ENZYMIC DEGRADATION OF PECTIC ACID

IV. Action of Carrot Exo-polygalacturonase on the Pectic Acids Prepared by Saponification of Pectin with Alkali and with Pectin Esterase.

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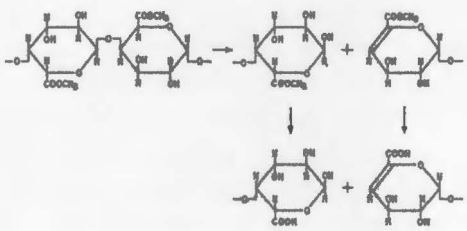
The instability of pectin in alkaline solutions is well known. Neukom and Deuel (8) suggested that the glycosidic bonds of pectin are split in alkaline solutions by a transelimination mechanism, unsaturated pectic acid (pectic acid having a 4, 5-unsaturated galacturonic acid unit at the non-reducing end of the molecule) being the major product of this reaction.



Since the pectic acid preparations commonly used for polygalacturonase (PG) assays are made by alkaline saponification, they probably contains the unsaturated pectic acid. This paper describes the results of a study made on the following subjects: (a) Rate of the formation of double bonds and the de-esterification in alkaline pectin solutions. (b) Activity of carrot exo-polygalacturonase (CPG) toward the unsaturated pectic acid. (c) Action of CPG on the pectic acids prepared by saponification of pectin with alkali at various temperatures and with pectin esterase (PE).

MATERIALS AND METHODS

1. *Enzymic Preparations*

CPG. This was prepared by a simplification of the method previously described (2). The steps omitted from the previous method were: (a) treatment with calcium phosphate gel, (b) elution with 0.005M solution of NaCl in 0.02M acetate buffer, pH 5, in the DEAE-cellulose chromatography and (c) CM-cellulose chromatography.

PE. The preparation was made from peels of *Citrus Unshiu* according

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to the method of Barrett and Northcote (1). It was dialyzed against 0.01M tris buffer, pH 8.2, containing NaCl (0.1M).

2. *Pectic Substances*

Pectin from peels of Citrus Unshiu. Peels of *Citrus Unshiu* were dipped in water (90°C) for 5 minutes and washed overnight with running tap water. They were then cut into small pieces and immersed in 1% ammonium oxalate under a layer of toluene. After standing overnight at room temperature the extract was filtered through diatomaceous earth. From the filtrate pectin was precipitated by adding an equal volume of ethanol. It was filtered on a Buchner funnel, washed successively with 50, 80 and 99% ethanol, and ether, and allowed to dry in air at room temperature.

Pectin from basts of hemp. Dried hemp basts, after being presoaked in cold water for a little while, were leached in batches by adding dilute hydrochloric acid and keeping the mixture at pH 2 for 2 hours, followed by decanting the leachings; the leaching was repeated three times at room temperature. Extraction of pectin and subsequent operations were done as described for the pectin from peels of *Citrus Unshiu.*

Citrus pectin. Citrus Pectin purchased from Nippon Kako Co. Ltd., was dissolved in water, precipitated by adding ethanol, washed with ethanol and ether as above, and dried in air at room temperature.

Pectic acid obtained by saponification of pectin with PE. Pectin was incubated with PE under the following conditions: substrate 0.25%, tris buffer (pH 8.2) 0.04M, NaCl 0.05%, temperature 15°C. To the mixture incubated for about 15 hours, was added an equal volume of ethanol. The precipitated pectic acid was washed well with 50% ethanol to remove enzymes and other impurities. It was further washed as described for pectin of *Citrus Unshiu* and dried at room temperature.

YPG•CPG-O. Procedure for preparation of YPG•CPG-O was the same as described previously (5) except that pectic acid was used in place of YGP-O and the incubation time was reduced from two to one week.

Unsaturated acid insoluble pectic acid. Citrus pectic acid was degraded by liquefying pectate transeliminase (9) until the reaction mixture gave a white amorphous precipitate with HCl. The reaction was stopped by adding acetic acid, the pH of the mixture being brought to 4.8. After the addition of CPG the reation mixture was further incubated for two days and then added to a DEAE-cellulose column, previously equilibrated with 0.1M carbonate buffer, pH 9.5. After washing with the same buffer unsaturated acid insoluble pectic acid was eluted from the column with 0.1M carbonate buffer, pH 9.73 (4). The eluate was brought to pH 5.0 with ion exchange resin and evaporated under reduced pressure. To the concentrate was added ethanol and the precipitate, after being collected with a centrifuge, dried at room temperature.

