STUDIES ON THE PECTIC ENZYMES
II. ON THE SPECIFICITY OF POLYGALACTURONASES

By

J. OZAWA and K. OKAMOTO

Introduction

In a previous paper (1) it was shown that the ratios of reducing group increase to viscosity decrease are by no means constant when the pectic substances are attacked by the enzymes. Therefore, different types of polygalacturonases obviously exist; some are mainly liquefying, and in others the saccharifying activity is predominant. We reported previously that the two polygalacturonases in the crude enzyme solution of Penicillium expansum can be separated more or less completely by alcoholic fractionation (2). Present work is an extension of former work and describes more precise mechanism of enzymic hydrolysis of pectin.

Materials and Methods

Pectin (A). The dried marc of Satsuma orange was extracted with hot water and pectin was precipitated with three volumes of 95% alcohol containing 0.05N hydrochloric acid. The precipitate was dissolved and precipitated again with alcohol. After repeated washing, the resulting pectin was dried at 45°C.

Norris pectic acid. The dried peel of Satsuma orange, after preliminary heating with 0.2% calcium chloride and washing with water, was extracted with dilute hydrochloric acid (pH 2.2) at boiling point for 15 minutes. The filtrate was passed through a column of cation exchange resin to remove calcium ion. Active carbon was used for decolorization of the extract. Pectin was precipitated with alcohol containing a small quantity of hydrochloric acid (The final alcohol concentration in the mixture was about 60%). The precipitate was filtered, washed with 70% alcohol and finally treated with absolute alcohol. The dried precipitate was dissolved to a concentration of 1% and an equal volume of N/10 sodium hydroxide was added to the solution. After standing for 1—1.5 hours at room temperature, the pectic acid was precipitated by adding the minimum quantity of hydrochloric acid necessary to effect complete precipitation. The precipitated gel was then redis-

solved in a minimum of dilute sodium hydroxide solution and reprecipitated with hydrochloric acid. It was then dissolved and precipitated three more times. After thoroughly washing with water and alcohol, the precipitate was dried at room temperature (3). The preparation was found to contain 96.1% of uronic anhydride.

Pectic acid (F_2). Pectin (A) was dissolved in 1% hydrochloric acid and maintained at 85°C. After 30 minutes, the mixture was cooled, made alkaline by adding sodium hydroxide solution to a concentration of 0.05N and maintained at 15°C for one hour. The gel precipitated by adding hydrochloric acid, was washed with water, redissolved in 0.2N sodium hydroxide and reprecipitated with hydrochloric acid. The precipitate was filtered and washed successively with water and alcohol. The precipitate was dried at room temperature. The uronic anhydride content of pectic acid (F_2) was 90.5%.

Pectic acid degraded by hydrochloric acid. 2% solution of pectin (A) was maintained at 85°C in the presence of 5% hydrochloric acid. After 6 hours a precipitate was collected and washed with 40% alcohol. This was dissolved and neutralized with dilute sodium hydroxide solution. Furthermore an equal volume of N/10 sodium hydroxide was added to the solution. After 45 minutes standing at 15°C, pectic acid was precipitated with hydrochloric acid. The precipitate was washed with 40% alcohol until the wash liquid was free from the chloride, and then dried at room temperature. The preparation was found to contain 93.2% of uronic anhydride.

Di- and trigalacturonic acid. The digest of pectic acid by the Rh. tritici polygalacturonase was treated with active carbon, passed through a column of cation exchange resin and concentrated under reduced pressure. To the concentrate, three volumes of alcohol was added to remove high molecular weight polyuronides. The filtrate was evaporated at 35°C and separated on a column of cellulose. n-Butanol. acetic acid. water (10 : 3 : 5) was used as the mobile phase. After an examination of small portions of the eluate on the paper chromatogram, the eluate was divided in such a manner as to lead to the highest possible recovery of each constituent (4). The solvent was removed at 15°C and neutralized with barium hydroxide and barium carbonate. Barium salts of di- and trigalacturonic acids were purified by the method of Link and Nedden (5). From these, free galacturonides were prepared by adding sulphuric acid and their sodium salts were used as substrates (6).

