ENZYMIC DEGRADATION OF PECTIC ACID

VI. Chromatography of Pectic Substances on DEAE-Cellulose Columns (2)

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DEAE-Cellulose chromatography of pectic substances, with sodium bicarbonate solution and carbonate-bicarbonate buffer as eluting agents, was described in previous work (1). It was shown that, by this chromatographic method, good separations were obtained among neutral polysaccharides (araban, galactan), galacturonic acid, oligogalacturonides and pectic acid. Oligogalacturonide homologues having chain length of 2—6 units were also well separated from one another. But only a poor separation was obtained between low and high molecular weight pectic acids, or between ASAP and pectic acid.

The present paper describes the chromatography of pectic substances on DEAE-cellulose columns with stepwise or gradient increase of concentration of acetate buffer (pH 6) and finally with 0.1 N NaOH. It is shown that ASAP are separated from pectic acid on DEAE-cellulose columns by eluting with the pH 6 acetate buffer.

MATERIALS AND METHODS

1. Preparation of Pectic Substances

Pectinic acid*. To 1 % solution of commercial citrus pectinic acid was added ethanol to a concentration of 60%. The precipitated pectinic acid was washed successively with 60, 80 and 99% ethanol, and then dried at room temperature.

Acid-soluble pectic acid. For two hours 2% solution of commercial citrus pectinic acid in 2% HCl was heated in a boiling water bath. After cooling this was filtered and the precipitate was used for the preparation of acid-insoluble pectic acid I. The filtrate was neutralized with barium hydroxide and the precipitated barium salt of acid-soluble pectic acid was collected by centrifugation. To dissolve acid-soluble pectic acid sulfuric acid was added to pH 2.0 and barium sulfate was removed away by filtration. Precipitation of acid-soluble pectic acid as barium salt and its solubilization with sulfuric acid were repeated and finally acid-soluble pectic acid was precipitated as sodium salt by the addition of ethanol. Its average degree of polymerization was 14.9.

Acid-insoluble pectic acid I. The above-mentioned precipitate of acid-insoluble pectic acid was washed with 40% ethanol acidified by hydrochloric acid

Abbreviations used: ASAP, acid-soluble acid polysaccharides; YPG, endopolygalacturonase of Saccharomyces fragilis; YPG-ASAP, ASAP produced from pectic acid by YPG action.
* Up to the 5th paper of this series this had been called pectin.
to pH 2, and dissolved in dilute sodium hydroxide, pH of the solution being brought to 5. After being mixed with one twentieth volume of 1 N NaOH the solution was allowed to stand for 90 minutes at 0°C. Hydrochloric acid was added to the solution and the precipitated acid-insoluble pectic acid was washed with 40% ethanol acidified with hydrochloric acid. This was dissolved in dilute sodium hydroxide, precipitated with ethanol and dried at room temperature (acid-insoluble pectic acid I). The average degree of polymerization was found to be 43.7.

**Acid-insoluble pectic acid II.** For six hours 2% solution of commercial pectinic acid in 5% HCl was heated at 80—85°C. From the precipitate, sodium salt of acid-insoluble pectic acid was prepared as in the case of acid-insoluble pectic acid I. This was dissolved in water and chromatographed on a DEAE-cellulose column as described in a previous paper (1). After removal of the pH 9.5 fraction the eluate with the pH 9.73 carbonate-bicarbonate buffer was collected. From this, acid-insoluble pectic acid II was prepared as sodium salt as described previously. Its average degree of polymerization was 70.5.

**Pectic acid.** This was prepared from commercial citrus pectinic acid. Saponification was for 90 minutes at 0°C in 0.05N NaOH (2).

**Pectic acid prepared by saponification at high temperature.** This was prepared from commercial citrus pectinic acid by saponification at a sodium hydroxide concentration of 0.05 N and 60°C for 10 minutes. It was insoluble in acid and specific viscosity of its sodium salt was 0.19 at a concentration of 0.2%. In contrast, specific viscosities of the pectinic acid and pectic acid were found to be 3.7 and 2.6, respectively.

