SUBSTRATE SPECIFICITY IN PECTIN SYNTHESIS*

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Upon examination of various glucose-linked nucleotides as glycosyl donors in plant cellulose synthesis, Elbein et al. (1964) showed that GDP-D-glucose was highly specific for this reaction. This finding stimulated an investigation to determine the nucleotide specificity involved in plant polygalacturonate synthesis. Although a pathway for the formation of UDP-D-galacturonate from UDP-D-glucose has been well documented in higher plants (Hassid et al., 1959) and UDP-galacturonate has been isolated from mung bean seedlings (Neufeld and Feingold, 1961), the mechanism of pectin synthesis has not been elucidated. The present communication provides initial evidence that UDP-galacturonate is the most active galacturonosyl donor.

Preparation of the radioactive galacturonic acid nucleotides - D-galactose-1-^{14}C (0.9 μc/μmole) was phosphorylated with ATP and galactokinase (from Saccharomyces fragilis) to give α-D-galactose-1-^{14}C-1-P (Trucco, et al., 1948). Galactose-1-^{14}C-1-P was subsequently oxidized to D-galacturonate-1-^{14}C-1-P (GalUA-1-P) by catalytic oxidation using the method of Marsh (1952) with minor modifications (Lin, 1965). The formation of galacturonic acid increased somewhat linearly with time for about 9 hours, at which time 63%

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of the galactose had been oxidized. Prolonged oxidation or reaction at higher temperature resulted in the production of significant amounts of two radioactive compounds migrating faster than GalUA-1-P on electrophoresis. The GalUA-1-P could be completely converted to radioactive GalUA either by hydrolysis at 100° for 15 minutes in 0.1 N HCl or by treatment with seminal phosphatase.

Radioactive ADP-, CDP-, GDP-, TDP-, or UDP-galacturonates were synthesized according to the proceedure of Roseman et al. (1961). The mixture was allowed to react for 5 days at 30° or 3 days at 37° and was then treated with ether and water containing sodium acetate. The aqueous solution was concentrated and the products were separated by paper electrophoresis in either 0.2 M ammonium formate buffer, pH 3.8 ($M_{picrate}$: ADP-GalUA 0.98, CDP-GalUA 0.92, GDP-GalUA 1.05, GalUA-1-P 1.34) or 0.05 M phosphate buffer, pH 7.5 ($M_{picrate}$: UDP-GalUA 1.38, TDP-GalUA 1.44, GalUA-1-P 1.87). All of these nucleotides were purified chromatographically in isobutyric acid: ammonium hydroxide: water (57:4:39). Average yields were 30-45% based on radioactivity of the GalUA-1-P. All of these products liberated GalUA-1-P as the sole radioactive compound upon treatment with snake venom phosphodiesterase.

Preparation and Assay of Enzyme - Mung bean (Phaseolus aureus) seeds were germinated in a moist chamber in the dark at room temperature as previously described (Barber et al., 1964). Three day old seedlings were collected and the roots (12 g) were ground with sea sand in an equal weight of 0.1 M Tris-HCl buffer, pH 7.5 at 0°. The homogenate was squeezed through two layers of cheesecloth and centrifuged at 105,000 x g for 1 hour. The precipitate suspended in a small volume (0.5 ml) of Tris buffer was used as the enzyme preparation. This fraction has previously been shown to incorporate glucose from GDP-glucose into cellulose (Elbein et al., 1964).

Similar particulate fractions were prepared from green tomatoes and turnip. The viscous pellet obtained from about 30 g of each material was suspended in about 1 ml of Tris buffer.