Pectic acid oxidized by hypoiodite. 20 ml. of N/10 I_2 solution and 30 ml. of N/10 sodium hydroxide solution were added to 66 ml. of 2% Norris pectic acid which had been neutralized previously with sodium hydroxide. After 30 minutes standing, the mixture was made to pH 5.0 with hydrochloric acid and precipitated with alcohol. The precipitate was dried at room temperature (7).

Methylglycoside of polygalacturonic methyl ester. This was prepared as described by Morell, Baur and Link (8).

Pectic acid degraded by B. mesentericus polygalacturonase. This was prepared by acting on 0.5% Norris pectic acid with the B. mesentericus enzyme at pH 7.0, until the degree of hydrolysis was 21.5%. At this stage, acetone was added to the digest. The precipitate was centrifuged, dissolved and reprecipitated, adding excess of $CaCl_2$ and alcohol. This was washed successively with 60% alcohol and 94% alcohol containing 0.05N hydrochloric acid, and then redissolved. The solution was passed through a column of cation exchange resin and precipitated with acetone. The precipitate was dried at room temperature. 1g. of this substrate was found to consume 11.0 ml. of 0.1N I_2 solution. The specific viscosity of 0.5% solution was 0.085 at pH 5.0.

Pectic acid degraded by carrot polygalacturonase. The carrot enzyme was allowed to act on Norris pectic acid, until the degree of hydrolysis was 13.0%. The digest was treated with the cation exchange resin, evaporated under reduced pressure and precipitated with 5 volumes of alcohol. The precipitate was washed with 80% alcohol and dried at room temperature. 1g. of this substrate consumed 3.9 ml. of 0.1N I_2 solution. The specific viscosity of 0.5% solution was 1.022 at pH 5.0.

B. mensentericus polygalacturonase. A crude enzyme solution was prepared by inoculating B. mesentericus in the potato decoction to which 0.5% peptone was added. The culture was allowed to grow at 35°C for 2 days. The cells were then removed by centrifugation. Partial purification was tried by alcoholic precipitation. The dried enzyme was dissolved in 100 times its weight of water.

Carrot polygalacturonase. Carrots were macerated, dispersed in 3—5 volumes alcohol, left standing in the ice chest for 2 hours, filtered, washed repeatedly with 80% alcohol and finally with 95% alcohol, and dried over $CaCl_2$. From this, the enzyme was extracted by adding 20 times its weight of water and standing for 12 hours at room temperature.

Tomato polygalacturonase. This was prepared by the same procedure as the carrot polygalacturonase.

Pen. expansum polygalacturonase. Pen. expansum was grown at 27°C on a medium which contained pectic acid (partially decomposed by the liquifying polygalcturonase of Rh. tritici) 2%, NH_4NO_3 0.5%, KH_2PO_4 0.3%, $MgSO_4 7H_2O$ 0.05% and yeast water 1/10 volume. After 5 days incubation, the mycelium was harvested, and the enzyme solution was prepared as described previously (2).

Reducing power. This was measured by the method of Willstätter and Schudel.

Viscosity. The viscosity determinations were performed at 25°C, using Ostwald viscosity pipets.

Paper chromatography. 0.02—0.05 ml. of hydrolysate was applied to the Toyo filter paper (No. 52, 2×40cm). The chromatogram was run with n-butanol·acetic acid·water (5 : 2 : 3) at 20°C, dried, sprayed with aniline hydrogen phthalate and heated at 95°C.

