

ENZYMIC DEGRADATION OF PECTIC ACID

VII. Acid-Soluble Acid Polysaccharides Produced from Pectic Acid by Endopolygalacturonase Action

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Even after the exhaustive degradation of pectic acid with YPG, YPG-ASAP remains undecomposed in the reaction mixture (1). Previous work showed that, by the action of carrot exopolygalacturonase, YPG-ASAP was further degraded giving birth to galacturonic acid (2). On the basis of this result it was considered that YPG-ASAP is, at least in part, present in pectic acid itself and not derived from contaminating materials. This concept was further supported by the previous experiments on chromatographic behavior of YPG-ASAP and pectic acid (3). Additional evidence that YPG-ASAP is an integral part of pectic acid will be presented in this paper.

The present paper also reports content of YPG-ASAP in pectic acid of *Citrus Unshiu* and of commercial citrus pectinic acid, uronic acid content of the YPG-ASAP, and manner of YPG-ASAP distribution among the individual molecules of the pectic acids.

MATERIALS

Pectic Acid

From peels of *Citrus Unshiu* and commercial citrus pectinic acid, pectic acid was prepared as described in previous papers (3, 4).

Preparation of YPG-ASAP

Pectic acid was incubated with YPG under the same conditions as described in a previous paper (1). After two weeks of incubation the mixture was freed from YPG by passing it through a Duolite CS-101 column and concentrated under reduced pressure. In the previous study (1), this concentrate was chromatographed on a DEAE-cellulose column and, after washing with 0.1 M carbonate-bicarbonate buffer, pH 9.5, the polysaccharide eluted with the pH 10 buffer was collected.

Up to the preceding paper the polysaccharides so obtained had been called YPG-ASAP. In this study the above concentrate was filtered through a Sephadex G-75 column and the polysaccharide fraction was collected. The polysaccharides thus prepared will be called YPG-ASAP in the present paper and hereafter.

Abbreviations used: YPG, endopolygalacturonase of *Saccharomyces fragilis*; YPG-ASAP, acid-soluble acid polysaccharides produced from pectic acid by the action of YPG.

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RESULTS

1. *YPG-ASAP Formed from the Pectic Acid Purified by DEAE-Cellulose Chromatography*

The recovery of YPG-ASAP from the mixture of YPG-ASAP and pectic acid. As was reported in the preceding paper, YPG-ASAP could be considerably well separated from pectic acid by the DEAE-cellulose chromatography with a concentration gradient of pH 6 acetate buffer and 0.1 N NaOH. But in that time tailing of YPG-ASAP could not perfectly be eliminated. In the present study the recovery of YPG-ASAP from the mixture of YPG-ASAP and pectic acid was examined first of all. YPG-ASAP was recovered in good yield, the percentages of recovery being 94.6 to 97.8 (Table 1). Fig. 1 shows the elution curves obtained in Expt. No 2, 3, and 5 (Table 1).

Purification of pectic acid by DEAE-cellulose chromatography. When the pectic acid preparation made from pectinic acid by alkali-saponification and alcohol precipitation was chromatographed on a DEAE-cellulose column, a small amount of polysaccharides was eluted with pH 6 acetate buffer. These could be removed by repeating the chromatography. Fig. 2 shows the results with pectic acid of *Citrus Unshiu*. Similar results were obtained with pectic acid from commercial citrus pectinic acid.

