium galacturonate hydrate crystallized after 24 hours at 25° C. Most of the galacturonic acid was thus removed and the remaining uronides were precipitated from solution in 60% ethyl alcohol and dried in air. This uronide fraction (about 15 grams) was dissolved in water and treated with 5 grams of Dowex 50 and the acidic solution was concentrated to a solids content of 30%. Two milliliters of the concentrate was streaked along the 60-cm. starting line of a 50 × 60 cm. sheet of thick filter paper and air-dried for 6 hours. The paper was irrigated, dried, and developed as described.

The acid bands from each sheet were excised and eluted with water by capillary flow (*7*). From 8 strips, about 100 ml. of solution was collected from each series of bands, mixed with 3 grams of Dowex 50 and 0.5 gram of decolorizing carbon, and filtered. The colorless solutions containing the apparent galacturonic, di-, and trigalacturonic acids were neutralized to pH 7.0 with 30% brucine in ethyl alcohol. Five grams of brucine galacturonate hydrate, first prepared by Ohle and Berend (*17*), 15 grams of dibrucine digalacturonate hydrate, and 10 grams of tribrucine trigalacturonate hydrate crystallized upon evaporation. The supposed tetragalacturonic acid had previously failed to yield good crystals with brucine and was evaporated to dryness in air as the free acid.

Results

In order to characterize these uronic acids it was desirable to remove the brucine. The free acids were, therefore, regenerated from their crystalline brucine salts by treatment with Dowex 50 and analyzed. A summary of the analyses is shown in Table II.

The ratio of carboxyls to reducing groups is near to the values calculated for the galacturonic and oligogalacturonic acids. Ehrlich's lead acetate

Table II. Analyses of Oligogalacturonides as Free Acids

Substance	Molar Ratio, —COOH / —CHO	$[\alpha]_D$ at Equilibrium	Lead Acetate Precipitate	R_{GA}
Galacturonic acid	1.01	+51.3	Red	1.00
Digalacturonic acid	2.02	153	Orange	0.50
Trigalacturonic acid	3.01	186	Light orange	0.25
Tetragalacturonic acid[a]	4.16	208	Yellow	0.12
Pectic acid[b]	10.4	270	Yellow	0

[a] Assumed to be tetragalacturonic acid from position on paper chromatogram.
[b] Average degree of polymerization 10, mol. wt. about 1900.

test (*5*) gave precipitates of brick red, orange, light orange, and yellow as the degree of polymerization increased. The specific rotations at equilibrium were all positive and increased with increasing degree of polymerization to a value of +270 (c = 1%), with a polygalacturonic acid of average degree of polymerization of 10.

Action of Polygalacturonase On Polygalacturonic Acids

Three substrates of different molecular weights were hydrolyzed at pH 5 and 26° C. in 1% concentration with polygalacturonase. Samples of the reaction mixture were removed and the enzyme was destroyed by heating to 100° C. for 1 minute. The rates were followed by measuring the increase in reducing power, and the results are shown in Figure 1. The experimentally determined points of the three substrates at the times indicated are superimposed within the circles shown in Figure 1.

Examination of Figure 1 shows that the initial rates and extent of hydrolysis of these polygalacturonic acids were substantially unaffected by differences in their molecular weights within this range. Jansen and MacDonnell (*8*) suggested from the kinetics that the second stage might be concerned with the hydrolysis of digalacturonic acid. In order to examine the products of reaction during the course of the hydrolysis aliquots of the reaction mixture were heated to boiling, cooled, treated with Dowex 50, concentrated, and qualitatively chromatographed on 45 × 55 cm. sheets of Whatman No. 1 filter papers. Irrigation, drying, and development of the paper sheets were done as described.

The qualitative results of the hydrolysis of acid-insoluble polygalacturonic acid with polygalacturonase are shown in the tracing of a typical chromatogram (Figure 2).

