Phytochemistry, Vol. 24, No. 4, pp. 699–702, 1985.
Printed in Great Britain.

0031–9422/85 $3.00 + 0.00

DECREASE OF POLYGALACTURONIC ACID SYNTHASE DURING XYLEM DIFFERENTIATION IN SYCAMORE

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(*Revised received* 25 *September* 1984)

Key Word Index—*Acer pseudoplatanus*; Aceraceae; sycamore; pectin synthesis; cell walls; secondary thickening.

Abstract—The activity of a polygalacturonic acid synthase, a membrane bound enzyme, was measured in particulate preparations obtained from homogenates of cambial cells, differentiating xylem cells and differentiated xylem cells isolated from actively growing trees of sycamore (*Acer pseudoplatanus*). The specific acitivity of the enzymic system fell at least 6–10-fold as cells differentiated from the vascular cambium to xylem. The percentage of radioactivity incorporated as galacturonic acid was 88% for cambium, 69% for differentiating xylem and 57% for differentiated xylem. The remainder of the radioactivity was in glucuronic acid. UDP-D-galacturonic acid-4-epimerase (EC 5.1.3.6) in the membrane preparation, therefore, probably enabled incorporation of radioactivity in the form of glucuronic acid by other synthases into polymers which are characteristic of secondary walls. Nevertheless, a considerable decrease of polygalacturonic acid synthase activity occurred which was correlated with the cessation of pectin deposition. This contrasts with the marked increase observed in xylan synthase activity as the cells differentiate.

INTRODUCTION

During the transition from primary to secondary wall there is a marked shift in the synthesis of cell wall polysaccharides characterized by the cessation of pectin deposition and enhanced deposition of hemicellulose and α-cellulose [1]. In sycamore trees, hemicellulose is represented by xyloglucan in the primary cell walls [2, 3], while in secondary cell walls it is mainly β-(1 → 4)-xylan with short side chains of 4-*O*-methyl-D-glucuronic acid [1]. Pectin, obtained from undifferentiated sycamore cells, consists mainly of acidic rhamno-galacturonan chains containing some neutral arabino-galactan side chains and a separate neutral arabino-galactan [4, 5]. Other minor pectin and hemicellulose components may be present in the primary cell wall [6].

The monomeric components of the polysaccharides are derived from the pool of nucleoside diphosphate sugars which are, to some extent, interconvertible [7]. In sycamore during the differentiation of cambial cells to xylem, the control of xylan synthesis is partly exerted at the level of the synthase and not at the stages involving interconversion of precursors by epimerases [7–10]. Similarly, xylan and arabinan synthesis are regulated in bean, in part, by the appearance of the requisite synthase within the endomembrane system [11–13]. It has not yet proved possible to demonstrate a decrease of arabinan synthase activity during differentiation in sycamore due to the presence of high levels of UDP-D-xylose-4-epimerase (EC 5.1.3.2) so the present study has focused attention upon the polygalacturonic acid synthesizing system. This enzyme has previously been characterized in mung bean [14–16]. We have examined the changes in this synthase in particulate preparations obtained from homogenates of sycamore cells at different stages of differentiation. A significant decrease in activity was observed as the cells differentiate.

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RESULTS AND DISCUSSION

Incorporation from UDP-D-[^{3}H]galacturonic acid into polymeric material

Particulate enzyme preparations from homogenates of cambial cells, differentiating xylem cells and differentiated xylem cells isolated from sycamore trees catalysed the formation of radioactive polymeric material using UDP-D-[1-^{3}H]galacturonic acid as substrate. The synthesized [^{3}H]polymer could be separated from the nucleoside diphosphate–uronic acid precursor by isolation of the material in 50% ethanol. This isolated material remained at the origin when it was subjected to PC for the separation of uronic acids [17]. Contamination of the polymer by free sugar or nucleotide sugar was, therefore, low but it was increased in the presence of Ca^{2+} so that attempts to facilitate precipitation of the polymer in the presence of this cation produced undesirable artefacts.