Quantitative paper chromatography of D-galacturonic acid. The pH of the digest was adjusted to 3.6 with acetic acid. 0.2—0.4 ml. of the digest was put on to the Toyo filter paper (No. 52, 20×40cm). The solution was applied in a line across the top of the paper about 5cm from the top edge, and some 10 cm long. The chromatogram was run by a single development with n-butanol·acetic acid·water (5 : 2 : 3). The developed chromatogram was air-dried at room temperature. The guide strips were cut off and sprayed with aniline hydrogen phthalate. From the unsprayed strip, the section corresponding to the spot of D-galacturonic acid was cut off and eluted with water (9). The eluate was concentrated at 45°C to give about 7.5 microgram of D-galacturonic acid per ml. To 2 ml. of the concentrated eluate, 2 ml. of 0.2% naphthoresorcinol solution and 2 ml. of concentrated hydrochloric acid was added. The mixture was heated in a boiling water bath for 45 minutes, then cooled in cold water for 10 minutes. 2 ml. of 95% alcohol and 15 ml. of ether (after washing with a 1% solution of ferrous sulfate to remove oxidizing agents) were added and the mixture was shaken vigorously in a glass-stoppered flask for 1 minute. The light transmission of the ether solution was measured by Pulfrich photometer. A blank and controls were run simultaneously (10).

Calcium pectate method. 2 ml. of the digest was diluted with water to a volume of 20 ml., and then 25 ml. of 1N acetic acid and 25 ml. of 1M calcium chloride solution were added. The mixture was boiled for 2 minutes and filtered through a glass crucible with a fritted glass bottom (G—3) which had been dried and weighed previously. The precipitate was washed with hot water until the wash liquid was free from the chlorides, and dried overnight at 100°C.

Results and Discussion

Specificity of polygalacturonases of tomatoes and B. mesentericus. The nature of tomato polygalacturonase differs from that of the fungal enzyme in some fundamental aspects. The tomato enzyme causes the complete viscosity loss of pectic acid solution at much lower reducing power values than fungal polygalacturonase. Furthermore, hydrolysis by the tomato enzyme proceeds only to what appears to be partial degradation. Kertesz thought that the tomato polygalacturonase is either specific for large polygalacturonic acid molecules or causes some type of fissures different from that effected by the fungal enzyme (11). We observed that the B. mesentericus polygalacturonase resembles the tomato enzyme in the abovementioned respects (7). In

order to investigate the specificity of the polygalacturonases of tomatoes and B. mesentericus, the products of pectic acid by both enzymes and their digalacturonase activity were tested. The results are shown in Tables 1 and 2.

Table 1. *Action of tomato polygalacturonase on pectic acid and digalacturonic acid* (N/50 I_2, ml.)

Reaction time (hrs.)	Pectic acid				Digalacturonic acid
	Relative viscosity	Reducing power	Digalacturonic acid	D-Galacturonic acid	
0	1.808	—	—	—	—
9	1.112	0.44	—	—	—
18	1.076	0.82	+	+	0
36	1.065	1.47	+	+	0

The digests were incubated at 35°C, at pH value of 5.0. They contained 2% pectic acid (F_2) (6.6 ml.) or 2% digalacturonic acid (5.0 ml.), N/10 acetate buffer (2.0 ml.), the tomato enzyme (2.0 ml.) and water made up to 20 ml. Toluene was used as an antiseptic. After 0, 9, 18 and 36 hrs. 2 ml. aliquots were withdrawn and the reducing power was measured. A control was run by omitting the substrate.

Table 2. *Action of B. mesentericus polygalacturonase on pectic acid and digalacturonic acid* (N/50 I_2, ml.)

Reaction time (hrs.)	Pectic acid				Digalacturonic acid
	Relative viscosity	Reducing power	Digalacturonic acid	D-Galacturonic acid	
0	2.224	—	—	—	—
2	1.068	0.81	—	—	—
9	1.058	1.08	+	+	0
24	1.058	1.10	+	+	0

The digests were incubated at 35°C, at pH value of 7.3, containing 2% pectic acid (F_2) (6.6 ml.) or 2% digalacturonic acid (5.0 ml.), N/10 phosphate buffer (2.0 ml.), B. mesentericus enzyme (1.5 ml.) and water made up to 20 ml.

As shown in Tables 1 and 2, digalacturonic acid was not hydrolyzed by both enzymes. This was confirmed by the chromatograms of hydrolysates. Mono- and digalacturonic acids were absent in the initial hydrolysates of pectic acid, but appeared thereafter. Trigalacturonic acid was also detected in both digests of pectic acid.