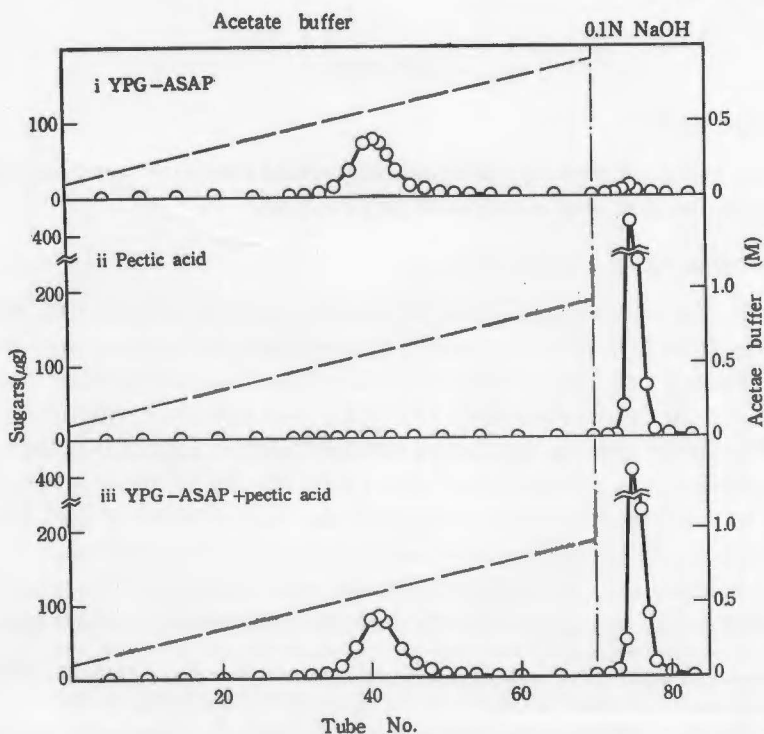


Fig. 1. Elution diagrams of pectic acid and YPG-ASAP on a DEAE-cellulose column.

TABLE 1

Recovery of YPG-ASAP from the mixture of pectic acid and YPG-ASAP by the DEAE-cellulose chromatography with a gradient of acetate buffer, pH 6 and 0.1 N NaOH

Expt. No.	Mixture	YPG-ASAP** (μ g)	Euate of acetate buffer (μ g, sugar)*	Recovery of YPG-ASAP (%)
1	1 % pectic acid*** (0.5 ml) + ca. 0.1 % YPG-ASAP (0.5 ml)	560****	570	94.6
2	1 % pectic acid*** (0.5 ml) + ca. 0.5 % YPG-ASAP (0.5 ml)	2,800****	2,780	97.8
3	0.5 % pectic acid*** (1 ml)	0	40	
4	ca. 0.05 % YPG-ASAP (1 ml)	560	468	83.5
5	ca. 0.25 % YPG-ASAP (1 ml)	2,800	2,600	92.8

Chromatography was done in the same manner as described in the previous paper (3). Tubes 20—60 (5 ml each, eluate of acetate buffer) were pooled.

* By the phenol-sulfuric acid method (1) sugar was determined in eluate and amount of sugar was expressed as μ g of galacturonic acid in the combined tubes.

** This was prepared from the preparation of YPG-ASAP described in the Methods section by DEAE-cellulose chromatography using 0.1 M sodium bicarbonate and 0.1 M carbonate-bicarbonate buffer (pH 10) as eluents. The fraction eluted with the former buffer was removed away and the latter fraction was collected.

*** The same purified pectic acid of *Citrus Unshiu* as used in the experiment of Table 2.

**** Calculated from the sugar content of YPG-ASAP solutions determined before mixing with pectic acid solution.

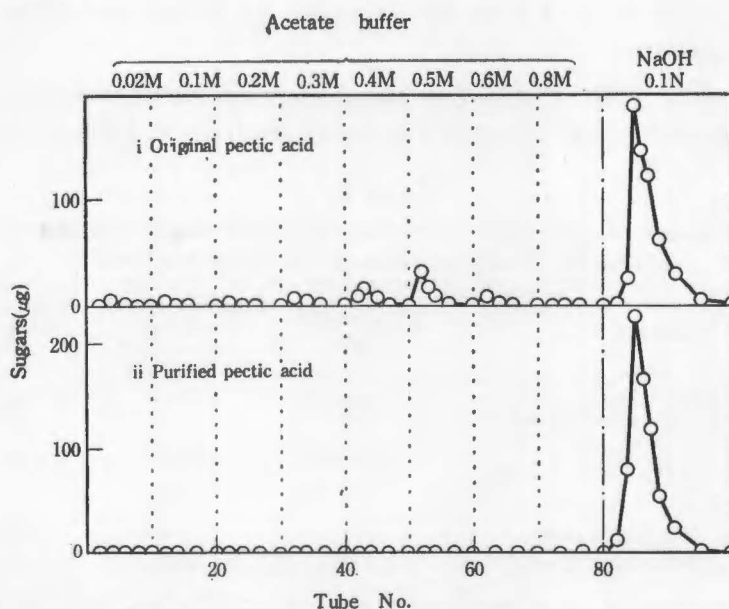


Fig. 2. Purification of pectic acid by the DEAE-cellulose chromatography eluting by stepwise increase of the concentration of acetate buffer, pH 6 and finally with 0.1 N NaOH. To a column (4×20 cm), equilibrated with 0.02 M acetate buffer, pH 6, was added 50 ml of 1 % pectic acid (*Citrus Unshiu*) solution and the column was washed with the same buffer. The effluent of 0.1 N NaOH was neutralized with acetic acid to pH 6 and rechromatographed after dialysis and concentration. Chromatography was done three times in all.