Analysis of incorporation

Incorporation into products other than polysaccharide and those soluble in lipid solvents were negligible (Table 1) and were even less than that found for arabinan and xylan synthases from bean [12]. These products did not appear to be glycolipids when analysed by TLC [11] and there is no evidence for involvement of lipid intermediates in the synthesis of the polygalacturonan.

Table 1. Distribution of radioactivity into radioactive aqueous and lipid solvent fractions after incubation of membranes with radioactive UDP-D-galacturonic acid

Fractions	Radioactivity incorporated	
	cpm	% of mean total incorporation
Material soluble in chloroform–methanol (3:2)	154 ± 43	0.7
Material soluble in the ethanol (50%) washes	183 000 ± 2000	
Material soluble in chloroform–methanol–water (10:10:3)	318 ± 123	1.5
Polymer	19 800 ± 4 500	97.7

Membranes isolated from cambial tissue were incubated with UDP-D-[1-^{3}H]galacturonic acid and analysed. Mean values ± s.d. for five incorporations are shown.

Analysis of [^{3}H]polymer formed by cambial cells

The isolated polymer synthesized by particulate preparations from cambial cells was hydrolysed by a pectinase containing α-(1 → 4)-polygalacturonase activity. The polymer (88% of the radioactivity) was digested after 16 hr to give soluble galacturonic acid identified by electrophoresis. The [^{3}H]polysaccharide was also hydrolysed by strong acid. Usually pectin of high MW is only hydrolysed with great difficulty but acid hydrolysis with 4% (w/w) sulphuric acid has proved successful for pectins of plasmolysed leaf cells [18] and sycamore roots [19]. The polysaccharide synthesized by the membranes from cambial cells was hydrolysed to the extent of 92% by this treatment and the content of the product determined by electrophoresis was 86 ± 4% galacturonic acid and 14 ± 2% glucuronic acid. Membranes isolated from cambial cells of sycamore can be shown, therefore, to possess an enzyme system capable of transferring galacturonic acid from UDP-D-galacturonic acid to polygalacturonic acid, probably α-(1 → 4)-linked.

Kinetics of synthase activity

Incorporation of radioactivity from the radioactive precursor was linear up to 15 min. Figure 1 shows the influence of substrate concentration on the reaction rate. From our results the apparent K_m was 0.77 mM. This does not agree with the value obtained for mung bean by Villemez *et al.* [15] (1.7 μM). However, in the earlier study [15], activity was measured over a low concentration range (0.3–10 μM) and the double reciprocal plot deviated markedly at higher substrate concentration. The enzyme system may be superficially similar to that found for the β-glucan synthase system which has two apparent K_m values [20], although in our study of the polygalacturonic acid synthase this was not apparent over the range of substrate concentration we used (0–2 mM).

Changes in activity of polygalacturonan synthase during differentiation

Characterization of the enzyme was carried out with membranes from cambial tissue. Comparison of the activities of membranes from cambium, differentiating xylem and differentiated xylem was made by using preparations from trees felled during three successive springs. A 6–10-fold decrease in specific activity during maturation of the cells was observed (Table 2). This compares with an eight-fold increase in xylan synthase involved in hemicellulose synthesis which was measured in the same membrane fractions from cells isolated from the tree felled in year 1 [10]. A decrease in polygalacturonic acid synthase observed in membranes isolated from cells from a tree felled in year 2 was accompanied by a 10-fold increase in phenylalanine ammonia lyase (EC 4.3.1.5) involved in lignin synthesis which accompanies secondary thickening [21].