**YPG-ASAP.** The PG fraction obtained from the culture liquid of *Saccharomyces fragilis* by the adsorption on calcium pectate gel (3) was added to a Duolite CS-101 column prepared in 0.02M acetate buffer, pH 5. After being washed with the same buffer the column was eluted with 1 M acetate buffer, pH 5. This was used as the enzyme solution of YPG after dialysis against the 0.02 M acetate buffer. Incubation of pectic acid with YPG was made first under the following conditions: pectic acid, 0.5%; YPG, 10 units per ml of reaction mixture; pH 5 acetate buffer, 0.04 M; 27°C; 1 week. Next, one-half of the YPG solution was added and the mixture was further incubated. After 1 week it was treated with Duolite CS-101 to remove YPG and added to a DEAE-cellulose column. The column was first washed with 0.1 M carbonate-bicarbonate buffer, pH 9.5, and then eluted with the pH 10 buffer as described in the previous paper (1).

**ASAP of hemp.** Dried bast of hemp (250 g) were immersed in dilute hydrochloric acid, pH 2, at 5°C for 3 to 15 hours, and the extract was decanted off. Extraction with the dilute hydrochloric acid was repeated five or six times. After being washed with water the bast were immersed in 0.05 M sodium acetate and kept overnight at room temperature under a layer of toluene. The extract was filtered through diatomaceous earth and the filtrate was held at 0°C for 90
minutes after being mixed with one twentieth volume of 1 N NaOH. To the mixture was added hydrochloric acid to precipitate pectic acid. The precipitate was filtered off and washed with 0.01 M acetic acid. The filtrate and washings were joined, brought to about pH 5 and mixed with one fifteenth volume of 15% CuSO₄. After standing overnight at 5°C the precipitate formed was collected by centrifugation, washed with water and suspended in water. This was treated with ion exchange resin to remove cupric ion and filtered through diatomaceous earth. The filtrate was neutralized with sodium hydroxide and mixed with ethanol to precipitate polysaccharides (about 0.9 g). The polysaccharides were chromatographed as described in the previous paper (1); after washing with 0.1 M carbonate-bicarbonate buffer, pH 9.5, the fraction eluted with the pH 10 buffer was collected (ASAP, 115 mg).

2. **DEAE-Cellulose Chromatography**

   **Stepwise elution.** To a column (1.7 × 5 cm) of DEAE-cellulose (0.9 meq./g, Brown Co.) equilibrated with 0.02 M acetate buffer, pH 6, was added 0.5 ml of an about 1% sample solution. Chromatograms were developed by stepwise elution by using in turn 0.02, 0.1, 0.2, 0.3, 0.4, 0.5, 0.6 and 0.8 M acetate buffer, pH 6, and finally 0.1 N NaOH. Approximately 5 ml per tube was collected.

   DEAE-Cellulose chromatography by stepwise elution was done also with sodium acetate solutions (0.02—0.6 M) and 0.1 N NaOH, the other conditions being the same as above.

   **Gradient elution.** To a DEAE-cellulose column (1.7 × 5 cm) equilibrated with 0.02 M acetate buffer, pH 6, was added 0.5 ml of an about 1% sample solution. After washing with the same buffer elution was carried out with a linear gradient of concentration of pH 6 acetate buffer: the mixer containing 235 ml of the 0.1 M buffer and the reservoir 235 ml of the 1 M buffer. After 350 ml of eluate was collected the eluent was changed to 0.1 N NaOH. Tube volume was 5 ml.

3. **Analytical Methods**

   Sugar and uronic acid were determined as described previously (1).

**RESULTS**

1. **Stepwise Elution**

   Chromatograms of pectic substances obtained by stepwise elution with pH 6 acetate buffer and 0.1 N NaOH are shown in Fig. 1. In all cases some peaks appeared during the elution with acetate buffer. The buffer concentration at which the highest peak appeared was found to be 0.2 M for pectinic acid, 0.4 M for acid-soluble pectic acid and 0.5 M for YPG-ASAP. In cases of acid-insoluble pectic acid II and pectic acid the highest peak appeared in the eluate of 0.1 N NaOH. The esterification degrees of the fractions of pectinic acid eluted with 0.2, 0.3
Fig. 1. DEAE-Cellulose chromatography of pectic substances by stepwise elution with pH 6 acetate buffers (0.1–1 M) and finally with 0.1 N NaOH. Tube volume, 5 ml.

