Enzymic Introduction of the Methyl Ester Groups of Pectin*

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SUMMARY

A particulate preparation obtained from Phaseolus aureus shoots contains an enzyme capable of transferring the 14C-labeled methyl groups from S-adenosyl-L-methionine to the carboxyl groups of polygalacturonic acid, which seems to be present in the same particulate material. The introduction of the methyl ester groups is optimal at pH 6.8 and does not require divalent ions. S-N-Methyltetrahydrofolate does not act as a donor of methyl groups. The apparent Michaelis constant of the enzyme for S-adenosyl-L-methionine is about $6 \times 10^{-5}$ M.

Pectic substances form an important part of the middle lamella and the primary cell wall of plants. They consist primarily of polygalacturonic acid chains, to which neutral sugars or polymers of neutral sugars are bound in covalent linkage (1, 2). The carboxyl groups of the polygalacturonic acid are of considerable significance for the chemical and biological properties of the pectic substances. Their ionic reaction is partly neutralized by being esterified with methanol. The degree of methylation, therefore, determines to a large extent the ion exchange and water-binding capacity of pectin and the possibility of getting involved in cross linkage by formation of salt bridges, ester linkage, and hydrogen bonding (2, 3).

It was recently shown with particulate enzyme preparations that the polygalacturonic acid chains of pectin are synthesized by repeated transfer of the $\beta$-galacturonic acid moiety from uridine diphosphate $\beta$-galacturonic acid and that this uronic acid nucleotide is the best uronic acid donor of any $\beta$-galacturonic acid nucleotide containing a base other than uracil (4-6). Although experiments in vitro indicated that the methyl ester groups of the pectic substances can be introduced from L-methionine or other related substances (7), nothing was known about the mechanism of methylation of the carboxyl groups. The results of the present investigation show that particulate enzyme preparations from mung bean shoots contain an enzyme capable of transferring methyl groups from S-adenosyl-L-methionine to polygalacturonic acid which is present in the same particulate material.

A preliminary report on this subject has been previously published (8).

EXPERIMENTAL PROCEDURE

Material—Mung beans were germinated in a dark chamber at about 100% humidity for 3 or 4 days after which the cotyledons were removed. 14C-Methyl-labeled S-adenosyl-L-methionine (specific activity, 53 mC per mmole) was purchased from New England Nuclear Corporation. The solution containing the S-adenosyl-L-methionine in dilute sulfuric acid was further diluted with 4 volumes of water; 5 ml of the resulting solution contained 1.06 mC/mole, and the radioactivity corresponded to 140,000 cpm. 5-N-Methyltetrahydrofolate labeled with 14C in the methyl groups was a gift of Dr. C. Kutzbach. Its specific activity was 5.3 mC per mmole (10 µmoles per ml of 0.1% ascorbate, pH 6.0).

Enzyme Preparation—All operations were performed in an ice bath. The mung bean shoots (25 g) were ground with sand in 25 ml of 0.05 M potassium phosphate buffer, pH 7.3, containing serum albumin and 10$^{-3}$ M EDTA (exception, Table II). The material was squeezed through cheesecloth and centrifuged at 1,000 x g for 15 min. The supernatant liquid was centrifuged at 34,000 x g for 45 min; the resulting pellet was suspended in 20 ml of the buffer which was used for grinding; and the mixture was centrifuged again at 34,000 x g for 45 min. The obtained particulate material was then suspended in 0.5 ml of 0.05 M potassium phosphate buffer, pH 6.8, containing 1% albumin and 0.4 M sucrose. Although the enzyme preparation lost only about 10% of its activity during storage at -20° for 3 days, the enzyme samples used for the experiments were prepared fresh every day.

Standard Enzyme Assay—The indicated amount of this particulate enzyme preparation and that of the incubation buffer (0.1 M cacodylate-HCl buffer containing 1% albumin and 0.4 M sucrose) plus a 5-µl solution of S-adenosyl-L-methionine were mixed at 0°. The pH value was then determined with duplicate samples, with the use of a Radiometer Copenhagen microelectrode. The mixture was incubated at 30° for different lengths of time. About the same amount of 20% trichloracetic acid as that of the total incubation mixture was added, followed after 20 min by 1.0 ml of water; the solution was then centrifuged at 10,000 x g for 20 min. The precipitate was washed twice with cold water, and then 1.0 mg of citrus pectin (Exchange