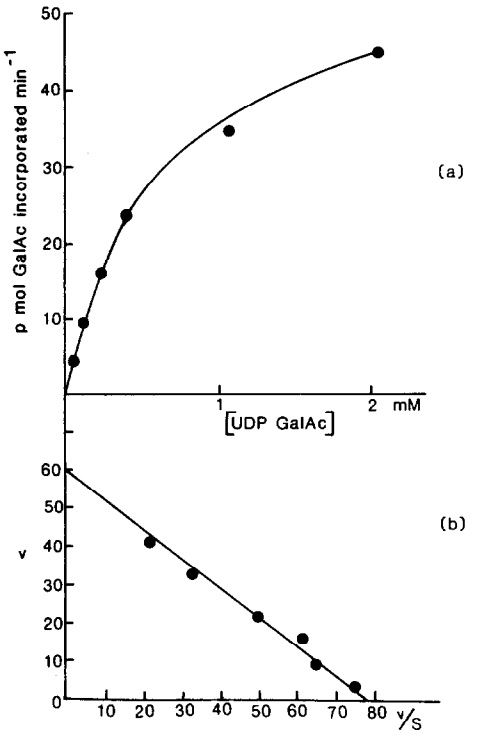

Fig. 1. (a) Kinetics of polygalacturonan synthase with varying UDPGalAc concentrations; and (b) analysed by a v against v/S plot (slope = K_m = 0.77 mM).

Variations in the apparent loss of activity of polygalacturonan synthase were observed in subsequent years and these may have been due to the stage of growth of the cells and environmental factors. However, comparisons of the product of the three cell types showed increased resistance to pectinase treatment and increased incorporation of radioactivity in the form of glucuronic acid as the cells differentiated (Table 3). The membranes contained similar levels of 4-epimerase activity so that sufficient UDP-D-glucuronic acid was produced during the incubation to allow incorporation into hemicellulose-like material by the membranes from differentiating and differentiated xylem. A single mixed polysaccharide heteropolysaccharide was unlikely to have been formed as the glucuronic acid incorporated was resistant to pectinase treatment which released only galacturonic acid.

Table 2. Synthase activities in differentiating sycamore cambium cells

Sources of particulate enzyme fraction	Polygalacturonan synthase: Sp. act. (nmol/min/mg protein)	Sp. act. corrected for K_m value (nmol/min/mg protein)	Sp. act. corrected for GlcAc content (nmol/min/mg protein)	Activity per cell (nmol/cell)	Sp. act. of xylan synthase (nmol/min/mg protein)	Sp. act. of phenylalanine ammonia lyase (nmol/min/mg protein)
Year 1 (1979) (a)						
Cambial cells	0.4	7.4	6.4	4.0×10^{-7}	0.5	—
Differentiating xylem	0.2	3.7	2.6	3.1×10^{-7}	1.1	—
Differentiated xylem	0.1	1.8	1.0	1.7×10^{-7}	3.9	—
Year 2 (1980) (b)						
Cambial cells	0.5	2.1	1.8	—	—	5.1
Differentiating xylem	0.3	1.3	0.9	—	—	22.3
Differentiated xylem	0.1	0.4	0.2	—	—	57.5
Year 3 (1981) (b)						
Cambial cells	0.7	2.9	2.5	—	—	—
Differentiating xylem	0.3	1.3	0.9	—	—	—
Differentiated xylem	0.1	0.4	0.2	—	—	—

The activities were measured on membranes isolated as particulate fractions (1000–100 000 *g*) from cell homogenates. The values for the activities measured using either 40 μM(a) or 240 μM(b) UDPGalAc have been corrected by factors [(18.5 × (a) and 4.2 × (b)] calculated from the K_m value of 0.77 mM for the synthase corrections for the amounts of glucuronic acid in the synthesized polymer were made from the data shown in Table 3.

Table 3. Uronic acid in the product synthesized by membranes from the tree felled in year 3

Source of particulate enzyme	Epimerization of substrate during incubation (%)	Materials degraded by pectinase (%)	Uronic acid in acid hydrolysate: GalAc (%)	GlcAc (%)
Cambial cells	12	92	86	14
Differentiating xylem	22	68	69	31
Differentiated xylem	24	57	57	43

The uronic acids were separated by electrophoresis at pH 3.5 and the epimerization was estimated by the relative amounts of GalAc and GlcAc in the precursors present in the supernatant [7, 8].