Recently Roboz et al. (12, 13) have reported on a new enzyme named polymethylgalacturonase which rapidly reduces the viscosity of pectin solution with the hydrolysis of some glycosidic bonds. This enzyme differs from other reported enzymes in that it degrades pectin without preliminary demethoxylation. The tomato polygalacturonase has never been reported to attack pectin without previous deesterification. Our experiment proved that the B. mesentericus polygalacturonase is distinct from polymethylgalacturonase because of inability to decompose the methylglycoside of polygalacturonic

methyl ester. Tomato and B. mesentericus enzymes differ from fungal PG in that they cause an incomplete hydrolysis of pectic acid. As described above, tomato and B. mesentericus enzymes have some fundamental properties in common. We are of opinion that the two enzymes are identical, and that they belong to a liquefying polygalacturonase. For this, a new name "polygalacturonase I" was provisionally proposed (6).

Specificity of carrot polygalacturonase.

The saccharifying capacity of carrot polygalacturonase, compared to the liquefying power, was found to be extremely higher than that of bacterial polygalacturonase or tomato polygalacturonase. In order to compare the properties of carrot polygalacturonase with those of other enzymes, the fission products of pectic acid were tested by the paper chromatography (6). The result are presented in Table 3.

Table 3. *Action of carrot polygalacturonase on pectic acid* (N/50 I_2, ml.)

Reaction time (hrs.)	Relative viscosity	Reducing power	Digalacturonic acid	D-Galacturonic acid
0	1.851	0	−	−
9	1.787	0.43	−	+
24	1.685	0.88*	−	+
48	1.622	1.24	−	+

The digest was incubated at 35°C and pH value of 5.0, contained 2% pectic acid (F_2) (6.6 ml.), N/10 acetate buffer (2.0 ml.) and the enzyme solution (11.4 ml.).

A spot corresponding to D-galacturonic acid was found from the beginning. But di- and trigalacturonic acids were not seen on all chromatograms. 1.5 ml. of the digest after 24 hours reaction (Table 3*) was put on to the Toyo filter paper (No. 52, 2×40 cm) and the chromatogram was run under the same conditions as described previously. Di- and trigalacturonic acids were not detected. But it should be clearly understood that the absence of a spot does not necessarily mean the complete absence of corresponding sugar, but that the quantity present was less than a certain amount. This, in the standard development procedure used, was about 10 and 20 microgram for digalacturonic acid and trigalacturonic acid respectively. Therefore it is concluded that 1.5 ml. of the digest (Table 3*) contains digalacturonic acid less than 10 microgram, trigalacturonic acid less than 20 microgram while D-galacturonic acid as much as 1200 microgram. This could be interpreted by either one of the following hypotheses:

(a) The carrot enzyme degrades the pectic acid chain from the end group liberating D-galacturonic acid.

(b) From the chain end of pectic acid, the carrot polygalacturonase liberates the digalacturonic acid which is converted to D-galacturonic acid by a second enzyme, digalacturonase.

(c) The carrot polygalacturonase attacks the glycosidic linkages of

pectic acid in a random manner forming low polymers and oligosaccharides which are hydrolyzed to the monomer by digalacturonase.

The hypothesis that the polygalacturonase liberates D-galacturonic acid directly (a), is supported by the evidence obtained from the following experiments.

Experiment 1. The hydrolysis of pectic acid by the carrot enzyme was followed by measurements of reducing power, amount of D-galacturonic acid formed and loss of substrate (14). The results obtained are given in Table 4.

Table 4. *Amount of fission products of the carrot polygalacturonase on pectic acid.*

Reaction time (hrs.)		10	24	30
Sugar produced (N/50 I_2, ml.)		0.57	0.85	0.87
D-Galacturonic acid (mg.)	calculated	1.10	1.65	1.69
	found	1.08	1.65	1.67
Pectic acid decomposed (as Ca-pectate, mg.)	calculated	1.1	1.7	—
	found	1.2	1.9	—

The digest was made up to contain 6.6 ml. of 2% Norris pectic acid, 11.4 ml, of the enzyme solution and 2 ml. of N/10 acetate buffer to adjust the solution to pH 5.0. The mixture was kept at 35°C.