For determination of the incorporation of radioactivity, 0.1 ml of the particulate suspension was incubated with 0.1 μmole of $MgCl_2$ and 0.1 μmole (0.09 μc) of one of the following: ADP-GalUA, CDP-GalUA, GDP-GalUA, TDP-GalUA, or UDP-GalUA. After 60 minutes at 30°, an equal volume of 95% ethanol was added and the precipitate collected by centrifugation. The precipitate was washed with 30% ethanol and an aliquot of the combined ethanol extract was taken for radioactivity measurement. The residues were then extracted 3 times with 0.5 ml of water at 100° for 10

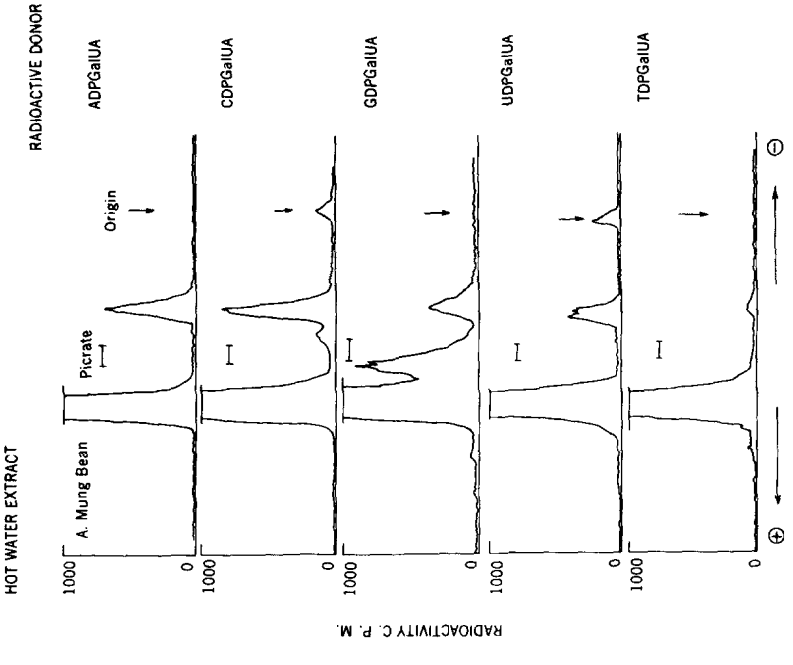

Fig. 1. Paper electrophoresis of the hot-water extracts of the reaction mixtures after incubating mung bean and tomato particulate preparations with D-galacturonosyl-1-^{14}C-diphosphate derivatives of adenosine, cytidine, guanosine, uridine, and thymidine. Each of the reaction mixtures contained 0.1 ml of particulate suspension in 0.1 M Tris-HCl, pH 7.5 (1.5-2.0 mg protein), 0.1 µmole of $MgCl_2$, and 0.1 µmole (0.09 µc) of one of the radioactive nucleotides in a total volume of 0.4 ml. The reaction mixtures were treated as described in the text. The hot-water extracts were concentrated to a small volume, and aliquots were subjected to paper electrophoresis in 0.2 M ammonium formate at pH 3.8. The radioactivity on paper was traced by a Vanguard 4π scanner.

minutes, and an aliquot of the combined water extract was removed for radioactivity determination. The remaining residue was spread on a planchet, dried and counted. After counting, the residue was resuspended in a small volume of 0.05 M acetate, pH 5.3 and incubated with fungal pectinase at 30° for 2 hours. The mixtures were centrifuged and the supernatant solutions were plated on planchets and counted.

The enzyme fraction was incubated in the absence of any galacturonate nucleotide and fractionated as described above. When each fraction was then analyzed for uronic acid content it was found that more than 80% of the carbazole-positive material resided in the hot-water extract. Thus, it is assumed that this hot-water soluble fraction contains the major polygalacturonate fraction or so called "pectic substance".

Results - As shown in Figure 1, the mung bean particulate enzyme catalyzed the incorporation of radioactivity from UDP-GalUA into a material which was insoluble in aqueous ethanol, soluble in hot water, but immobile on paper electrophoresis at pH 3.8. Less, but still significant, radioactivity

