Acta Bot. Neerl. 30(1/2), February 1981, p. 1–7.

ELECTRON MICROSCOPIC AUTORADIOGRAPHY OF β-GLUCANS SYNTHESIZED BY A MEMBRANE FRACTION FROM POLLEN TUBES OF PETUNIA HYBRIDA

J. P. F. G. HELSPER

Botanisch Laboratorium, Toernooiveld, 6525 ED Nijmegen

SUMMARY

Alkaline extraction of a membrane fraction from *Petunia* pollen tubes left an alkali-insoluble fraction that consisted of microfibrils embedded in amorphous material. The amorphous material was partially separated from the microfibrils by disaggregation with ultrasound or by solubilization with hydrogen peroxide/glacial acetic acid. Previously it was shown that incubation of the membrane fraction with UDP-[U-^{14}C]glucose gave an alkali-insoluble fraction containing labelled β-glucans. EM-autoradiography of this fraction, after labelling with UDP-[6-^{3}H]glucose, showed that *in vitro* synthesized β-glucans did not self-assemble but bound strongly to pre-existing microfibrils. Applicability of EM-autoradiography for confirmation of *in vitro* synthesis of microfibrils is discussed.

1. INTRODUCTION

The microfibrillar component of plant cell walls is a cellulosic β-1,4-glucan. The *in vitro* synthesis of β-glucans has been investigated extensively in the past two decades with membrane systems from *Acetobacter xylinum* (GLASER 1958; COOPER & MANLEY 1975), *Acanthamoeba* (POTTER & WEISMAN 1971) and higher plants (ELBEIN et al. 1964; VAN DER WOUDE et al. 1974). *In vitro* synthesis of cellulose microfibrils by a particulate fraction from *Acetobacter xylinum* was described by FORGE (1977). With the platinum-shadowing and X-ray diffraction techniques used in this study pre-existing microfibrils, whose presence cannot be excluded, would be indistinguishable from newly-synthesized microfibrils.

With UDP-glucose as a substrate a vesicular membrane fraction isolated from *Petunia* pollen tubes was shown to synthesize alkali-insoluble β-glucans (HELSPER et al. 1977). By means of X-ray diffraction ENGELS (1974) showed the presence of cellulose in a similar membrane fraction after extraction with chloroform and alkali. No microfibrils were observed in the unextracted membranes. No mention was made of the presence of cellulose microfibrils in alkali-insoluble material.

In the present report membrane-derived alkali-insoluble material from *Petunia* pollen tubes is investigated for the presence of contaminating microfibrils. EM-autoradiography is applied to establish whether β-glucans, *in vitro* synthesized from UDP-[6-^{3}H]glucose, assemble to new microfibrils separate from possibly pre-existing microfibrils.

2. MATERIALS AND METHODS

Isolation of membranes: membranes were isolated from pollen tubes of *Petunia hybrida* L. in the interspace of the 0.5 M and 1.0 M sucrose layers of a discontinuous gradient as described previously (HELSPER et al. 1977). Denatured membranes were obtained by heating for 10 min at 100°C.

Incubation and extraction procedures: membrane material, equivalent to 10 mg of protein, was incubated for 90 min at 25°C with 100 μCi UDP-[6-^{3}H]glucose (3.7 Ci/mmole) in 0.1 M TRIS-HCl (pH 8.0), containing 20 mM $MgCl_2$, 10 mM cellobiose, 4 mM Na_2EDTA and 1 mM dithiothreitol (final volume 10 ml). The reaction was stopped by heating the reaction mixture to 95°C. Insoluble material was precipitated and extracted three times with 5 ml water (95°C), once with 5 ml chloroform-methanol (1:2 v/v), once with 5 ml methanol, twice with 5 ml 1 N NaOH (95°C) and once with 5 ml water. The final pellet was suspended in 0.85 ml water.

A 0.2 ml sample of this suspension was treated for 30 sec in a MSE PG-100 ultrasonic disintegrator. A second sample was treated with 1.8 ml H_2O_2/CH_3COOH (30% H_2O_2-glacial acetic acid 1:1 v/v) for 2 h at 100°C. The insoluble material was washed three times with 1 ml water. A third sample was given both treatments, ultrasonic disintegration followed by the H_2O_2/CH_3COOH treatment. Following the treatments with H_2O_2/CH_3COOH pellets were washed and resuspended in 0.2 ml water.