Unsaturated acid soluble pectic acid. This was prepared in the same manner as above with the exception that (a) the incubation with liquefying pectate transeliminase was stopped at the stage where the reaction mixture gave no precipitate with HCl and (b) in the chromatography the column was washed with 0.1M $NaHCO_3$ and eluted with 0.1M carbonate buffer, pH 9.5.

Acid insoluble pectic acid. The crude acid insoluble pectic acid prepared from the acid hydrolyzate of citrus pectin (11) was fractionated by DEAE-cellulose chromatography in the same manner as described for the unsaturated acid insoluble pectic acid.

Acid soluble pectic acid. The crude acid soluble pectic acid (11) was fractionated as described for the unsaturated acid soluble pectic acid.

3. *Analytical Methods*

Methoxyl content. The chromotropic acid method (13) was devised so that many samples could be examined rapidly. The pectic acid resulting from saponification of pectin was precipitated with $CuSO_4$ and methanol in the filtrate estimated without being distilled. To 0.5% pectin solution (1% solution was used for low methoxyl pectin) was added an equal volume of 0.1N NaOH to saponify pectin. The mixture was left in the well-stoppered flask at room temperature for 40 minutes. A volume of 5% $CuSO_4$ in 0.2N H_2SO_4 equal to the original pectin solution was added with vigorous stirring and the mixture filtered through Toyo No. 2 filter paper. To 0.5 m*l* or 1 m*l* of the filtrate were added 2 m*l* of 3% $KMnO_4$ in 15% H_3PO_4 and, 4 minutes later, about 150mg of $NaHSO_3$. After the purple color of the solution had disappeared, 0.5 m*l* of 2% chromotropic acid solution (supernatant fluid) and 10 m*l* of concentrated H_2SO_4 were added successively, and the mixture was heated for 10 minutes in a boiling water bath. This was diluted with water to 50 m*l* after being cooled in a bath of cold water. The optical density of the diluted solution was measured on a Hitachi photoelectric photometer with No. 57 filter. Control experiments were run in which pectic acid (0.5 or 1%) plus methanol (0~1mg/m*l*) was treated as above.

Other estimation. Reducing sugar was determined by a modification of Willstätter-Schudel's method (6). Galacturonic acid was estimated by the carbazole method (heating time, 20 minutes) or by the naphthoresorcinol method (12). Viscosity was measured using Ostwald viscosity pipets. Absorption spectra were measured on a Shimadzu QR-50 photoelectric spectrophotometer.

RESULTS

1. *Conditions for Alkaline Saponification of Pectin*

pH. When the temperature was maintained at 15°C, pectin was hardly de-esterified at pH 8.0 for at least 15 hours. At pH 11, 60 to 70% methoxyl groups of pectin were released for 2 hours. At pH 12 pectin was almost completely saponified for 30 minutes (Fig. 1).

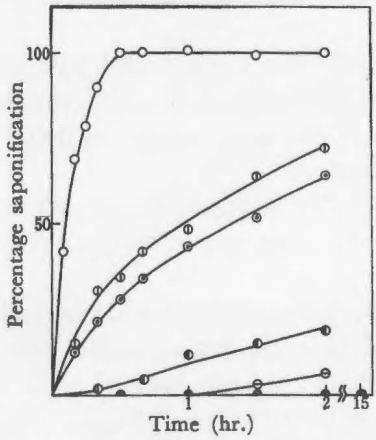

Fig. 1. Effect of pH on saponification of pectin. Mixtures containing 1 m*l* of 0.4% pectin (pH 7) and 1 m*l* of buffer were kept at 15°C. After a given period of time 1 m*l* of 5% $CuSO_4$ in 0.2N H_2SO_4 was added to precipitate the product of saponification. Methanol in the filtrate was determined by the method described in the Materials and Methods section. Buffers used were: 0.1 M phosphate buffer, pH 8 (●—●); 0.1 M carbonate buffers, pH 9 (⊖—⊖), pH 10 (◐—◐), pH 11 (ⓘ—ⓘ); 0.1 M Na_2HPO_4 — 0.1 N NaOH, pH 11 (⊙—⊙), pH 12 (○—○).