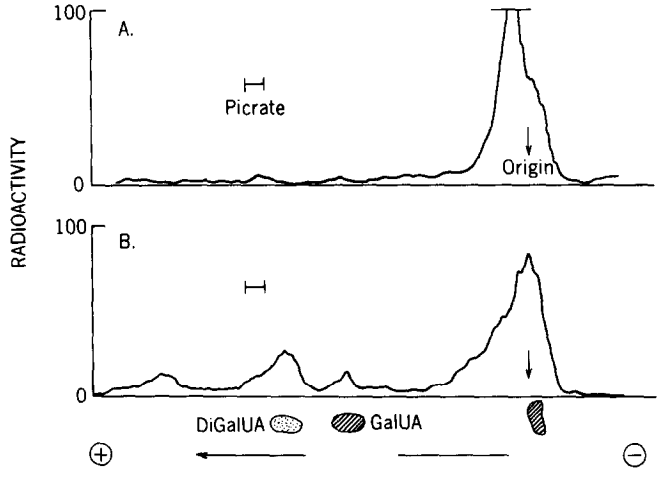

Fig. 2. Paper electrophoresis of the pectinase-partial hydrolyzate of the product isolated from the hot-water extract after incubation of mung bean particles with UDP-galacturonate-1-^{14}C. The condition of pectinase treatment is described in the text. The upper curve represents tracing of the incorporated material after re-electrophoresis, and the lower indicates release of radioactive galacturonic acid and digalacturonic acid by the enzyme. No low molecular weight saccharides could be detected by paper chromatography in pyridine: ethyl acetate: H_2O from the radioactive residue still located at the origin after enzymic digestion, but more radioactive galacturonic acid was liberated from it by prolonged incubation with pectinase. The outlined areas are tracings of the bands obtained after staining with $AgNO_3$ on the electrophoretogram of citrus pectin treated with pectinase.

was found in this area when CDP-GalUA was used as substrate. The other nucleotides were inactive. Particles previously heated for 3 minutes did not catalyze the incorporation. Tomato and turnip particles also incorporated GalUA from UDP-GalUA, but in these cases TDP-GalUA was also active. (In one experiment in which 2 x 10^4 c.p.m. of each nucleotide was used, the c.p.m. remaining at the origin were as follows: UDP-GalUA, 340; TDP-GalUA, 115; CDP-GalUA, 20; GDP-GalUA, 10; ADP-GalUA, 5; counted with a thin window Geiger counter.) The extent of incorporation of radioactivity from UDP-GalUA into this electrophoretically immobile material was low and amounted to an incorporation of about 0.1 mμmole/hour/mg protein. However, the incorporation could be greatly improved and demonstrated reproducibly by using UDP-GalUA with a higher specific activity (7 μc/μmole), and by using the assay described in Figure 4.

The radioactive eluates from the origin of the electrophoretograms (Figure 2) were suspended in 25 μl of 0.1 M acetate buffer, pH 5.5 and treated with 25 μl of 2% fungal pectinase at 37° for 2 hours. Citrus pectin was used as a control. As shown in Figure 2, after this treatment, electrophoretically mobile, labeled components with the same mobility as authentic galacturonic and digalacturonic acids were formed. The DiGalUA was further identified in ethyl acetate: acetic acid: water (3:1.2:3) and the number of galacturonic acid residues (determined by the borate-carbazole method) per mole of material (based on reducing value). It is not known whether both galacturonic acid residues are labeled. A considerable amount of activity remained at the origin after pectinase treatment and of this 60% became mobile after exhaustive enzymic hydrolysis for 48 hours.

Incorporation of ^{14}C from UDP-GalUA into the ethanol-insoluble, hot-water soluble fraction was proportional to time for 10 minutes as shown in Figure 3 and then decreased sharply. This decrease may be due to hydrolytic enzymes which degrade the pectin substance. This possibility seems more likely in view of the fact that during the course of incubation a number of ^{14}C-electrophoretically-mobile components were formed. Preliminary evidence indicates that these are galacturonate derivatives. However, possible transformation of the initially incorporated galacturonosyl derivative into a hot water insoluble form cannot be excluded (Jansen et al., 1959). The incorporation of radioactivity within 15 minutes was also proportional to the amount of protein. No incorporation occurred when either GalUA, GalUA-1-P or UDP-glucuronic acid (all ^{14}C-labeled) were used in place of UDP-GalUA.