Fig. 2. DEAE-Cellulose chromatography of pectic acid and pectinic acid by stepwise elution with sodium acetate solutions and finally with 0.1 N NaOH.
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and 0.4 M acetate buffer were 39.3, 70.7 and 7.7\%, respectively (Fig. 1).

When sodium acetate solution was used as eluent in place of acetate buffer, the highest peaks of pectinic acid and pectic acid both appeared at 0.2 M concentration of sodium acetate (Fig. 2).

2. **Gradient Elution**

When pectic substances were chromatographed on DEAE-cellulose columns with a gradient of concentration of pH 6 acetate buffer, only one peak appeared in each eluate (Fig. 3). The peak seems to correspond to the highest one observed in case of stepwise elution with the acetate buffer. Uronic acid content of the peak eluted with the gradient was found to be 40.2\% for YPG-ASAP and 48.0\% for ASAP of hemp, and those of 0.1N NaOH were 38.0 and 54.6\%, respectively.

![Fig. 3. DEAE-Cellulose chromatography of pectic substances with a concentration gradient of pH 6 acetate buffer and next with 0.1N NaOH. Dash line, concentration of buffer.
* Prepared by saponification of pectinic acid at 60°C.](image)

3. **Separation of Different Forms of Pectic Substance**

(a) **Separation of pectinic acid and acid-insoluble pectic acid or pectic acid.** Chromatograms obtained with a mixture of pectinic acid and acid-insoluble
pectic acid I or pectic acid are shown in Figs. 4 and 5. As the first peaks shown in Fig. 4 i and ii and the peak eluted with 0.3 M acetate buffer in Fig. 5 contain methoxyl group, it is considered that they correspond to pectinic acid. On mixing with hydrochloric acid the second peak in Fig. 4 i becomes turbid; this indicates that it contains acid-insoluble pectic acid. The positions of peak of pectinic acid and acid-insoluble pectic acid I are almost the same as those observed when they

![Graph](https://i.imgur.com/4yZ5.png)

**Fig. 4.** Separation of pectinic acid, and acid-insoluble pectic acid I and pectic acid by the DEAE-cellulose chromatography with acetate buffer gradient and 0.1 N NaOH.

![Graph](https://i.imgur.com/5yZ5.png)

**Fig. 5.** Separation of pectinic acid and pectic acid by the DEAE-cellulose chromatography by stepwise elution with acetate buffers, pH 6 and finally with 0.1 N NaOH.
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Fig. 6. Separation of acid-soluble pectic acid, acid-insoluble pectic acid I and pectic acid by the DEAE-cellulose chromatography with acetate buffer gradient and 0.1 N NaOH.

Fig. 7. Separation of YPG-ASAP and ASAP, and acid-insoluble pectic acid I and pectic acid by the DEAE-cellulose chromatography with acetate buffer gradient and 0.1 N NaOH.
are chromatographed separately. From this result it seems clear that no interaction occurs between pectinic acid and acid-insoluble pectic acid I.

(b) *Separation of acid-soluble pectic acid, acid-insoluble pectic acid and pectic acid.* These could be considerably well separated from one another by the DEAE-cellulose chromatography (Fig. 6). On adding hydrochloric acid the materials in the first peaks in Fig. 5 form no precipitate at 0.5% concentration, indicating that these peaks correspond to acid-soluble pectic acid.

(c) *Separation of YPG-ASAP or ASAP of hemp and acid-insoluble pectic acid or pectic acid.* YPG-ASAP or ASAP of hemp and acid-insoluble pectic acid or pectic acid were well separated from each other (Fig. 7). By the addition of hydrochloric acid the materials in the first peaks (Fig. 7) were not precipitated but precipitation occurred from the eluates with 0.1 N NaOH.