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TABLE I
Liberation of labeled methanol by different treatment of high polymer product

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Radioactivity remaining on planchets</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>320</td>
</tr>
<tr>
<td>4 N NH₄OH</td>
<td>11</td>
</tr>
<tr>
<td>0.05 N NaOH + 0.2% dimeron</td>
<td>0</td>
</tr>
<tr>
<td>Boiled pectin esterase</td>
<td>330</td>
</tr>
<tr>
<td>Pectin esterase</td>
<td>16</td>
</tr>
</tbody>
</table>

Fig. 1. Radio gas chromatography of the labeled product liberated from the high polymer material by alkaline hydrolysis. —— signal from the gas chromatograph; ——, pattern from the radioactivity measurement. Details are under “Experimental Procedure.”

Lemon Products) in 0.2 ml of water was added to the mixture; the pectin was then extracted two times with 1.0 ml of 0.5% ammonium oxalate at 100° for 10 min. This extraction yielded about 90% of the total alkali-labile radioactivity present in the particulate material. Sufficient absolute ethanol was then added to the solution to bring the concentration to 75% (v/v) and the precipitate was collected by centrifugation after standing for 1 hour at room temperature.

The pectin was solubilized in 1.0 ml of water, and aliquots of 200 µl were applied on strips of chromatography paper (Whatman No. 1, 2.5 x 5 cm). After drying with a stream of warm air, the strips were counted in toluene with Liquifluor (New England Nuclear) in a Tri-Carb scintillation counter at about 40% efficiency. To determine the proportion of esterified methanol, similar strips of paper were wetted with 7.5 N NH₄OH and kept for 1 hour in a closed container over 7.5 N NH₄OH to saponify the ester; they were then counted in the same manner.

In some experiments, alternatively, the strips were wetted with a solution of pectin methyl esterase (Nutritional Biochemicals Corporation, 10 mg/10 ml of 0.2 M sodium phosphate buffer, pH 6.7) and kept for 24 hours in a chamber over water and toluene.

Protein Determination—For determination of the protein content of the particulate enzyme preparation, 100 µl of the suspension were centrifuged and the pellet was suspended in 400 µl of water. Aliquots of 5 µl of this suspension were used for protein determination according to the method of Lowry et al. (9), with serum albumin as a standard. The particulate enzyme preparations from 4- or 3-day-old shoots contained 20 to 25 mg of protein per ml.

Radio Gas Chromatography—An Aerograph A 700 with a Carbowax 300 column, 10 feet long, was used. The carrier gas was helium (16 ml per min), and the oven temperature 85°. This gas chromatograph was followed by a Nuclear-Chicago flow counter set. A dry sample of labeled high polymer material to which 0.2 mg of citrus pectin was added was solubilized in 10 or 15 µl of 0.5 N NaOH. After 30 min, 5 µl of authentic carrier methanol were added, and the mixture was injected into the gas chromatograph.

RESULTS

Incubation of the particulate enzyme preparation with labeled S-adenosyl-L-methionine resulted in the formation of radioactive material which was water-soluble and was precipitated in 75% ethanol, an indication that it might be a carbohydrate of a high molecular weight. Table I shows that the labeled methyl groups are easily hydrolyzed by alkali at room temperature within a short time, which shows that the methyl group of the radioactive product must be linked as an ester rather than as an ether. The product of the hydrolysis volatilized from alkaline or slightly acidic solutions and is therefore a neutral, low molecular substance. This volatile substance cannot be formaldehyde, because it was not trapped by dimeron. The volatile substance liberated by alkaline hydrolysis was further identified by radio gas chromatography (Fig. 1). The peak representing the authentic methanol carrier coincides with the peak from the radioactivity measurement. The retention times for several other compounds that were suspected to be produced were as follows: carbon dioxide, 2.0; methanol, 5.0; ethanol, 6.2; propanol, 10.0; and water, 13.4 min. A solution of formaldehyde in water gave only the signal of water under the conditions used.

That almost all of the methanol is bound in ester linkage to the carboxyl groups of polygalacturonic acid is evident from the fact that the enzyme, pectin methyl esterase (Table I), also liberates the methanol. This enzyme is known to be specific for the methyl ester of polygalacturonic acid (2). The treatment with esterase usually liberates from 2 to 4% less methanol than the alkaline hydrolysis. This may be due to the fact that pectin esterase acts only very slowly on single methyl ester groups (2) or to the presence of a small proportion of methyl esters of acids other than polygalacturonic acid. These results show that the product formed by transmethylation is 14C-methyl-labeled pectin.