Temperature. In 0.05N NaOH, almost complete saponification of pectin could be achieved under the following conditions: 50°C 2 minutes, 30°C 7 minutes, 15°C 10 minutes and 0°C 20 minutes (Fig. 2).

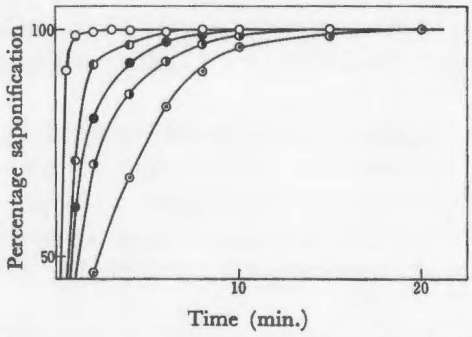

Fig. 2. Effect of temperature on saponification of pectin. Reaction mixture contained 1 m*l* of 1% pectin (pH 7) and 1 m*l* of 0.1 N NaOH. After a given period of time methanol in the reaction mixture was determined as described for Fig. 1. ⊙—⊙ 0°C, ◑—◑ 10°C, ●—● 15°C, ◐—◐ 30°C, ○—○ 50°C.

2. *Saponification of Pectin with PE*

Orange flavedo has been reported to be devoid of PG activity. But

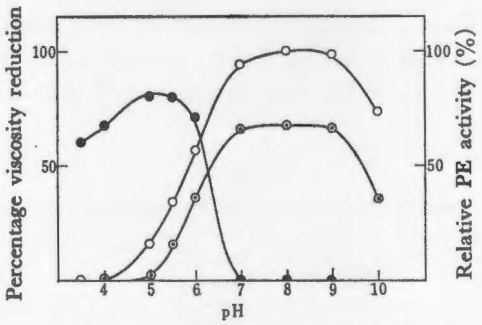

Fig. 3. PE and PG activity of the PE preparation. PE activity.—Incubation mixture: 1 m*l* of 0.4% pectin (pH 7), 1 m*l* of buffer and 0.5 m*l* of enzyme solution (dialyzed against 0.1 M NaCl). Temperature, 15°C. After 20 min. (⊙—⊙) and 40 min. (○—○) methanol in the reaction mixture was determined as described for Fig. 1. PG activity. —Incubation mixture: 1 m*l* of 1% pectic acid (pH 5), 1 m*l* of buffer and 0.5 m*l* of enzyme solution (dialyzed against water). Temperature, 27°C. After 16 hr. viscosity of the incubation mixture was measured (percentage viscosity reduction ●—●). Buffer used.—0.1 M acetate buffers, pH 3.5, 4.0, 5.0; 0.1 M phosphate buffers, pH 5.5, 6.0, 7.0, 8.0; 0.1 M carbonate buffers, pH 9.0, 10.0.

TABLE 1

Saponification of pectin by PE

Source of pectin	Time of reaction	Percentage saponification	Percentage esterification*
Citrus Pectin (No. 1)	15 hr.	100.3	59.8
〃	20 min.	62.3	
〃	40 min.	92.5	
〃	60 min.	96.3	
〃	90 min.	99.4	
〃	120 min.	100.2	
〃	240 min.	99.8	
Citrus Pectin (No. 2)	15 hr.	99.6	57.0
Peel of *Citrus Unshiu*	15 hr.	100.4	47.2
Bast of hemp	15 hr.	99.4	16.9

Pectin was incubated with PE as described in the Materials and Methods section. Up to 240 min. methanol in the incubation mixture was determined as described for Fig. 1. Percentage saponification = 100 × methoxyl liberated in the incubation mixture/ total methoxyl in the incubation mixture. From 240 min. onward methoxyl content of the products obtained by the ethanol precipitation was determined as described in the text. Percentage saponification = 100 × (methoxyl content of the original pectin — methoxyl content of the products)/methoxyl content of the original pectin.

* 100 × galacturonic acid unit methyl-esterified/total galacturonic acid unit.

the present study revealed the presence of PG in peels of *Citrus Unshiu*. Our attempt to remove this enzyme from the PE preparation was not successful. However, the PE could be studied independently of the associated PG by taking advantage of the fact that the latter enzyme was quite inactive above pH 7.0, whereas the former had a broad optimum pH from 7 to 9 (Fig. 3). In this study incubations of PE were made at pH 8.2.

