Cooperation of Enzymes Responsible for Polymerisation and Methylation in Pectin Biosynthesis

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Some more properties of a particulate enzyme preparation from mung bean shoots capable of the formation of polygalacturonate as well as its methyl ester are reported.

1. When the preparation was freed of traces of endogenous nucleotide uronic acids by digestion with phosphodiesterase or by preincubation up to 2 hours, the methylation was still possible. This sustains the recently made proposal that the methylation proceeds by transmethylation directly to polygalacturonate and not intermediary to nucleotide uronic acids.

2. The formation of the polygalacturonate chains from radioactive UDP-galacturonate is independent of the methylation process.

3. The polygalacturonate methyl transferase does not methylate exogenous polygalacturonate. In contrast, the rate of pectin methyl ester formation is strongly increased if additional polygalacturonate is pregenerated in the enzyme preparation by incubation with UDP-galacturonate followed by digestion with phosphodiesterase. This may indicate that the enzymes for polymerization and methylation as well as the pectic substances are localized intimately together in structural compartments.

The carboxyl groups of the polygalacturonate chains in the pectic substances are fully or partly esterified with methanol. The degree of their methylation determines to a great extend the chemical and biological properties of the polysaccharides, which constitute a part of the cementing material in the middle lamella and primary cell wall of plants. Biosynthesis and degradation of these methyl ester groups must be an important process in the formation and elongation of the plant cell wall and were therefore even thought to play a direct role in the plant growth process.

It was formerly suggested by several authors, that the methylation may occur at a nucleotide level and that a methylated uronic acid residue is transferred to the polymer. We have recently shown that a particulate enzyme preparation was able to introduce labelled methyl ester groups from radioactive S-adenosyl-L-methionine into pectic substances. Making the assumption that the particulate preparation did not contain considerable amounts of nucleotide sugars, it was proposed that the introduction of the methyl ester groups may proceed by methylation of preformed polysaccharides at the macromolecular level.

In the present work some more experiments with the particulate enzyme preparation on the biosynthesis of pectic substances are reported.

Experimental Procedures

Material: Radioactive and nonradioactive UDP-galacturonic acids were prepared according to reference 7. All other materials were obtained from commercial sources: the different pectic substances from NBCo and Exchange Lemon Products, the [14C]-SAM from NEN, UDP-glucuronic acid and crystalline bovine serum albumin from Sigma and all the other nucleotides from Calbiochem.

Pectin methyl esterase from tomatoes (NBCo, 10 mg/ml) was stirred in cold 0.2 M phosphate buffer, pH 6.8, and the supernatant after centrifugation was used as enzyme. Snake venom phosphodiesterase (1.5 U/mg) in 50% glycerol was bought from Boehringer und Soehne, Mannheim, and diluted with one volume of water. A 25% glycerol solution was added in all control experiments.

Abbreviations: SAM = S-adenosyl-L-methionine, [14C]-SAM = SAM-[14C]-methyl, Galacturonosyl transferase = Uridine diphosphate galacturonate: 2-1,4-polygalacturonate 4-galacturonosyl-transferase; Polygalacturonate methyl transferase = S-adenosyl-L-methionine: 2-1,4-polygalacturonate carboxyl-methyltransferase; PDE = Phosphodiesterase.

1 H. DEETZ and E. STUTZ, Advances in Enzymol. 20, 341 [1958].


experiments when this enzyme was used. The particulate material, containing the enzymes galacturonosyl transferase and polygalacturonate methyl transferase was prepared as described previously. The only alterations were the omission of EDTA from the buffer used for washing the particles and the pH value of the suspension buffer was changed to 6.55. The hypocotyls and roots of 3—4 day old Phaseolus aureus (mung bean) seedlings grown in the dark were used for the preparation. Mung bean seeds were bought as "Katjang Idjoe" from N. V. Conimex, Baarn, Netherlands.