Amount of D-galacturonic acid and loss of substrate measured were in good agreement with the quantities calculated from the reducing power of the reaction mixture.

Experiment 2. The hydrolytic activities of carrot enzyme on several substrates were investigated (7). The results are shown in Table 5.

Table 5. *Hydrolysis of pectic acid, digalacturonic acid, pectic acid oxidized by hypoiodite, pectin and methylglycoside of polygalacturonic methyl ester, by the enzyme solution of carrots* (N/50 I_2 ml.)

Reaction time (hrs.)		0	5	12	22
		2.089	1.975	1.895	1.860
Pectic acid	0.66%	—	0.28	0.52	0.65
	0.17%	—	0.16	0.22	0.23
	0.20%	—	—	0.13	0.20
Digalacturonic acid	0.05%	—	—	0.05	0.07
Pectic acid oxidized by hypoiodite		—	0.27	0.50	0.64
Pectin		—	—	—	0.05
Methylglycoside of polygalacturonic methyl ester		—	—	—	0

The digests, incubated at 35°C and pH 5.0, contained Norris pectic acid, digalacturonic acid, or other substrates (0.66%), N/10 acetate buffer (2.0 ml.), carrot enzyme (11.4 ml.) and water made up to 20 ml. Relative viscosity was determined after adding 1/10 volume of 1% ammonium oxalate.

As shown in Table 5, the carrot polygalacturonase hydrolyzed only about 14% of the glycosidic bonds in Norris pectic acid. This observation

might indicate that the carrot enzyme degrades the pectic acid from the end group forming a "limit polygalacturonide" comparable to the "β- dextrin" obtained from starch.

Experiment 3. The pectic acid (F_2) in the digest (Table 3*) was replaced by 0.0815% of digalacturonic acid and the same enzyme solution was allowed to act on the latter under the same conditions as in the case of the pectic acid. (2 ml. of this reaction mixture contains 1.63 mg. of digalacturonic acid. From 1.63 mg. of digalacturonic acid, 1.71 mg. of D-galacturonic acid is produced by the complete hydrolysis. 1.71 mg. of D-galacturonic acid consumes 0.88 ml. of N/50 I_2 solution.) After 24 hours action, chromatogram showed that the digalacturonic acid had not disappeared. This favours hypothesis (a) and can not be satisfactorily acounted for by the hypothesis that pectic acid is first hydrolyzed to digalacturonic acid and the latter then converted to D-galacturonic acid by the carrot enzymes (b).

Experiment 4. Since the degree of hydrolysis of pectic acid by the carrot enzyme did not exceed 14%, it was thought to be of interest to determine whether the addition of liquefying enzyme of tomatoes would increase the action of carrot enzyme on pectic acid. A small amount of tomato enzyme was added to carrot enzyme and allowed to act on pectic acid. The degree of hydrolysis was compared with that by each enzyme. The results obtained are given in Table 6.

Table 6. *Effect of added tomato polygalacturonase on the hydrolysis of pectic acid by carrot polygalacturonase* (N/50 I_2, ml.)

Reaction time (hrs.)	5	15	25
Carrot polygalacturonase (10.0 ml.)	0.30	0.35	0.36
Tomato polygalacturonase (0.1 ml.)	0.02	0.05	0.10
Carrot polygalacturonase (10.0 ml.) + Tomato polygalacturonase (0.1 ml.)	0.31	0.88	1.25

Enzymes were incubated with Norris pectic acid (0.5%) and N/10 acetate buffer (2.0 ml.) at 35°C and pH 5.0; total volume of the digests, 20 ml.

The results show that the addition of tomato enzyme increases the action of carrot enzyme on pectic acid at a rate greater than the sum of the component rates and that the degree of hydrolysis of pectic acid by carrot enzyme exceeded 14%. These results support hypothesis (a) and can not be interpreted by the hypothesis that pectic acid is first degraded to low polymer by the liquefying polygalacturonase and the latter then converted to D-galacturonic acid by digalacturonase (c).