In the early stages up to 40% hydrolysis, galacturonic acid and a series of oligouronides are produced. From 45 to 57% hydrolysis, spots which correspond to galacturonic, di-, and trigalacturonic acids and probably tetra- and pentagalacturonic acids are shown. Tetragalacturonic acid is still present up to at least 72% and trigalacturonic acid disappears at 95% hydrolysis. Digalacturonic acid can still be demonstrated by paper chromatography after about 97% of the bonds are hydrolyzed.

In order to obtain further information on the course of the reaction in the early stages of hydrolysis, quantitative determinations of the separated products of partial hydrolysis of polygalacturonic acid with polygalacturonase were made. Five-milliliter aliquots of a digest were removed, heated rapidly to boiling to inactivate the enzyme, cooled, treated with Dowex 50, evaporated to dryness in air, and dissolved in a known volume of water. Five microliters of concentrate (adjusted to contain about 2.5% reducing oligouronides) was placed in duplicate 8 cm. apart on the starting line 2.5 cm. above the solvent on a 45 × 55 cm. sheet of Whatman No. 1 filter paper. Standards of known concentrations of galacturonic and oligogalacturonic acids were run simultaneously. The irrigated sheets were dried in air and test strips were excised from the sheet and developed with the aniline–trichloroacetic acid solution. Areas 2.5 × 8 cm., similar in position to those indicated by the test strips, were excised and eluted between microscope slides by capillary flow. Less than 1 and more than 0.5 ml. was collected from each strip. This eluate was made to 1.00 ml. and the uronic acids were determined by a colorimetric carbazole method. From 20 to 80 micrograms of uronic acid per spot were determined as anhydrouronic acid.

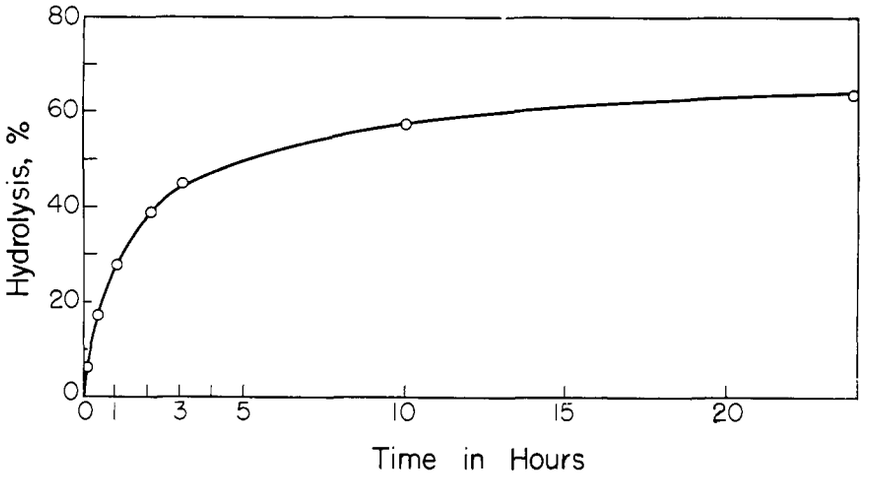
The early stages of the course of the action of polygalacturonase on polygalacturonic acid are shown in Figure 3. Galacturonic, di-, tri-, and probably tetragalacturonic acids appear in the very early stages of hydrolysis. At slightly above 50% hydrolysis, as measured by reducing power, the tetragalacturonic acid begins to decline in concentration. The concentration of trigalacturonic acid appears at its maximum near 62% hydrolysis and then slowly declines. Meanwhile, the concentration of di- and galacturonic acids slowly increases at the expense of the other oligogalacturonides. These results are in agreement with the qualitative results, demonstrating the reaction course of glycosidic hydrolysis of polygalacturonic acid by polygalacturonase.