The product synthesized by membranes from cambial cells isolated from a tree felled in year 3 did, however, appear to be mostly polygalacturonic acid. The small amount of glucuronic acid present may have been due to contaminating membranes from differentiating cells or it may reflect the state of differentiation of the cambial layer itself. Taking into account the level of incorporation into glucuronic acid, the specific activity of the cambium was *ca* 2–3-fold greater than that in differentiating xylem and 6–10-fold greater than that in differentiated xylem. These results indicate that one of the control points of the loss in pectin production during differentiation of xylem cells is exerted by a decrease in polygalacturonic acid synthase activity. Although the rate limiting steps were not identified, this decrease may be compounded by an increase in the rate of conversion of UDPGalAc → UDPGlcAc and increased levels of a polyglucuronic acid synthase concerned with hemicellulose production.

EXPERIMENTAL

Plant material. Trees of sycamore (*Acer pseudoplatanus*) (16–20 years old) *ca* 10–15 m tall and 200 mm in diameter were cut down during late May and early June 1979, 1980 and 1981 from Madingley Wood, Cambridge, U.K. Logs (1 m) were cut from each tree and transferred to a cold room for all further manipulations. Cambial cells, differentiating xylem cells and differentiated xylem cells were obtained as described in refs [7, 10] and the number of cells in the samples were counted as previously described.

Chemicals and radiochemicals. D-[1-^{3}H]Galacturonic acid (7.4 GBq/nmol) was obtained from the ^{3}H-labelling service of Amersham International, U.K. UDP-D-[1-^{3}H]galacturonic acid was synthesized using enzymes isolated from mung bean [22, 23]. The sp. act. of the radioactive UDP-galacturonic acid was determined after ion exchange chromatography on Amberlite/R45. The nucleotide was bound to the column at

pH 7.0 and eluted with 0.1 M HCOOH. Non-radioactive UDP-D-galacturonic acid and pectinase were obtained from Sigma.

Preparation of membrane fractions. The particulate membrane fractions were prepared as described in ref. [10].

Enzyme assays. Incorporation of radioactivity from 7.4 kBq of UDP-D-[1-^{3}H]galacturonic acid was measured after incubation at 26° in 50 mM NaPi buffer, pH 7.2, 1 mM dithiothreitol, 10 mM $MgCl_2$ and 0.4 M sucrose with varying amounts of UDP-D-galacturonic acid with membrane in a total vol. of 25 μl. Incubations were terminated by the addition of 500 μl $CHCl_3$–MeOH (3:2). The ppted material was pelleted by centrifugation at 10000 *g* for 2 min. The pellet was then supplemented with 0.5 ml bean callus pectin (acidic fraction [4]) and washed with 50% EtOH until the washes were free of radioactivity. The polysaccharide residue was resuspended in 500 μl $CHCl_3$–MeOH–H_2O (10:10:2) for 30 min before centrifugation, resuspended in H_2O before addition of 3 ml scintillant [6 g PPO (2,3-diphenyloxazole), 30% (v/v) Triton X-100 in 2 l. toluene] and counted in a Searle Mark III liquid scintillation system (model 6880). The enzyme activity was estimated by comparison with boiled controls. Xylan synthase was estimated by the method described in ref. [9] and the phenylalanine ammonia lyase (EC 4.3.1.5) by the method described in ref. [26].

Characterization of products. Glycolipids. Incorporation of radioactivity into material soluble in $CHCl_3$–MeOH (3:2) and $CHCl_3$–MeOH–H_2O (10:10:3) was estimated and analysed as described in ref. [11].