From the foregoing results, it is reasonable to conclude that the carrot polygalacturonase degrades the pectic acid from the non-reducing end group liberating D-galacturonic acid. The carrot enzyme differs from previously described pectic enzymes. It differs from fungal polygalacturonase (PG) in

its mode of hydrolysis of pectic acid. The latter has been reported to cause complete hydrolysis of pectic acid at random scission. Carrot polygalacturonase differs from polymethylgalacturonase because it is incapable of attacking pectin without previous deesterification (Table 5). Carrot polygalacturonase is also distinct from polygalacturonase I in the end products of hyrdolysis. At the present state of purification of carrot polygalacturonase, there can be no critical evidence that the same enzyme is responsible for the hydrolyses of pectic acid and digalacturonic acid. However, no separation of these properties has been observed during fractional alcoholic precipitation and adsorption by active carbon. We considered carrot polygalacturonase to be a new enzyme and proposed to call it 'polygalacturonase II" (6).

Action of polygalacturonases on degraded pectic acids *Enzymatic hydrolysis of the pectic acid previously degraded by hydrochloric acid.* There is some indication, from physical and chemical measurements, that the polygalacturonic acid units, formed exclusively by 1.4 linkages, are further combined in some manner. This might include branching or laminated structures such as were shown to exist in starch, or any other type of association between the individual polygalacturonic acids. Kertesz (15) holds the view that the primary polygalacturonic acid chains $(G)_n$ associate into larger units $[(G)_n]_m$ by linkages which are thought to be more vulnerable to destruction than the 1, 4 glycosidic linkages connecting the adjoining individual anhydrogalacturonic acid units. The formation of such more complex structures or molecular associations is the property of long chains containing more than a minimum number of anhydrogalacturonic acid molecules. When such chains are reduced in length below this minimum, the secondary structure may also be destroyed. Kertesz's work on the hydrolysis of pectic acid by the tomato enzyme indicates the formation of some limit polygalacturonides reminiscent of the limit dextrins obtained from starch. Roboz et al. have reported that by the action of polymethylgalacturonase on pectic acid, units of polygalacturonic acid giving about 7 or 22 anhydrogalacturonic acid residues can be obtained. This observation might be regarded as a further indication of the existence of different types of interpolygalacturonic acid linkages in pectin. But much systematic study is needed before we shall have any definite knowledge of all types of linkages which participate in the building of large polygalacturonic acid molecules. In the present paper, the molecular structure of pectin is discussed from the enzymological standpoint. Tables 7 and 8 give the results which were obtained from the study on the enzymatic decomposition of pectic acid previously degraded by hydrochloric acid (16).

The results, presented in Tables 7 and 8, show that the degraded pectic acid is hydrolyzed more rapidly than Norris pectic acid by all the enzymes, and that the degree of hydrolysis of the former by the carrot enzyme exceeds 14%.

Enzymatic hydrolysis of the pectic acid previously degraded by poly-

Table 7. *Action of the enzyme solutions of B. mesentericus and tomatoes on pectic acid previously degraded by hydrochloric acid* (N/50 I_2, ml.).

Reaction time (hrs.)		3	14	21
B. mesentericus enzyme	Degraded pectic acid	1.63	1.76	1.87
	Norris pectic acid	0.64	1.02	1.10
Tomato enzyme	Degraded pectic acid	0.73	1.60	1.80
	Norris pectic acid	0.48	1.43	1.63

The digests were incubated at 35°C, at pH values of 7.0 for the B. mesentericus enzyme and 5.0 for the tomato enzyme, and contained substrate (100 mg.), buffer of McIlvaine (2ml.) for the B. mesentericus enzyme and N/10 acetate buffer (2 ml.) for the tomato enzyme, enzyme (8 ml.); total volume of the digests, 20 ml.

Table 8. *Action of the enzyme solutions of carrots and Pen. expansum on pectic acid degraded by hydrochloric acid* (N/50 I_2, ml.)

Reaction time (hrs.)		14	28	40
Carrot enzyme	Degraded pectic acid	0.64	1.23	1.69
	Norris pectic acid	0.39	0.67	0.69
Pen. expansum enzyme	Degraded pectic acid	4.50	4.50	4.50
	Norris pectic acid	1.29	1.98	2.38

The enzymes of carrots and Pen. expansum were allowed to act on the substrates under the same conditions as in the case of tomato enzyme in Table 7. The Pen. expansum enzyme was in general of the saccharifying polygalacturonase type, but it appeared to contain a small amount of liquefying polygalacturonase.

galacturonases.