YPG-ASAP formed from the purified pectic acid. From the pectic acid purified as described in the preceding paragraph YPG-ASAP was produced by the action of YPG. On DEAE-cellulose chromatography similar elution curve to that shown in Fig. 1 was obtained with this YPG-ASAP preparation. The yields from pectic acid of *Citrus Unshiu* and commercial pectinic acid were both about 10% (Table 2). Almost the same yield was obtained with the original pectic acid. The purified pectic acids resembled each other also in uronic acid content of YPG-ASAP; the values were both about 40% and, furthermore, quite similar to those of the original pectic acids.

TABLE 2
Yield of YPG-ASAP from the pectic acids purified
by DEAE-cellulose chromatography

Pectic acid	YPG-ASAP (%)	Uronic acid content of YPG-ASAP (%)
Purified (<i>Citrus Unshiu</i>)	9.5	41.5
Purified (commercial citrus pectinic acid)	10.0	40.0
Original (<i>Citrus Unshiu</i>)	9.7	39.9
Original (commercial citrus pectinic acid)	10.9	37.5

2. YPG-ASAP Formed from the Fractions of Pectic Acid Obtained by Acid Precipitation

Recovery of YPG-ASAP from the mixture of YPG-ASAP and pectic acid by precipitation and washing with acid. As shown in Table 3, by precipi-

TABLE 3
Recovery of YPG-ASAP from the mixture of pectic acid and
YPG-ASAP by precipitation and washing with acid

Mixture	YPG-ASAP* (μ g)	Supernatant and washings (μ g, sugar)**	Recovery of YPG-ASAP (%)
1% pectic acid (0.5 ml) + ca. 0.1% YPG-ASAP (0.5 ml)	530***	774	96.0
1% pectic acid (0.5 ml) + ca. 0.5% YPG-ASAP (0.5 ml)	2,740***	29970	98.8
0.5% pectic acid (1 ml) ca. 0.05% YPG-ASAP (1 ml)	0 530	265 534	 100.8
ca. 0.25% YPG-ASAP (1 ml)	2,740	2,970	102.9

To the mixture (1 ml), or to the solution of pectic acid or YPG-ASAP (1 ml) was added with stirring 5 ml of 0.2 M $\text{CH}_3\text{COONa-HCl}$ buffer, pH 1.5. After 20–30 min. the precipitate was centrifuged down and washed twice with 5 ml of the same buffer.

* The same YPG-ASAP as described in Table 1.

** By the phenol-sulfuric acid method sugar was determined in the combined solution of supernatant and washings.

*** Calculated from the sugar content of YPG-ASAP solution before mixing with pectic acid solution.

tation and washing with acid YPG-ASAP was recovered from the mixture of YPG-ASAP and pectic acid in as good a yield as by the DEAE-cellulose chromatography.

Fractionation of pectic acid by solubility in acid. Below pH 1.8 pectic acid precipitates almost completely, while above pH 2.15 its solubility increases with pH and the polysaccharides even of higher uronic acid content becomes soluble (Table 4).

Yield of YPG-ASAP from the acid-soluble and acid-insoluble fractions of pectic acid. YPG-ASAP was prepared respectively from the fractions of pectic acid precipitated and not precipitated at pH 2.15 and 2.6. Its yield and uronic acid content of YPG-ASAP are shown in Table 5. Among all of the fractions no

TABLE 4
Fractionation of pectic acid by solubility in acid

pH	Fraction precipitated (%)	Fraction not precipitated (supernatant) (%)	Uronic acid content of fraction not precipitated (%)
1.51	97.7	2.3	43.6
1.80	96.5	3.5	50.4
2.15	73.1	26.9	92.5
2.32	60.5	39.5	94.8
2.45	30.8	69.2	95.1
2.60	23.7	76.3	95.3

To 250 ml of 0.2 M $\text{CH}_3\text{COONa-HCl}$ buffer was added with stirring 20 ml of 1% pectic acid solution. After 30 min. standing and pH determination with a glass electrode, the mixture was centrifuged. Sugar was determined in the supernatant.