**Assay of polygalacturonate methyl transferase:** A new assay method was used which is less time consuming and more reliable than the one previously employed since it does not include extraction and precipitation steps.

The standard incubation mixture contained the following: 20 µl of the incubation buffer (0.1 m Na-cacodylate-HCl, pH 6.7, 1% albumin, 0.4 m sucrose, 0.006 m MnCl₂), 5 µl of [¹⁴C]-SAM (180 000 counts/min, 1.9 mmole), and 5 µl of the particulate enzyme preparation (20 to 25 mg protein/ml), total volume 40 µl. Immediately after adding the enzyme the tubes were transferred from 0° to 30° and incubated for the indicated time. The reactions were stopped with 20 µl of 0.2 m formic acid (adjusted to pH 2.0 with 1 N HCl). In the case that an additional enzyme was used in the incubation mixture, 20 µl of 5% TCA were used to stop the reaction. For zero time controls, the stopping agent was added at 0°, before the addition of the enzyme preparation.

The tubes were kept at 0° or stored over night at —20°. 50 µl of the mixture were applied on strips of Schleicher and Schüll 2043 b paper, 2.5 cm broad and 43 cm long, and were electrophoresed at 900 volts in a 0.2 m ammonium formate buffer pH 2.0 (adjusted with conc. HCl) until a picrate standard had moved about 20 cm. The paper was dried with a stream of cold air and cut 2.5 cm at both sides of the starting line. The radioactivity on the accordion pleated strip was determined at about 70% efficiency in 15 ml of a 5 g PPO/toluene solution with a Beckman LS 100 liquid scintillation counter. The strip was then removed from the scintillation fluid, dried, wetted with 7.5 N NH₄OH and placed for 2—3 hours in a closed container over 7.5 N NH₄OH. After drying at room temperature the strips were recounted using the same scintillation vial containing the fluid from the first counting. The incorporation of methyl ester groups was calculated from the difference in both countings. The counting procedure was checked with [¹⁴C]-glucose standards. The errors caused by different placement of the strips in the counting equipment and by the treatment of the strips with alkali were within the 1% counting error.

The formation of methyl ester groups was proportional to the amount of enzyme preparation up to 7 µl when the standard assay mixture was incubated for 2 minutes.

To ensure that the radioactivity liberated with alkali was due to pectin methyl ester groups, some strips from each type of experiment were treated after the first counting not with alkali but with a solution of tomatoe pectin methyl esterase and kept for 48—72 hours in a chamber over water and chloroform. The amount of methanol liberated enzymically was the same as with the alkali treatment.

**Polygalacturonate transferase:** The formation of polygalacturonic acid was determined according to reference 8 with the alterations indicated in the legends of table 1 and table 5, respectively.

**Results and Discussion**

**Destruction of endogenous nucleotide uronic acids**

The first aim of the experiments has been to show that the introduction of the methyl ester groups of pectin indeed occurs at the polysaccharide level and not at the level of a nucleotide precursor. Therefore, we have worked out conditions under which the particulate enzyme preparation should be definitely free of endogenous nucleoside diphosphate uronic acids.

One treatment used for this purpose was preincubation of the preparation with phosphodiesterase. The effectiveness of this treatment in hydrolysing nucleotides is demonstrated by the data given in Fig. 1. About one minute after the addition of the phosphodiesterase no more polysaccharide was form-

<table>
<thead>
<tr>
<th>conditions</th>
<th>methylester formed [counts/min]</th>
</tr>
</thead>
<tbody>
<tr>
<td>1) enzyme etc.+water 5° + 25% glyc.</td>
<td>2° + [¹⁴C]—SAM 30°</td>
</tr>
<tr>
<td>2) enzyme etc.+water 5° + PDE in 25% glyc.</td>
<td>2° + [¹⁴C]—SAM 30°</td>
</tr>
<tr>
<td>3) enzyme etc.+ UDP-GalUA 5° + PDE in 25% glyc.</td>
<td>2° + [¹⁴C]—SAM 30°</td>
</tr>
</tbody>
</table>