The effect of increasing the time of incubation on the amount of alkali-labile methyl groups which are incorporated into pectin is shown in Fig. 2. The curve is linear up to about 10 min; from
then on the rate of synthesis decreases with further increase of incubation time. After about 2 hours the reaction practically stops. This might be due to a progressive inactivation of the enzyme or, more likely, to the limited amount of polygalacturonic acid present in the particulate material. Between 2 and 4 hours of incubation, the amount of methyl ester groups in the particulate material remains constant. The enzyme responsible for the introduction of the methyl ester groups into pectin has an optimal pH value of 6.8 (Fig. 3). A similar value was previously reported (8) with the particulate enzyme prepared without EDTA.

The addition of manganese or magnesium ions has no effects on the rate of incorporation of methyl ester groups when compared with the effects of potassium and sodium chloride (Table II). It is obvious that the enzyme does not require divalent ions. Other metal salts decrease the incorporation rate. The addition of EDTA therefore increases the incorporation due to the formation of complexes with traces of metal ions. Because of these results, all the enzyme samples for the experiments in this paper (except Table II) were prepared in the presence of EDTA. Starting with the same plant material, the particulate enzyme was about 20 to 30% more active when prepared in the presence of EDTA and proved to be more stable during storage at -20°.

Table III shows that the enzyme responsible for the transmethylation appears to be specific for S-adenosyl-L-methionine. With 5-N-methyltetrahydrofolate as a donor of methyl groups, no labeled pectin is formed. Identical results were obtained whether the incubation was done at a pH value of 6.8 or 8.1, or the particulate enzyme was prepared in the presence or absence of EDTA.

Fig. 4. shows that the rate of incorporation of methyl ester

![Graph](image_url)

**Fig. 2.** Incorporation of alkali-labile *14*C-methyl groups with increasing time of incubation. Standard assay with 50 µl of enzyme preparation, 1.96 mmole of S-adenosyl-L-methionine, and 50 µl of incubation buffer, pH 6.8, in a total volume of 105 µl.

**Fig. 3.** Incorporation of alkali-labile *14*C-methyl groups at different pH values. Standard assay with 50 µl of enzyme preparation, 50 µl of incubation buffer, and 1.96 mmoles of S-adenosyl-L-methionine in a total volume of 105 µl. Incubation time, 5 min.

### Table II

<table>
<thead>
<tr>
<th>Cofactor added</th>
<th>Radioactivity incorporated</th>
</tr>
</thead>
<tbody>
<tr>
<td>None (control)</td>
<td>30350 µc/min</td>
</tr>
<tr>
<td>NaCl</td>
<td>2970 µc/min</td>
</tr>
<tr>
<td>KCl</td>
<td>2847 µc/min</td>
</tr>
<tr>
<td>MnCl₂</td>
<td>2973 µc/min</td>
</tr>
<tr>
<td>MgCl₂</td>
<td>2835 µc/min</td>
</tr>
<tr>
<td>CaCl₂</td>
<td>2604 µc/min</td>
</tr>
<tr>
<td>AlCl₃</td>
<td>2433 µc/min</td>
</tr>
<tr>
<td>CoCl₂</td>
<td>2204 µc/min</td>
</tr>
<tr>
<td>FeCl₃</td>
<td>1974 µc/min</td>
</tr>
<tr>
<td>ZnCl₂</td>
<td>1864 µc/min</td>
</tr>
<tr>
<td>NiCl₂</td>
<td>847 µc/min</td>
</tr>
<tr>
<td>EDTA</td>
<td>3971 µc/min</td>
</tr>
</tbody>
</table>

### Table III

<table>
<thead>
<tr>
<th>Donor present</th>
<th>Amount (µmole)</th>
<th>Radioactivity incorporated (µc/min)</th>
<th>Amount incorporated (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>S-Adenosyl-L-methionine</td>
<td>9.8</td>
<td>2856</td>
<td>39</td>
</tr>
<tr>
<td>5 N Methyltetrahydrofolate</td>
<td>50</td>
<td>10</td>
<td>&lt;1.4</td>
</tr>
</tbody>
</table>

### Table II

Influence of cations and EDTA on introduction of alkali-labile *14*C-methyl groups

Standard assay mixture consists of 50 µl of enzyme (prepared without addition of EDTA to the buffer used for grinding the shoots and washing the particles), 50 µl of incubation buffer, 1.96 mmole of S-adenosyl-L-methionine, and 2.5 × 10⁻⁴ M metal salts or 2 × 10⁻⁴ M EDTA in a total volume of 110 µl. Incubation time, 5 min, pH 6.8.
with different enzyme preparations were in the range of 4 to 7
zyme preparation.