Polysaccharides. The final residue following extraction with $CHCl_3$–MeOH–H_2O (10:10:3) was hydrolysed by enzymic degradation. Incubations contained 50 μl 10% (w/v) pectinase from *Aspergillus niger* (Sigma) in acetate buffer, pH 4.4, for 16 hr at 25°. The reaction was stopped by boiling and the samples were centrifuged at 10000 *g* for 5 min. Samples of the supernatants were electrophoresed at pH 3.5 (5 ml pyridine, 50 ml HOAc made up to 1 l. with H_2O) at 4 kV for 45 min to separate uronic acids [24]. The residue was also hydrolysed with 4% (w/w) H_2SO_4 at 120° 103 kPa for 1 hr [18, 24]. Hydrolysates were neutralized with methyl di-*N*-octylamine [25] and then evaporated to dryness under red. pres. The sample, redissolved in H_2O, was electrophoresed at pH 3.5 at 4 kV for 45 min. In both hydrolysates, radioactive sugars were detected by scintillation counting of paper strips cut from the electrophoretogram as described in ref. [25].

REFERENCES

1. Northcote, D. H. (1972) *Annu. Rev. Plant Physiol.* **23**, 113.
2. Aspinall, G. O., Molloy, J. A. and Craig, J. W. T. (1969) *Can. J. Biochem.* **47**, 1063.
3. Bauer, W. D., Talmadge, K. W., Keegstra, K. and Albersheim, P. (1973) *Plant Physiol.* **51**, 174.
4. Barrett, A. J. and Northcote, D. H. (1965) *Biochem. J.* **94**, 617.
5. Stoddart, R. W. and Northocote, D. H. (1967) *Biochem. J.* **102**, 194.
6. Darvill, A., McNeil, M., Albersheim, P. and Delmer, D. P. in *The Biochemistry of Plants, The Plant Cell* (Tolbert, N. E., ed.) Vol. 1, p. 91. Academic Press, London.
7. Dalessandro, G. and Northcote, D. H. (1977) *Biochem. J.* **162**, 281.
8. Dalessandro, G. and Northcote, D. H. (1977) *Biochem. J.* **162**, 267.
9. Dalessandro, G. and Northcote, D. H. (1981) *Planta* **151**, 53.
10. Dalessandro, G. and Northcote, D. H. (1981) *Planta* **151**, 61.
11. Bolwell, G. P. and Northcote, D. H. (1981) *Planta* **152**, 225.
12. Bolwell, G. P. and Northcote, D. H. (1983) *Biochem. J.* **210**, 497.
13. Bolwell, G. P. and Northcote, D. H. (1983) *Biochem. J.* **210**, 509.
14. Villemez, C. L., Lin, T.-Y. and Hassid, W. Z. (1965) *Proc. Natl. Acad. Sci. U.S.A.* **54**, 1626.
15. Villemez, C. L., Swanson, A. L. and Hassid, W. Z. (1966) *Arch. Biophys. Biochem.* **116**, 446.
16. Lin, T.-Y., Elbien, A. D. and Jong-Ching, S. (1966) *Biochem. Biophys. Res. Commun.* **22**, 650.
17. Fisher, F. G. and Dorfell, H. (1955) *Hoppe-Seyl. Z.* **301**, 224.
18. Boffey, S. A. and Northcote, D. H. (1975) *Biochem. J.* **150**, 433.
19. Wright, K. and Northcote, D. H. (1974) *Biochem. J.* **139**, 525.
20. Ray, P. M. (1979) in *Plant Organelles* (Reid, E., ed.) p. 135. John Wiley, New York.
21. Rubery, P. H. and Northcote, D. H. (1968) *Nature (London)* **219**, 1230.
22. Neufeld, E. F., Ginsburg, V., Putman, E. W., Fanshier, D. and Hassid, W. Z. (1957) *Arch. Biochem. Biophys.* **69**, 602.
23. Neufeld, E. F., Feingold, D. S. and Hassid, W. Z. (1960). *J. Biol. Chem.* **235**, 906.
24. Wright, K. and Northcote, D. H. (1975) *Phytochemistry* **14**, 1793.
25. Harris, P. J. and Northcote, D. H. (1970) *Biochem. J.* **120**, 479.
26. Bevan, M. and Northcote, D. H. (1979) *J. Cell Sci.* **39**, 339.