Matus has demonstrated that fungal polygalacturonase seems to possess increased affinity toward pectic acids of high average molecular weights. Our results, obtained from the study on affinity of polygalacturonases to the pectic acids degraded by enzymes, are shown in Tables 9 and 10.

As in Table 9, the pectic acid previously degraded by the carrot enzyme was hydrolyzed by B. mesentericus and tomato enzymes as readily as Norris pectic acid, whereas by carrot enzyme it was decomposed with great difficulty. As shown in Table 10, the enzymes of tomatoes and Pen. expansum hydrolyzed the pectic acid previously degraded by the B. mesentericus enzyme,

Table 9. *Enzymatic hydrolysis of the pectic acid previously degraded by carrot enzyme* (N/50 I_2, ml)

Reaction time (hrs.)		8	20	40
B. mesentericus enzyme	Degraded pectic acid	0.88	0.88	0.88
	Norris pectic acid	0.96	0.99	0.99
Tomato enzyme	Degraded pectic acid	1.25	1.79	2.06
	Norris pectic acid	1.25	1.90	2.19
Carrot enzyme	Degraded pectic acid	0.08	0.09	0.09
	Norris pectic acid	0.36	0.62	0.64

Table 10. *Enzymatic hydrolysis of the pectic acid previously degraded by B. mesentericus enzyme* (N/50 I_2, ml.)

Reaction time (hrs.)		7	24	40
B. mesentericus enzyme	Degraded pectic acid	0.05	0.06	0.06
	Norris pectic acid	0.46	0.81	0.81
Tomato enzyme	Degraded pectic acid	0.45	0.84	0.86
	Norris pectic acid	1.01	1.80	1.98
Carrot enzyme	Degraded pectic acid	0.12	0.17	0.17
	Norris pectic acid	0.28	0.60	0.61
Pen. expansum enzyme	Degraded pectic acid	1.10	1.85	2.00
	Norris pectic acid	1.24	2.61	3.13

The digests in Tables 9 and 10 were set up as stated in Tables 7 and 8, and incubated at 35 C°.

less readily than Norris pectic acid. Furthermore, the enzymes of B. mesentericus and carrots were found to decompose the former substrate very slowly or almost with negligible velocities.

The results in Tables 7 and 8 can be accounted for either one of the following possibilities:

(a) All the enzymes possess increased affinity to pectic acids of low molecular weights.

But this will not be the case, considering the results in Tables 9 and 10.

(b) The anomalous linkages in pectin molecule are split off by acid treatment.

This view seems to be supported by the results in Tables 9 and 10.

Norris pectic acid is not completely hydrolyzed; a more or less definite limit of saccharification is reached. Incomplete hydrolysis of Norris pectic acid by the carrot enzyme is not likely due to the low molecular weight of remaining polygalacturonides, because the remainder was found to possess the typical properties of high molecular polygalacturonides which might be regarded as a limit polygalacturonide reminiscent of the β-dextrin obtained from starch. On the other hand, the remainder is readily hydrolyzed by the liquefying polygalacturonase such as B. mesentericus and tomato enzymes. These results may be explained as follows:

Some anomalous linkages exist in the pectin molecule. The carrot enzyme degrades pectic acid chain from the end group and its action is arrested by the presence of anomalous linkages forming a limit polygalacturonide. The liquefying polygalacturonase can hydrolyze the normal linkages of inner chains of pectic acid between the anomalous linkages.

As shown in Table 10, the B. mesentericus enzyme also forms a limit polygalacturonide reminiscent of α-dextrin. Furthermore, the remaining polygalacturonide is not appreciably degraded by the carrot enzyme. It

appears therefore that both actions of carrot and B. mesentericus enzymes are arrested at the same anomalous linkages in pectin molecule.