Electron microscopy and autoradiography: 0.5 μl droplets of the 0.2 ml suspensions were dried on collodion-coated slides with continuous shaking. The dried material was shadowed with platinum and coated with a thin layer of carbon. For preparation of EM-autoradiographs a monolayer of photographic emulsion (Ilford L 4) was applied by means of a semi-automatic coating apparatus (VRENSEN 1970). After exposure for 14 days at 4°C the autoradiographs were developed according to WISSE & TATES (1968). Copper grids were placed on the specimens. The grids with the autoradiographs were floated on a water surface, picked up with parafilm and air-dried.

The features on the autoradiographs were itemized as amorphous material, microfibrils or vacant space, the latter showing only the collodion support. For statistical evaluation of the autoradiographs the method as described by KROH & VAN BAKEL (1973) was used. The radius of the circle within which 50% of the electrons, emitted in the direction of the emulsion layer, will cause a group of silver grains was calculated to be 100 nm. The significance of the difference (P value) between the silver grain groups/area ratios was determined by a test four a fourfold table (χ^2 or binomial approximation, depending on the marginal totals).

Chemicals: All chemicals were reagent grade. UDP-D-[6-^{3}H]glucose (3.7 Ci/mmole) was obtained from the Radiochemical Centre, Amersham.

Analytical measurements: Radioactivity was determined in 1.5 ml water with 3.5 ml Lumagel as a scintillation cocktail. Protein was measured according to LOWRY et al. (1951) with bovine serum albumin as a standard.

3. RESULTS

Sequential extraction with hot water, chloroform-methanol, methanol and hot alkali solubilized virtually all material in the membrane fraction that was incubated with UDP-[6-^{3}H]glucose. The remaining material represented less than 0.1% of the initial membrane volume and 1.3% of the originally applied radioactivity (*table 1*). Electron microscopy of the residue revealed microfibrils with diameters of about 10 nm embedded in a densily packed amorphous material (*fig. 1a*). Similar results were obtained from residues of extracted membranes prior to incubation or from residues of denatured membranes treated with UDP-[6-^{3}H]glucose. Alkali-insoluble material from denatured membranes incubated with UDP-[6-^{3}H]glucose was devoid of label.

After treatment with ultrasound the densily packed amorphous material was disaggregated exposing more microfibrils (*fig. 1b*; *table 2*, compare data on the relative areas of 'vacant space plus amorphous material' with 'vacant space plus microfibrils' in the columns a and b). H_2O_2/CH_3COOH treatment of control and sonicated alkali-insoluble material solubilized about 55% of the radioactivity (*table 1*). Electron micrographs of the remaining pellets show much less amorphous material and more microfibrils in comparison to the untreated alkali-insoluble material (*fig. 1; table 2*, compare data on the relative areas of 'vacant space plus amorphous material' with 'vacant space plus microfibrils' in columns a and c, and b and d).

The accumulation of silver grain groups above amorphous material and microfibrils (*fig. 1*) indicates that both structures were radioactive. From comparison of individual autoradiographs it was observed that when microfibrils extended from amorphous material it showed a higher accumulation of silver grains.

Statistical evaluation of the autoradiographs (*table 2*, silver grain groups

Table 1. Influence of various treatments of alkali-insoluble material on the radioactivity (μCi) and on the morphologic appearance of the residue. Membrane material, equivalent to 10 mg protein, was incubated for 90 min with 100 μCi UDP-[6-^{3}H]glucose.

Treatment	Radioactivity (μCi) in residue	Morphologic appearance of residue
None	1.3	Microfibrils, embedded in densily packed amporhous material (*fig. 1a*).
Ultrasound	1.3	Many loose microfibrils between disaggregated amorphous material (*fig. 1b*).
H_2O_2/CH_3COOH	0.5	Less amorphous material, more microfibrils (*fig. 1c*).
Ultrasound plus H_2O_2/CH_3COOH	0.6	Less amorphous material, more microfibrils (*fig. 1d*).