Table 1. Influence of preincubation with phosphodiesterase and UDP-galacturonic acid on the formation of polygalacturonate methyl ester. The incubation mixture consisted of 25 µl incubation buffer, 5 µl particulate enzyme suspension, 5 µl water or UDP-galacturonic acid (0.96 mmole), 5 µl 25% glycerol or phosphodiesterase in 25% glycerol and 5 µl [¹⁴C]-SAM (180 000 counts/min, 1.9 mmole), final volume 55 µl, final pH 6.6. The constituents were added at different times in the indicated sequence, the mixtures incubated after the addition of [¹⁴C]-SAM for 30 minutes, stopped and assayed as in the standard assay procedure. The given values are averages from each four identical samples.
ed from UDP-[14C]-galacturonic acid. This was due to complete transformation of the nucleotide to the respective uronic acid-L-P04, as verified by paper electrophoresis of the products in 0.2 M ammonium formate buffer, pH 3.6.

![Graph](image)

**Fig. 1.** Prevention of polysaccharide formation from UDP-[14C]-galacturonic acid by addition of phosphodiesterase. The electrophoresis assay for polygalacturonate formation according to reference 8 was used with the following alterations: 3 x 10^{-3} M MnCl2, 8500 counts/min corresponding to 0.35 mMoles UDP-[14C]-galacturonic acid, final pH 6.7, final volume 40 μl. 5 μl of phosphodiesterase in 25% glycerol or 5 μl of 25% glycerol (control) were added 30 seconds after the start of incubation. The reactions were stopped with 20 μl of 5% TCA.

The data reported in Table 1, lines 1 and 2, show that preincubation of the incubation mixture with phosphodiesterase does not decrease the formation of pectin methyl ester groups from [14C]-SAM. In the contrary, the value is even slightly higher as compared with the control. This fact cannot be explained in the moment but was consistent in all experiments.

Another treatment employed to eliminate the possibility that the preparation contained nucleotides was prolonged preincubation of the assay mixture and then measuring the rate of methyl group formation (Fig. 2). The enzyme activity dropped only slightly and the enzyme retained still 50% of its initial activity even after 2 hours of preincubation at 30°C. During this preincubation period the particular preparation would have converted appreciably high amounts of nucleotide uronic acids, in part by the formation of polysaccharides and in a greater part by epimerization, decarboxylation and hydrolysis. The respective enzymes are very potent in the particulate preparation, which is demonstrated as they strongly interfere in sugar transfer experiments by causing a rapid run-out of nucleotide sugars (8, 9 and own unpublished results). It is, therefore, unlikely that any trace of nucleotide sugar, which still might have been in the preparation shortly after the grinding of the tissues, would have survived a two hours preincubation period.

It must be concluded from both types of experiments just described, that endogenous nucleotide uronic acids cannot be the acceptors for the methyl ester groups found in the pectic substances and that polygalacturonate molecules themselves might be the acceptors. That such polysaccharides are present in similar preparations is evident from analytical data 10.

**Methylation of exogenous pectic substances**

It was next intended to determine the influence of different exogenous pectic substance preparations on the amount of methyl ester groups formed. The data in Table 2 show that there is definitely no increase and no decrease. To explain this observation, we first assumed that an endogenous pectin methyl esterase, which is present in the same enzyme preparation 11, might have immediately split the newly

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9 H. Kauss, BBA 118, 372 [1967].
Polygalacturonate prep. added  | methyl ester formed [counts/min]
--- | ---
water (control) | 1898
polygalacturonate | 1949
pectin N.F. | 1829
citrus pectin (about 30% methylated) | 1905

Table 2. Influence of different polygalacturonate preparations on the formation of pectin methyl ester by the particulate enzyme preparation. Standard assay + 5 μl of the polygalacturonate preparation in water (2.5 mg/ml, pH 6.8), incubated for 15 minutes. Average values from two identical samples in each case.