The equivalent weight of polygalacturonide preparations has never reached the calculated values. (The equivalent weight was determined by titrating 0.5% solution of pectic acid to pH 7.6 with N/10 NaOH.) This may be taken as an indication that the secondary linkages involving the carboxyl groups exist in the macromolecule of pectin. We found that the equivalent weight of Norris pectic acid was larger than that of the pectic acid previously degraded by hydrochloric acid. The susceptibility of the latter to enzyme action (Tables 7 and 8) may be the result of the liberation by acid treatment of carboxyl groups which were engaged in the macromolecule of pectin.

Summary

1. It is verified that the nature of the two types of polygalacturonase named polygalacturonase I and polygalacturonase II, was quite different. The former enzyme attacked the middle parts of polygalacturonide-chain, while D-galacturonic acid was produced by the latter enzyme with which glycosidic linkages on the end of the chain was decomposed.

2. The existence of anomalous linkages in pectin molecule was suggested from the enzymological standpoint.

Acknowledgements

The authors wish to express their gratitude to Prof. H. Katagiri of Kyoto University for his advise and criticism.

Literature Cited

(1) Ozawa, J., et al. (1948) On the microbiological decomposition of pectin, VI. Nogaku Kenkyu, 38, 43. (In Japanese)

(2) Ozawa, J. (1951) Studies on the pectic enzymes, I. Action of pectinase. Ber. Ohara Inst., 9, 431.

(3) Norris, F. R. and Resch, C. E. (1937) The pectic substances of plants, V. Further observations on the nature of pectin and pectic acid. Biochem. J., 31, 1945.

(4) Hirst, E. L., Hough, L. and Jones, J. K. N. (1951) Constitution of mucilage from the bark of Ulmus Furva (slippery elm mucilage), III. The isolation of 3-Monomethyl D-galactose from the products of hydrolysis. J. Chem. Soc., p. 323.

(5) Link, K. P. and Nedden, R. (1931) Improvements in the preparation of D-galacturonic acid. J. Biol. Chem., 94, 307.

(6) Ozawa, J. (1952) Studies on the pectic enzyme, I, On the specificity of polygalacturonase. J. Agr. Chem. Soc. Japan, 26, 505. (In Japanese)

(7) Ozawa, J., Takeda, A. and Okamoto, K. (1953) On the microbiological decomposition of pectin, XVI. On the specificity of polygalacturonases. Nogaku Kenkyu, 41, 21. (In Japanese)

(8) Morell, S., Baur, L. and Link, K. P. (1934) The methylglycosides of the naturally occurring hexuronic acids, III. Polygalacturonic acid-methylglycosides derived from

pectin, J. Biol. Chem., 105, 1.
(9) Fukinbara, T. and Muramatsu, K. (1952) On the sugars in saké, I. Detection and determination by the Somogyi's procedure and paper chromatography. J. Agr. Chem. Soc. Japan, 26, 583. (In Japanese)
(10) Kertesz, Z. I. (1951) The pectic substances, p. 40.
(11) Kertesz, Z. I. (1951) The enzymes, 1, 745. Ed. by Sumner, J. B. and Myrbäck, K.
(12) Roboz, E., Barratt, R. W. and Tatum, E. L. (1952) Breakdown of pectic substances by a new enzyme from Neurospora crassa. J. Biol. Chem. 195, 459.
(13) Seegmiller, C. G. and Jansen, E. F. (1952) Polymethylgalacturonase, an enzyme causing the glycosidic hydrolysis of esterified pectic substances. J. Biol. Chem., 195, 327.
(14) Ozawa, J. and Okamoto, K. (1954) On the microbiological decomposition of pectin, XIX. The affinity of polygalacturonases toward degraded pectic acids (2). Nogaku Kenkyu, 41, 157. (In Japanese)
(15) Kertesz, Z. I. (1939) Pectic enzymes, IV. Structural considerations in connection with the enzymic hydrolysis of pectin. J. Am. Chem. Soc., 61, 2544.
(16) Ozawa, J. and Okamoto, K. (1954) On the microbiological decomposition of pectin, XVIII. The affinity of polygalacturonases toward degraded pectic acids. (1) Nogaku Kenkyu, 41, 128. (In Japanese)