The values determined
with various enzyme preparations were in the range of 4 to 7
X 10^-4 M.

Fig. 5 shows the effect of the age of the seedlings on the activity of
the particulate enzyme preparations calculated on a protein
basis. On the 2nd day after the beginning of the imbibition,
the activity of the seedlings is low; it reaches a high level on the
3rd day and remains at this level for at least 3 more days. This
level coincides with the phase of the rapid elongation of the hypo-
cotyl. Calculated on the basis of fresh weight of the mung bean
shoots used for the preparation of enzyme, the curve drops
slightly after the 3rd day.

DISCUSSION

Several authors (10-12) suggested that the methylation reac-
tion in the biosynthesis of pectin may occur prior to polymeriza-
tion and involves a nucleotide-bound methyl-D-galacturonic acid.
In the experiments reported in this paper, no nucleoside diphos-
phate-D-galacturonic acid was included in the incubation mix-
ture. It is very unlikely that the washed particulate enzyme
preparation contains such a low molecular endogenous precursor
in amounts sufficient for synthesis of a methylated nucleotide.
It can therefore be concluded that the methyl groups of S-adenos-
yl-methionine are directly transferred to the carboxyl groups
of polygalacturonic acid. This acceptor for the methyl groups
must be present in the particulate material. That similar partic-
ulate preparations from mung bean shoots may contain poly-
galacturonic acid is indicated by the fact that acid hydrolysis
resulted in the formation of di- and trigalacturonic acid (13).
The conclusion that the methylation occurs at a high molecular
level is in agreement with the observation that under conditions
in which the uronic acid moiety of uridine diphosphate-D-galac-
turonic acid is transferred to form polygalacturonic acid, the
respective labeled carboxyl methyl ester is not incorporated into
high polymer products (5).

The enzyme responsible for the biosynthesis of the methyl
ester groups of pectin has properties differing from those of the
enzyme which introduces the 4-O-methyl ether groups into the
polysaccharide unit of glucuronoxylan. This enzyme was re-
cently demonstrated in particulate preparations from growing
corn cobs (14). It had a pH optimum of 8.1, is stimulated by
Co++, Mn++, and is not inhibited by other metal ions such as
Ni++, or Zn++.

The introduction of the methyl ester groups into macromole-
cular carbohydrates is analogous to the well known fact that the C-
and N-methyl groups of RNA and DNA are transferred at the
macromolecular level (5). A similar transfer was recently
shown for the formation of the 4-O methyl ether groups of glu-
curonoxylan (14).

The carboxyl groups are of considerable significance for the
chemical properties of the pectic substances. As previously
stated, the ion exchange and water-binding capacity, and the
possibility of their getting involved in cross-linkages by cations,
ester, and hydrogen bonds depend to a large extent on the degree
of methylation (2, 3). These properties seem to be of interest
with respect to the biological function of the pectic substances,
and are therefore considered by a number of investigators to
play a role in connection with the growth of plant cell walls (16-
18). Regardless of whether or not the carboxyl groups of pectic
substances play a direct role in plant growth, their partial or full
methylation by the enzyme reported in the present work appears
to be a part of the complex process of cell wall formation.

In this connection it is of interest to note that plant meriste-
matic tissues contain a relatively high proportion of unesterified
polygalacturonic acid (19). This might possibly indicate that
the degree of methylation differs during the course of develop-
ment of plant tissues. It is also of interest to note that growing
tissues always contain pectin methyl esterase (2), a hydrolytic enzyme which is the reverse in its action of the enzyme that transfers the methyl ester groups to polygalacturonic acid.

Acknowledgments—We thank Miss A. L. Swanson for excellent technical assistance; Dr. C. Kutsbach for a gift of labeled 5-N-methyltetrahydrofolic acid; and Dr. C. E. Ballou and Dr. T. S. Stewart for help in performing the radio gas chromatography.

REFERENCES

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