The radioactivity detected in the polymer was shown to be due to

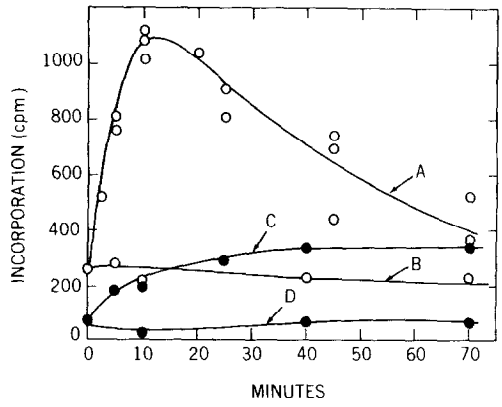

Fig. 3. Time course of UDP-D-galacturonate-^{14}C incorporation into ethanol-insoluble, hot-water soluble fraction by mung bean particulate preparations. The reaction mixtures consisted of 0.1 ml of particulate suspension in 0.1 M Tris-HCl, pH 7.5, 0.1 μmole of $MgCl_2$, 0.1 μmole of UTP, and 0.3 mμmole of UDP-D-galacturonic acid (ca. 2 X 10^4 c.p.m.). The amount of protein in each reaction mixture was about 0.8 mg (particles prepared in Tris buffer) or 0.6mg (particles prepared in Tris buffer containing 5% PVP and 0.5% ascorbate). At the indicated times of incubation at 36° the reaction mixtures were analyzed by the procedure described in the text except that the first precipitate was washed five times with 50% ethanol. Citrus pectin (0.5 mg) was then added and the mixture extracted with hot water. The hot water solution was then precipitated by the addition of 5 volumes of ethanol and the precipitate collected and counted in hyamine in a liquid scintillation spectrometer.

A - Tris-HCl buffer preparation
B - Heated preparation
C - Tris-PVP-ascorbate preparation
D - Heated Tris-PVP-ascorbate preparation

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incorporation and not to surface adsorption as follows: UDP-GalUA (7.5 x 10^5 c.p.m.) was incubated for 15 min. with mung bean particles (12 mg protein). The ethanol-insoluble, hot water-soluble material was isolated, mixed with 10 mg citrus pectin and treated with 1 ml of predialyzed pectinase. The mixture was then heated at 60° for 15 minutes and centrifuged. The precipitate was washed twice with 1 ml of water and the combined supernatant solution passed through a column of Dowex AG 50 (H^+)(1 x 5 cm). The effluent was concentrated and applied to a column (1 x 8 cm) of Dowex AG 1 (formate) resin. The column was eluted with 50 ml each of water, 0.1 N, 0.2 N, 0.5 N and 1 N formic acid. As indicated in Table 1, when these eluates were chromatographed in 1-

Table I

Fractionation of Pectinase-Hydrolyzate on Dowex AG 1 (Formate) Column

Eluant (50 ml)	c.p.m.	Radioactive components
Water	1650	---
0.1 N Formic acid	2604	GalUA, A low R_f compound
0.2 N Formic acid	14420	DiGalUA, GalUA, TriGalUA A high R_f compound
0.5 N Formic acid	13650	TriGalUA, A high R_f compound
1 N Formic acid	3150	Low R_f and high R_f compounds

propanol: ethyl acetate: H_2O (6:1:3) along with authentic GalUA, DiGalUA and TriGalUA, radioactive compounds with the same mobility as DiGalUA and TriGalUA were found in the 0.2 N and 0.5 N formic acid eluate. The low R_f compounds observed in these eluates may be more highly polymerized galacturonic acid compounds whereas the components with a high R_f may be methylated derivatives. However, more work must be done before the nature of these compounds can be determined.

Summary - Particulate preparations from higher plants incorporate galacturonic acid from UDP-galacturonate into a "pectin-like" material. The enzymatic reaction is fairly specific for UDP-GalUA although some incorporation of TDP-GalUA was observed with tomato particles, and a slight incorporation of CDP-GalUA with mung bean particles. The radioactive product formed from UDP-GalUA liberated GalUA, DiGalUA, and TriGalUA when treated with pectinase, indicating that it was polygalacturonic acid. More extensive studies on the nature of the product are being communicated elsewhere (Villemez, et al., 1965).

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