TABLE 2

Viscosity of pectic acids prepared by alkaline saponification

Conditions for saponification	Specific viscosity	Relative value (%)
(Original pectin)	3.699	100
0° 90 min.	2.594	70.1
10° 50 〃	2.126	57.5
15° 40 〃	1.665	45.0
30° 30 〃	0.995	26.9
50° 15 〃	0.359	9.7
100° 5 〃	0.035	1.0
pH 12, 15° 90 〃	1.753	47.4

Viscosity of 0.2% pectic acid solution was measured at 16°C.

TABLE 3

Viscosity of PE-PA, PE·Alkali-PA, Alkali-PA and pectin

Source of pectin	Pectic acid	Specific viscosity	Relative value (%)
Citrus Pectin (No. 1)	Original pectin	2.986	100
	PE-PA	2.174	72.8
	PE·Alkali-PA	1.845	61.8
	Alkali-PA	1.609	53.9
Citrus Pectin (No. 2)	Original pectin	3.280	100
	PE-PA	2.470	75.3
	PE·Alkali-PA	2.182	66.5
	Alkali-PA	1.528	46.6
Peel of *Citrus Unshiu*	Original pectin	3.850	100
	PE-PA	2.722	70.7
	PE·Alkali-PA	2.452	63.7
	Alkali-PA	1.539	40.0
Bast of hemp	Original pectin	5.580	100
	PE-PA	4.511	80.8
	PE·Alkali-PA	4.372	78.4
	Alkali-PA	3.802	68.1

Viscosity of 0.2% pectic acid solution was measured at 16°C with EDTA added.

In contrast to the results reported by other workers, almost complete saponification was easily achieved with the PE of *Citrus Unshiu* (Table 1).

3. *Relation between Saponification and Transelimination of Pectin*

Viscosity reduction. Pectic acids obtained by alkaline saponification were dissolved in water and diluted to a concentration of 0.2%. At room temperature viscosity of the solutions was measured. It was found that the higher the temperature of saponification, the lower is the viscosity of the pectic acid obtained (Table 2). The percentages of specific viscosity of the pectic acid to that of the original pectin were: 0°C 90 minutes, 70; 15°C 40 minutes, 45; 100°C 5 minutes, 1. It was 47 for the pectic acid obtained at pH 12 and 15°C for 90 minutes.

Alkaline saponification, even at 0°C, caused a greater decrease in viscosity than saponification with PE (Fig .4). In the latter case the viscosity reached a constant value at the stage of 50% de-esterification. In the case of alkaline saponification, it was not until 90% methoxyl groups were released that the viscosity ceased to change.

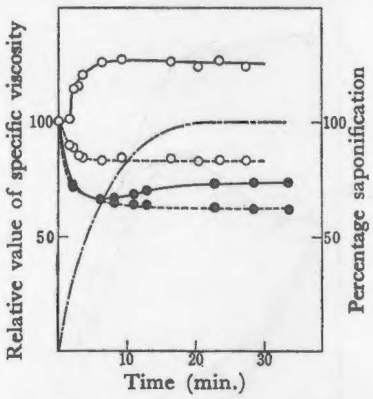

Fig. 4. Relation between saponification and viscosity reduction of pectin. ○ Products of saponification of pectin with PE, ● products of saponification of pectin with alkali (0.05 N NaOH, 0°C). ——— Viscosity of the product solution, ····· viscosity of the product solution containing 0.6% EDTA and 0.01 M phosphate buffer, pH 7.5. Viscosity of 0.2% product solution was measured at 16°C. — · — · — Percentage de-esterification of pectin.

Pectin preparations are far from free from ash impurities which probably influence the viscosity of pectin or pectic acid solution. To minimize the effect of the ash, EDTA was added to the solution of the products the viscosity of which was to be measured. It was found that the addition of EDTA decreased the viscosity of the solutions (Fig. 4). All the products of saponification with alkali or with PE showed a viscosity lower than the

TABLE 4

Viscosity of pectic acids treated with alkali

Pectic acid	Conditions for alkali-treatment		Specific viscosity	Relative value (%)
PE-PA	(Original pectic acid)		2.380	100
	0°	90 min.*	1.862	78.2
	15°	40 〃	1.859	78.1
	30°	30 〃	1.856	78.0
	50°	15 〃	1.774	74.5
Alkali-PA	(Original pectic acid)		1.723	100
	0°	90 〃	1.725	100.1
	15°	40 〃	1.726	100.2
	30°	30 〃	1.717	99.7
	50°	15 〃	1.686	97.9

Viscosity was measured as described for Table 3.