formed ester groups. If this had been the case, the overall process should have resulted in the formation of radioactive methanol from the [14C]-SAM. This, however, was not observed as demonstrated by the experiments reported in Table 3. It must, therefore, be concluded that none of the different exogenous polygalacturonate preparations was methylated by the methyl transferase. This could have two reasons. It could be that the pectic substances did not have the right chemical structure. This is unlikely as the homomeric parts of the pectic substances from different plants seem to have quite similar structure. The more likely explanation is that the exogenous polysaccharides could somehow not come in the proper way to the active site of the methylating enzyme. It has been demonstrated by other experiments that the enzymes galacturonosyl transferase and polygalacturonate methyl transferase as well as the newly formed pectic substances are localized together in structural compartments containing lipid membranes as integral parts.

Table 3. Amount of total radioactivity in the assay mixture after incubation with and without pectic substances. To demonstrate that no radioactivity was liberated as methanol, the standard assay mixture for polygalacturonate methyl transferase but with about 40 000 counts/min [14C]-SAM and with 5 μl of the pectic substance (2.5 mg/ml, pH 6.8) or 5 μl of water was incubated. After the indicated time 20 μl of the stopping formate buffer was added and a 50 μl aliquot applied on a paper strip, dried and counted as in the standard assay. The given values are averages of four identical incubations. The amount of pectin methyl ester groups formed in 30 minutes by a similar incubation mixture was determined in a parallel experiment to be about 2000 counts/min.

Methylation of endogenous polygalacturonate

Although exogenous pectic substances did not stimulate the methylating process, it seemed of interest to determine the influence of alterations in the amount of available endogenous polygalacturonate. The data in Table 4 show that the amount of pectin methyl ester produced is more than doubled when UDP-galacturonate is present in the incubation mixture. UDP-glucuronate has a similar, but smaller effect. This is most likely due to the fact, that it can be epimerized to UDP-galacturonate. The presence of the respective enzyme in the particulate preparation can be inferred from the data in references and from unpublished own experiments. No other nucleotide sugar or any of the possible breakdown products of UDP-galacturonate gives a similar stimulation.

<table>
<thead>
<tr>
<th>cofactor [10^{-3} M]</th>
<th>methyl ester formed [counts/min]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (no cofactor added)</td>
<td>2093</td>
</tr>
<tr>
<td>UDP-galacturonic acid</td>
<td>4858</td>
</tr>
<tr>
<td>UDP-glucuronic acid</td>
<td>3520</td>
</tr>
<tr>
<td>UDP-glucose</td>
<td>2111</td>
</tr>
<tr>
<td>UDP-xylose</td>
<td>1967</td>
</tr>
<tr>
<td>UDP</td>
<td>1695</td>
</tr>
<tr>
<td>UMP</td>
<td>1645</td>
</tr>
<tr>
<td>galacturonic acid-1-P0_4</td>
<td>1955</td>
</tr>
<tr>
<td>galacturonic acid</td>
<td>1736</td>
</tr>
</tbody>
</table>

Table 4. Influence of different nucleotides and their possible breakdown products on the incorporation of methyl ester groups into pectin. Standard assay, 10 minutes incubation time.

The explanation for this stimulation is clear from data in Table 1, line 2 and 3. The UDP-galacturonate was offered to the particulate preparation for 5 minutes, then any remaining nucleotide split by phosphodiesterase before the methylating process was started. This treatment, too, resulted in a definite increase in the amount of pectin methyl ester synthesized. The uronic acid unit of the UDP-galacturonate obviously led to the formation of new or to the elongation of already existing polygalacturonate chains whose carboxyl groups represented new acceptor sites in addition to those still available from the original cell. The additional methyl ester groups introduced in this experiment must be located in the newly formed parts of the polygalacturonate chains. The experiment does not allow, however, any conclusion in respect to the problem, whether in the regular particulate preparations the methylation only
occurs at chain ends "in construction" or all over the macromolecule. This question has to await future studies.