TABLE 5
Yield of YPG-ASAP from the fractions of pectic acid obtained by acid precipitation

pH	YPG-ASAP		Uronic acid content of YPG-ASAP	
	Fraction precipitated (%)	Fraction not precipitated (%)	Fraction precipitated (%)	Fraction not precipitated (%)
2.15	10.4	11.4	40.2	36.7
	9.8**		41.3**	
2.60	10.8	9.4	39.4	37.0
Control*	9.7		39.9	

The fractions of pectic acid precipitated with 0.2 M $\text{CH}_3\text{COONa-HCl}$ buffer at pH 2.15 and 2.60 (Table 4) was washed four times with the same buffers of pH 2.15 and 2.60 respectively. The washed precipitates were dissolved in dilute alkaline solution. These solutions and the fractions not precipitated (Table 4) were dialyzed against water. YPG-ASAP was prepared from the dialyzed solution.

* Original pectic acid. ** Prepared by five washings.

appreciable difference was observed in yield of YPG-ASAP and its uronic acid content. Similar values were also obtained with the original pectic acid (Table 5).

DISCUSSION

As described in the preceding paper YPG-ASAP and pectic acid can be separated from each other by DEAE-cellulose chromatography using successively a concentration gradient of pH 6 acetate buffer and 0.1 N NaOH, YPG-ASAP being eluted with the former and pectic acid with the latter. In the present study it was found that YPG-ASAP was recovered in good yield from the mixture of YPG-ASAP and pectic acid by the above DEAE-cellulose chromatography, though tailing of band of YPG-ASAP could not perfectly be eliminated. By repeating the chromatography pectic acid can be freed from the material which is eluted in the same chromatographic position as YPG-ASAP. When YPG is acted on the purified pectic acid thus obtained YPG-ASAP is produced which emerges in the same chromatographic position as shown in the preceding paper. YPG-ASAP can be recovered in good yield from the mixture of YPG-ASAP and pectic acid also by precipitation and washing with acid. All these results, together with those obtained in previous studies, indicate that YPG-ASAP is not a contaminant but a product formed by the action of YPG on pectic acid and that pectic acid contains neutral sugar blocks which remain undecomposed after the prolonged incubation of pectic acid with YPG.

Yield of YPG-ASAP from the pectic acid purified by the DEAE-cellulose chromatography was about 10% and uronic acid content of YPG-ASAP about 40%; these values were almost the same as those of the original pectic acid, respectively.

Pectic acid becomes increasingly soluble with pH from 2.15 and uronic acid content of the soluble fraction increased almost to the level of the original pectic acid. In the present study the soluble and precipitated fractions were prepared from pectic acid by precipitation and washing at pH 2.15 and 2.6. It was found that yields of YPG-ASAP from these fractions resembled each other, the values being about 10% and quite similar to those obtained for YPG-ASAP from the original pectic acid and the pectic acid purified by DEAE-cellulose chromatography. Furthermore, uronic acid contents of all YPG-ASAP preparations including that of the original pectic acid were found to be almost the same, the values being about 40%. These results suggest that pectic acid of *Citrus Unshiu* and that from commercial citrus pectinic acid are considerably homogeneous in respect of the YPG-ASAP distribution among the individual molecules.

SUMMARY

YPG-ASAP could be recovered in good yield from the mixture of YPG-ASAP and pectic acid by DEAE-cellulose chromatography and by precipitation and washing with acid. Pectic acid purified by DEAE-cellulose chromatography, fraction obtained from pectic acid by precipitation and washing with acid, and original pectic acid all closely resembled each other in contents of YPG-ASAP (about 10%) and uronic acid of YPG-ASAP (about 40%). These results indicate that YPG-ASAP is an integral part of pectic acid and that pectic acid of *Citrus Unshiu* and that from commercial citrus pectinic acid are considerably homogeneous in respect of the YPG-ASAP distribution among the individual molecules.

LITERATURE CITED

1. Hatanaka, C and Ozawa, J. 1966. Enzymic degradation of pectic acid. II. Chromatography of pectic substances on DEAE-cellulose columns. (in Japanese) J. Agr. Chem. Soc. Japan 40 : 98—105.
2. Hatanaka, C. and Ozawa, J. 1966. Enzymic degradation of pectic acid. III. Sugar constituents of pectic acids. (in Japanese) J. Agr. Chem. Soc. Japan 40 : 106—109.
3. Hatanaka, C. and Ozawa, J. 1968. Enzymic degradation of pectic acid. VI. Chromatography of pectic substances on DEAE-cellulose columns (2). (in Japanese) J. Agr. Chem. Soc. Japan 42 : 645—650.
4. Hatanaka, C. and Ozawa, J. 1964. Enzymic degradation of pectic acid. I. Limited hydrolysis of pectic acids by carrot exopolysaccharuronase. Agr. Biol. Chem. 28 : 627—632.