* The product will be called PE・Alkali-PA.

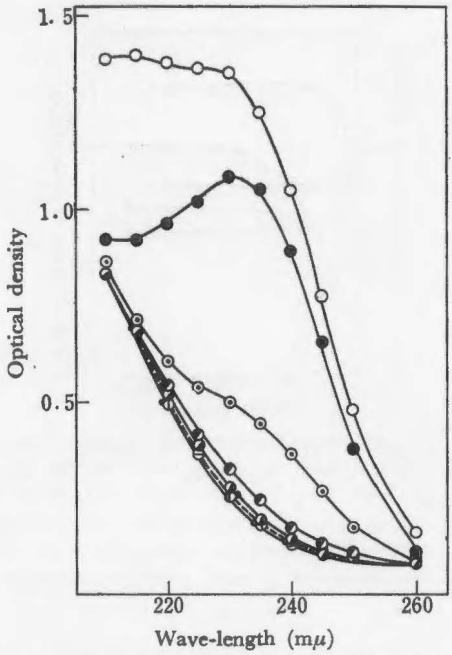

Fig. 5. Ultraviolet absorption of pectic acids (0.1%) and 4, 5-unsaturated digalacturonic acid (ca. 0.2 μmol./ml). Pectic acids examined.—Pectic acids prepared by alkaline saponification under the conditions, 0°C 90 min. (•—•), 10°C 50 min. (◐—◐), 15°C 40 min. (◑—◑), 30°C 30 min. (◕—◕), 50°C 15 min. (⊙—⊙), 100°C 5 min. (○—○) and pH 12 15°C 90 min. (•···•): PE・Alkali-PA (⊖—⊖): PE-PA (◒—◒). Pectin (◕—◕). 4, 5-unsaturated digalacturonic acid (●—●).

original pectin. Alkali-treatment further reduced the viscosity of PE-PA, while it caused no change in the viscosity of Alkali-PA (Table 4).

Pectic acids were prepared from four sources by the following methods: (a) saponification with PE (PE-PA), (b) saponification with PE followed by alkali-treatment (PE·Alkali-PA) and (c) saponification with alkali (Alkali-PA, 0°C). These preparations, regardless of the source of pectin, showed the viscosity in the following order (Table 3):

PE-PA>PE·Alkali-PA>Alkali-PA, 0°C

Ultraviolet-absorption. The peak at 230 mμ, indicative of 4, 5-unsaturated uronic acid, was observed with the products of alkaline saponification in warm solutions. But there was no difference in absorption around 230 mμ between the products of saponification with alkali below 15°C or with PE and the original pectin (Fig. 5).

Thiobarbituric acid test. Products obtained by alkaline saponification showed a stronger absorption at 550 mμ than the original pectin. The intensity of this peak increase with the temperature of saponification. No peak was observed at 550 mμ with PE-PA and PE·Alkali-PA.

The thiobarbituric acid test (10, 14) given by Alkali-PA (saponified at

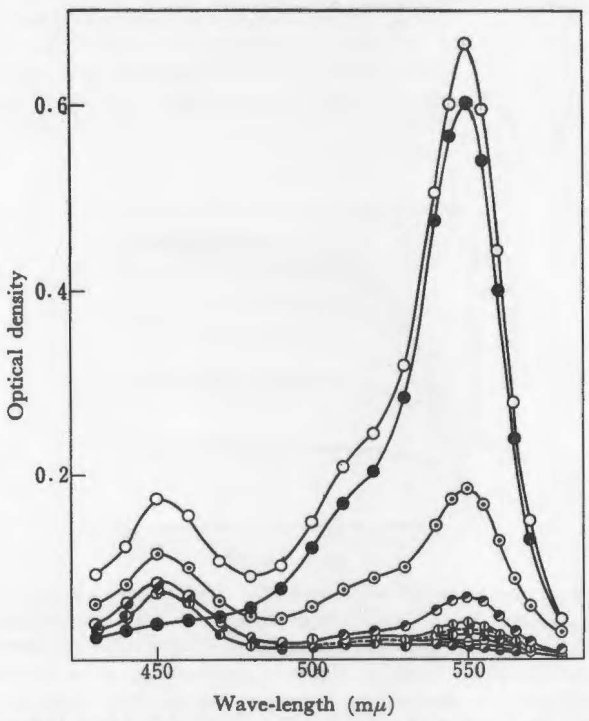

Fig. 6. Thiobarbituric acid test of pectic acids (0.2%) and 4, 5-unsaturated digalactururonic acid (ca. 0.1 μmol./m*l*). Periodate oxidation was for 15 min. at 80°C (10). Symbols are the same as described for Fig. 5.