Figure 3 demonstrates that the increase in available acceptor molecules really affects the initial rate of the reaction. It can be concluded, therefore, that

\[
\text{t} + \text{U} \text{C} - \text{S} \text{A} \text{M} \quad [\text{min}]
\]
\[
\text{t} + \text{P} \text{D} \text{E}
\]

Fig. 3. Time course of the incorporation of methyl ester groups into pectin by particulate enzyme preparations pre-incubated with (B) or without (A) UDP-galacturonic acid. Two incubation mixtures were composed: A) 280 \( \mu l \) incubation buffer + 70 \( \mu l \) particulate enzyme preparation + 70 \( \mu l \) water. B) the same, but 13.4 \( \mu m \) moles of UDP-galacturonic acid in the 70 \( \mu l \) of water. Both mixtures were incubated for 1 minute and then 70 \( \mu l \) of PDE in 25% glycerol was added and the mixtures incubated for another 2 minutes at 30°. The mixtures were then cooled to 0°, aliquots of 30 \( \mu l \) transferred to small tubes and 5 \( \mu l \) of water added to each of them. The methylation was started by addition of 5 \( \mu l \) of \( [1^4\text{C}]\)-SAM (180 000 counts/min, 1.9 \( \mu m \) moles) and immediate transfer of the tube to 30° for the indicated time. The reaction was stopped and the pectin methyl ester formed was assayed as in the standard assay.

in the regular particulate preparation under the conditions of the standard assay the amount of the polygalacturonate present is a limiting factor, whereas the available activity of the methylating enzyme itself is not fully employed.

The latter is evident, too, from the fact that the initial rate in regular preparations can be increased at least up to threefold if the \( [1^4\text{C}]\)-SAM concentration is higher as in the standard assay (Fig. 4 in reference 6). The observed slight decrease in the initial rate after preincubation of the incubation mixture (Fig. 2) can, therefore, not account for the non-linearity with time in the formation of pectin methyl ester groups (Fig. 3 A, and reference 6), at least in the first 15 minutes. From Fig. 3 and Table 1 it can rather be concluded that the limited amount of available acceptor may be one of the factors accounting for the non-linearity. An important additional factor seems to be the occurrence of several other enzymes in the particulate preparation which produce unknown products from \( [1^4\text{C}]\)-SAM and cause the disappearance of most of the substrate contained in the standard incubation mixture within one hour.

**Influence of methylation on the formation of polygalacturonate**

It is obvious from the data discussed so far that the formation of the polygalacturonate chain is a prerequisite for the methylation process, and it was of interest now whether or not the synthesis of the polysaccharide backbone on the other hand is influenced by the introduction of the methyl ester groups. Table 5 shows that the rate of polygalacturonate formation from UDP-galacturonate is independent from the methylation process, with a pH value optimal for the methylation as well as optimal for polygalacturonate synthesis. Similar results gave experiments, which were run up to 10 minutes under the conditions used in Fig. 2 (or Fig. 1 of reference 8) where the formation of polysaccharide was not linear with the incubation time. This means that the formation of the polygalacturonate backbone of pectin proceeds independently of the introduction of its methyl ester groups.

All these experiments sustain the idea that the biosynthesis of the methylated polygalacturonate chain

<table>
<thead>
<tr>
<th>conditions</th>
<th>polygalacturonate formed [counts/min]</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 min, pH 6.05 -AM</td>
<td>168</td>
</tr>
<tr>
<td>1 min, pH 6.05 +AM</td>
<td>154</td>
</tr>
<tr>
<td>1 min, pH 6.65 -AM</td>
<td>105</td>
</tr>
<tr>
<td>1 min, pH 6.65 +AM</td>
<td>96</td>
</tr>
<tr>
<td>5 min, pH 6.65 -AM</td>
<td>496</td>
</tr>
<tr>
<td>5 min, pH 6.65 +AM</td>
<td>491</td>
</tr>
</tbody>
</table>