Discussion

Polygalacturonase, a glycoside-splitting enzyme that degrades polygalacturonic acid to galacturonic acid, occurs in microorganisms, yeast, and higher plants. The enzyme appears to have different pH optima, depending upon the source. A series of oligogalacturonides produced from fungal pectic enzymes acting on pectic substances was first demonstrated by Jermyn and Tomkins (*9*), using paper chromatography. The fungal polygalacturonase purified by the method of Jansen and MacDonnell (*8*) was shown to act on polygalacturonic acids of either low or high molecular weights and to produce galacturonic and a series of oligogalacturonic acids in a like manner. The action of the polygalacturonase used in this work resembles that of the polygalacturonase of Altermatt and Deuel (*1*).

Recently Schubert (*22*) postulated that polygalacturonase preparations from mold were complexes containing four distinct enzymes, one of which resembles the polymethylgalacturonase (PMG) of Seegmiller and Jansen (*23*). Ayers *et al.* (*3*) and Dingle *et al.* (*4*) using chromatographic and "cup plate assay" methods

Figure 1. Production of reducing groups *vs.* time during polygalacturonase hydrolysis of polygalacturonic acid of varying molecular weights



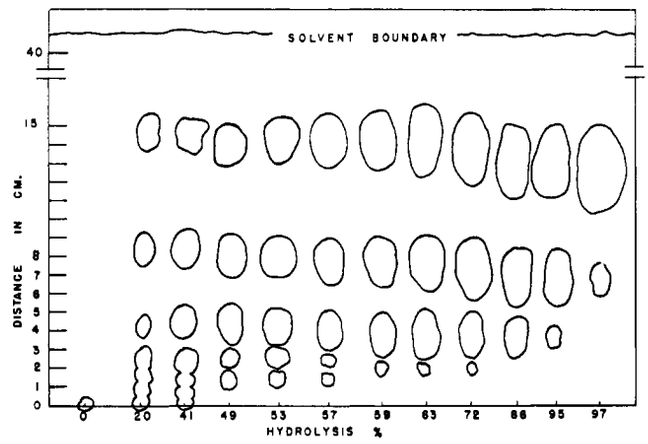


Figure 2. Tracing of qualitative paper chromatogram showing uronides produced during polygalacturonase hydrolysis of polygalacturonic acid

for analyzing mold enzyme preparations concluded that their crude polygalacturonase preparations contained at least three pectin-degrading enzymes. One seemed to be similar to the polymethylgalacturonase of Seegmiller and Jansen (*23*) and Roboz *et al.* (*20*); two other enzymes were also involved in the degradation of pectic acid to galacturonic acid. One enzyme seemed to convert pectic acid to di- and trigalacturonic acids; the other hydrolyzed only di- and trigalacturonic acids to galacturonic acid. The action of the first enzyme slightly resembled yeast polygalacturonase described by Luh and Phaff (*10*).

The polygalacturonase used in the present work was substantially free of polymethylgalacturonase, and hydrolyzed high polymers, as well as di- and trigalacturonic acids, to galacturonic acid. There seems to be no justification at present for postulating the presence of more than one active polygalacturonide-degrading enzyme in this preparation.

Matus in 1948 (*15*) demonstrated that the extent of hydrolysis of polyuronic acids with a pectic enzyme was greater with high-molecular-weight substrates than with the lower ones. A drop in velocity of hydrolysis accompanied a drop in molecular weight of substrate. In the present work no differences were observed in rate or apparent course of the hydrolysis of polygalacturonic acid with polygalacturonase from the range of molecular weights of 1900 to 35,000.

The apparent differences between this work and that of Matus may be explained by the differences in the substrates. Matus used partially enzymically hydrolyzed substrates which contained substantial amounts of low-molecular-weight oligogalacturonides. There seems to be no measurable reaction-product inhibition under the conditions of the reaction, yet the rate of hydrolysis of di- and trigalacturonic acids is only 5% of that of a high-molecular-weight polygalacturonic acid (*13*). The polygalacturonic acid substrates used in work reported contained no measurable low-molecular-weight oligouronides.

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Figure 3. Production of galacturonic, di-, tri-, and tetragalacturonic acids during initial rapid stage of polygalacturonase hydrolysis of polygalacturonic acid