0°C) was so slight that its discrimination from that of PE·Alkali-PA was very difficult (Fig. 6). To increase the intensity of the color YPG·CPG-Os were prepared from these products and the thiobarbituric acid test was made

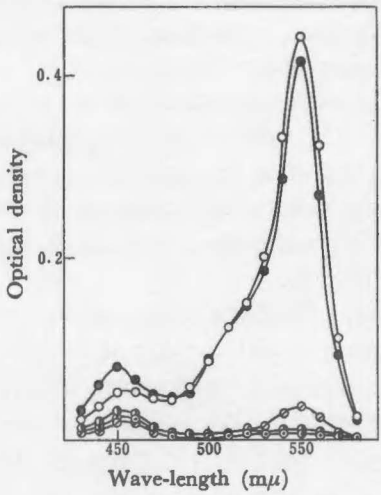

Fig. 7. Thiobarbituric acid test of YPG·CPG-Os (0.05%). Periodate oxidation was for 15 min. at 80°C. YPG·CPG-Os examined.—YPG·CPG-Os from pectic acids prepared by alkaline saponification under the conditions, 100°C 5 min. (○—○), 50°C 15 min. (●—●) and 0°C 90 min. (⊘—⊘): YPG·CPG-O from PE·Alkali-PA (⊙—⊙). Oligogalacturonide (◑—◑).

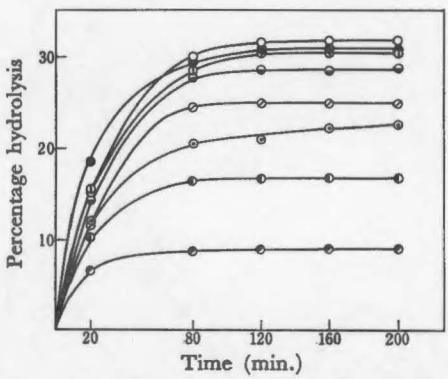

Fig. 8. Degradation of pectic acids by CPG. Incubation of enzyme and pectic acid was at 35°C in 0.02 M acetate buffer, pH 4.65, the concentration of pectic acid being 0.05%. Galacturonic acid formed was determined by the naphtoresorcinol method. Pectic acids used as substrates.—Pectic acids prepared by alkaline saponification under the conditions, 0°C 90 min. (●—●), 10°C 50 min. (◫—◫), 15°C 40 min. (◒—◒), 30°C 30 min. (⊘—⊘), 50°C 15 min. (◐—◐) 100°C 5 min. (◪—◪): PE-PA (⊙—⊙): PE·Alkali-PA (○—○).

on these YPG·CPG-Os. The oligogalacturonide prepared as described in the previous paper (3) was used as a control. The control and the YPG·CPG-O from PE·Alkali-PA gave no thiobarbituric acid test, while the YPG·CPG-Os of the products of alkaline saponification, even that of Alkali-PA prepared at 0°C, gave absorption maxima at 550 mμ (Fig. 7). Their absorptions at 550 mμ were stronger than those of their respective starting pectic acids, the ratios being: ca. 3 (saponified at 100°C), ca. 8 (saponified at 50°C) and ca. 10(saponified at 0°C).

4. *Action of CPG on the Pectic Acids Prepared by Saponification with Alkali and with PE*

With the pectic acids obtained by alkaline saponification, degradation limit by CPG was found to decrease with the temperature of saponification (Fig. 8).

TABLE 5

Limits of degradation of pectic acids by CPG

Source of pectin	PE-PA	PE·Alkali-PA	Alkali-PA, 0°C
Citrus Pectin (No. 1)	24.0	32.1	31.1
Citrus Pectin (No. 2)	28.5	35.1	34.9
Peel of *Citrus Unshiu*	34.1	39.7	36.6
Bast of hemp	21.1	32.5	25.2

Pectic acids (final concentration, ca. 0.05%) were incubated with CPG in 0.02M acetate buffer, pH 4.65, at 27°C. After 15 hr. the incubation mixture was diluted to a suitable volume. Galacturonic acid formed was determinated by the naphtoresorcinol method and total galacturonic acid by the carbazole method.