Table 5. Influence of AM (2.5 \( \times 10^{-3} \) m) on the formation of polygalacturonate from UDP-[\( 1^4\text{C}]\)-galacturonic acid. The electrophoresis assay according to reference 8 was used with minor alterations to give conditions for which the assay was linear with time (5 \( \mu l \) of the 1 : 5 diluted particulate enzyme preparation, 3 \( \times 10^{-4} \) M MnCl\(_2\), 17 000 counts/min corresponding to 0.7 \( \mu m \) moles UDP-[\( 1^4\text{C}]\)-galacturonic acid, final volume 40 \( \mu l \)). Average values from two identical incubation mixtures.
in the pectic substances occurs by methylation of preformed polysaccharide molecules. It is now general agreed that the degree of methylation in native pectic substances can vary considerably \(^1,2\), possibly depending on the type and the developmental stage of the tissue investigated \(^2\). This may indicate that the degree of methylation indeed is of importance in respect to the biological function of the pectic substances. Unfortunately no information is available in the moment whether the methyl groups are distributed randomly or arranged in a certain pattern over the molecule chains. It appears difficult, therefore, to propose, by which mechanism the extend of methylation is finally regulated.

This work was supported by a grant from the Deutsche Forschungsgemeinschaft. We wish to thank Dr. M. H. Zenk for valuable help during the course of the studies.

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**Weitere Untersuchungen zur Aminosäuresequenz des Melittins**

**IV.: Messung der optischen Rotationsdispersion**

**Joachim Jentsch**

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Melittin is the (main) toxic peptide of bee venom having a molecular weight of 2840, with a known sequence (s. fig. 1). Optical rotatory dispersion of non-crystalline melittin in aqueous solution suggests that the polypeptide chain is random, although 7\% \(\alpha\)-helix has been determined. These results are in agreement with the amino acid sequence of melittin and the assumption that the biological activity is attributable to its surface active character.

Die zellschädigenden Eigenschaften des Melittins und die Erniedrigung der Oberflächenspannung des Wassers wurden aus der extrem ungleichmäßigen Verteilung der hydrophoben und hydrophilen Aminosäurereste erklärt.

Nach gelungener Aufklärung der Aminosäuresequenz des Melittins ist die Kenntnis der Sekundärstruktur von großem Interesse. In der vorliegenden Arbeit wird daher über den \(\alpha\)-Helixgehalt des Melittins in Lösung berichtet, gemessen durch die optische Rotationsdispersion im sichtbaren und UV-Bereich.

**Experimenteller Teil**

Das spezifische Drehvermögen wird bei Raumtemperatur mit dem lichtelektrischen Präzisionspolarimeter * \(0,005°\) der Firma Zeiss (Oberkochen) bei den Wellenlängen 365, 405, 436, 546 und 578 m\(\mu\) gemessen. \(\varepsilon = 4,09 \times 10^{-3}\) g/ml Wasser.

Die optische Rotationsdispersion des Melittins im UV-Spektralbereich (Cotton-Effekt) wird mit dem Cary-Spektralpolarimeter Modell 60 ** gemessen. \(c = 0,084\) mg/ml Wasser. Die Messung erfolgt im Wellenlängenbereich von 195—300 m\(\mu\). Der volle Skalencode beträgt \(0,2°\), Dämpfung = 3 Sek., Temperatur = 27 \(^\circ\) C, Schichtdicke = 1,0 cm und die Schreibgeschwindigkeit = 0,14 m\(\mu\)/Sekunde.

* Herrn Doz. Dr. R. Jänicke, Institut für physikalische Biochemie, Universität Frankfurt, danken wir für die Messungen im lichtelektrischen Polarimeter.

** Für die Messung der optischen Rotation im Cary-Spektralpolarimeter danken wir Herrn Dr. A. Stock, Institut für Biochemie, Universität Frankfurt.

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\(^2\) II. Mitt.: J. Jentsch, unveröffentlicht.