4. **Effect of the Amount of Pectic Substance on its Distribution between the Eluates with Acetate Buffer and 0.1 N NaOH**

The amount of a sample applied on the column of a fixed diameter and height affects its distribution between the eluates with the pH 6 acetate buffer and 0.1 N NaOH. As the sample is decreased, the ratio of its amounts eluted with the acetate buffer and 0.1 N NaOH becomes lower (Table 1). This may be accounted for mainly by tailing of the fraction eluted with acetate buffer.

**Table 1**

<table>
<thead>
<tr>
<th>Pectic substance</th>
<th>Sample (mL)</th>
<th>Acetate buffer fraction</th>
<th>0.1 N NaOH fraction</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Amount (mg)</td>
<td>Percentage</td>
</tr>
<tr>
<td>YPG-ASAP</td>
<td>0.1</td>
<td>0.878</td>
<td>86.5</td>
</tr>
<tr>
<td></td>
<td>0.5</td>
<td>5.280</td>
<td>95.4</td>
</tr>
<tr>
<td></td>
<td>1.0</td>
<td>10.477</td>
<td>97.6</td>
</tr>
<tr>
<td>Pectic acid</td>
<td>0.1</td>
<td>0.873</td>
<td>71.5</td>
</tr>
<tr>
<td></td>
<td>0.5</td>
<td>6.079</td>
<td>91.5</td>
</tr>
<tr>
<td></td>
<td>1.0</td>
<td>13.236</td>
<td>91.9</td>
</tr>
</tbody>
</table>

Sample (ca. 1% solutions of YPG-ASAP and pectinic acid) were added to DEAE-cellulose columns (1.7 × 5 cm), equilibrated with 0.02 M acetate buffer, pH 6. Each column was eluted with a gradient of pH 6 acetate buffer (0.1—1 M) and next with 0.1 N NaOH.

**DISCUSSION**

When pectinic acid, ASAPs and various forms of pectic acid were chromatographed respectively on DEAE-cellulose columns by eluting successively with a concentration gradient of pH 6 acetate buffer and 0.1 N NaOH, they were eluted in the specific chromatographic positions. Tailing could not perfectly be eliminated
even by the gradient elution. When mixture of two of them were chromatographed, they were separated from each other; the shape and position of each peak were not influenced by the presence of the other. It is of interest that ASAPs and pectic acid were successfully separated from each other, because these had not been resolved with sodium bicarbonate solution and carbonate-bicarbonate buffer as eluents in previous work (1). The peak obtained with the gradient of acetate buffer was divided into several peaks by the stepwise elution with the same buffer; similar results had been obtained with sodium bicarbonate solution and carbonate-bicarbonate buffer. This may suggest that the above preparations of pectic substances, even though they were eluted as a single peak respectively with the gradient of acetate buffer, are not homogeneous. From the results in this paper, however, it may be said that the pectic substances differing somewhat sharply from each other in molecular weight, or content of neutral sugar units or methoxyl group are resolved by the DEAE-cellulose chromatography with pH 6 acetate buffer and 0.1 N NaOH. When sodium acetate solution was used in place of the acetate buffer, pectinic acid and pectic acid were eluted at the same concentration of the eluent and not resolved from each other.

The results obtained in the present work together with those of the previous paper are summarized as follows:

By DEAE-cellulose chromatography separations can be achieved (1) between neutral polysaccharides occurring naturally with pectic substances (araban and galactan) and galacturonic acid, (2) among galacturonic acid and oligogalacturonides, (3) between oligo- and polygalacturonides and (4) among the pectic substances differing sharply in molecular weight, and in content of neutral sugar units and methoxyl group.

**SUMMARY**

The pectic substances differing from each other in molecular weight, or in content of neutral sugar units or methoxyl group were resolved by the DEAE-cellulose chromatography with a gradient of increasing concentration of pH 6 acetate buffer and 0.1 N NaOH. YPG-ASAP and pectic acid, which had been reported not to be resolved with sodium bicarbonate solution and carbonate-bicarbonate buffer, were separated from each other by the use of the above eluents. Tailing could not perfectly be eliminated even by the gradient elution. When sodium acetate solution was used in place of the acetate buffer, pectinic acid and pectic acid were eluted at the same concentration of the eluent.
LITERATURE CITED