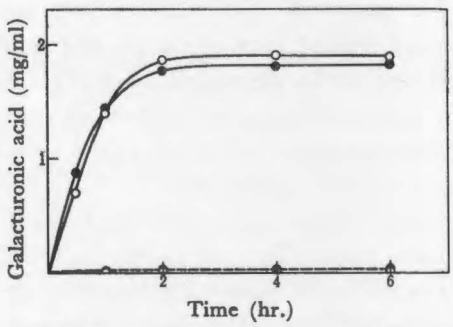

Fig. 9. Action of CPG on saturated and unsaturated pectic acids. Reducing power was determined by a modification of the Willstätter-Schudel method and expressed as mg of galacturonic acid per m*l* of the reaction mixture. Incubation: 0.2% pectic acid; 0.015 M acetate buffer, pH 4.65; 27°C. ○—○ saturated acid insoluble pectic acid, ○···○ unsaturated acid insoluble pectic acid, ●—● saturated acid soluble pectic acid, ●···● unsaturated acid soluble pectic acid.

Limited hydrolysis by CPG was observed with all the pectic acids examined (Fig. 8). The order of limit values of the pectic acids was as follows (Table 5):

PE•Alkali-PA>Alkali-PA, 0°C>PE-PA

As shown in Fig. 9, both unsaturated acid soluble and acid insoluble pectic acid were not degraded by CPG.

DISCUSSION

Pectin breaks down rapidly in warm alkaline solutions by way of a trans-elimination. The rate of the breakdown increases with rising temperature. Needless to say, the reaction is accompanied by de-esterification of pectin. In the cold the de-esterification predominates over the transelimination. At 0°C the latter can hardly occur, while the former proceeds to completion. Pectic acids obtained by (A) alkaline saponification at 0°C, (B) enzymic saponification and (C) enzymic saponification and alkali treatment showed a viscosity (determined after addition of EDTA) lower than that of the original pectin, the order of the viscosity decreases being (A)>(C)>(B). For these viscosity reductions some factors other than transelimination should be considered. These results oppose the view of Neukom and Deuel who attributed the viscosity reduction of pectin by alkaline saponification to the trans-elimination alone.

The pectic acid prepared by the method involving enzymic saponification and alkali treatment was the most susceptible of all the pectic acid preparations to CPG action. But even in this case the degradation was far from complete. Pectic acid resulting from the alkaline saponification became more resistant to CPG as the temperature of saponification was raised from 0 to 100°C. Unsaturated acid soluble and acid insoluble pectic acid, prepared by partial degradation of pectic acid with pectate endo-trans-eliminase, were found not to be degraded by CPG. It is considered from these results that the incompleteness of hydrolysis of pectic acid molecule by CPG is caused by the presence of the terminal unsaturated galacturonic acid unit together with neutral sugar units.

The degradation limit of pectic acids by CPG was lower than that reported in the previous paper (2). As shown in Table 5 pectic acid prepared by saponification with PE showed a lower limit value than those of alkaline saponification though the latter have more unsaturated bonds than the former. These discrepancies may lie in some factors other than the neutral sugar and unsaturated galacturonic acid units in the pectic acid molecule.

The chromotropic acid method was used for the determination of methoxyl content with suitable modifications. Using this method we ob-

served that almost complete de-esterification was achieved with PE prepared from peels of Citrus Unshiu. This contrasts markedly with the results reported by other workers.

SUMMARY

Glycosidic bonds of pectin are rapidly cleaved in warm alkaline solutions by a transelimination mechanism. In the cold, however, the reaction proceeds slowly and the de-esterification of pectin predominates over the transelimination. At 0°C, the latter can hardly occur, while the former proceeds to completion. In this case an unaccountable drop in viscosity is observed.

The pectic acid having an unsaturated galacturonic acid unit at the non-reducing end of the molecule, which is a product of pectin transelimination, was resistant to CPG action. The incompleteness of hydrolysis of pectic acid molecules by CPG must be caused by the presence of this terminal unsaturated galacturonic acid unit together with neutral sugar units.

In contrast to the reported partial de-esterification of pectin achieved by PE, almost complete de-esterification was obtained with PE prepared from peels of *Citrus Unshiu*.

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