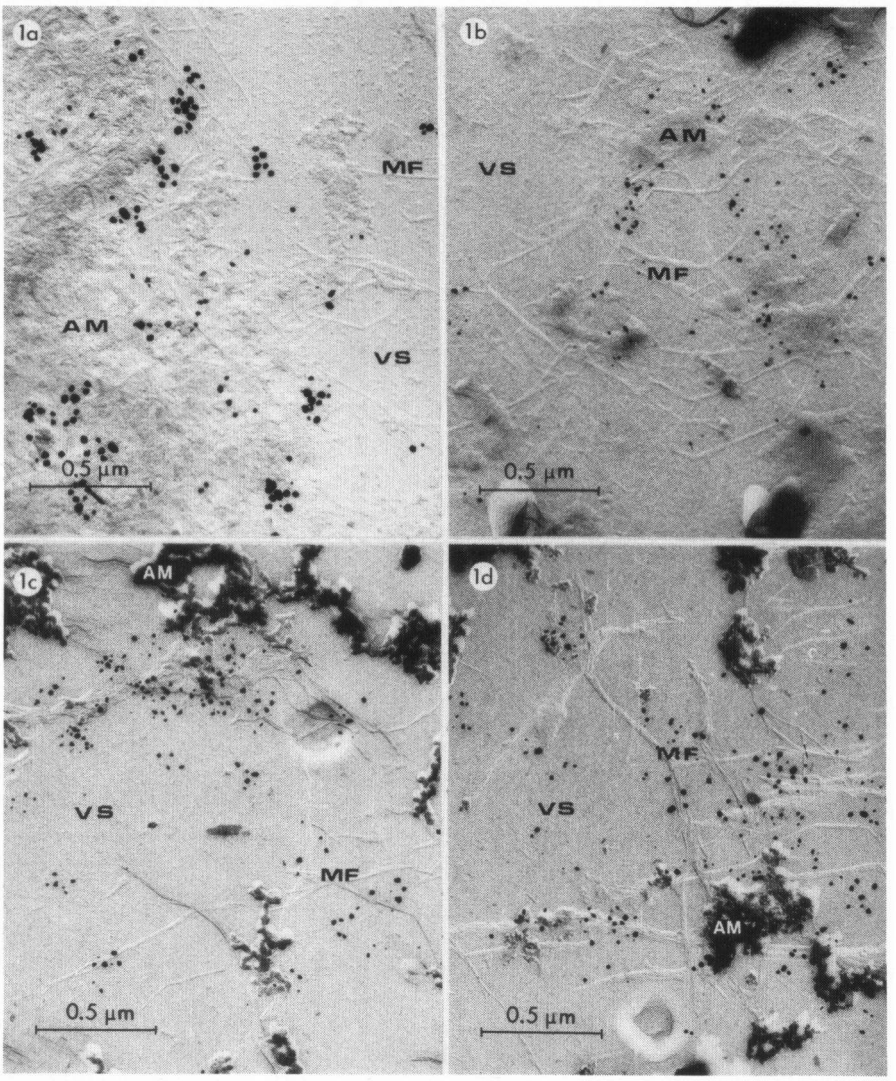

Fig. 1. Em-autoradiographs of platinum-shadowed alkali-insoluble material a. untreated and after treatment with b. ultrasound, c. H_2O_2/CH_3COOH and d. ultrasound plus H_2O_2/CH_3COOH. Exposure time: 14 days. VS = vacant space, AM = amorphous material, MF = microfibrils.

area) confirms that the vacant space is less radioactive than any feature containing microfibrils and/or amorphous material ($p < 10^{-6}$). Comparison of the features 'vacant space plus microfibrils' and 'vacant space plus amorphous material' shows that microfibrils are more heavily labelled than amorphous material ($0.085 > p > 10^{-6}$). There is no significant influence of the treatment with ultrasound and/or H_2O_2/CH_3COOH on the silver grain groups/area ratios.

Table 2. Evaluation of 30 EM-autoradiographs of alkali-insoluble material a. untreated and after treatment with b. ultrasound, c. H_2O_2/CH_3COOH and d. ultrasound plus H_2O_2/CH_3COOH. – = not detected. Relative area corresponds to 'Number of circles' in KROH & VAN BAKEL (1973).

Morphological feature(s)	Number of silver grain groups				Relative area				Silver grain groups/area			
	a	b	c	d	a	b	c	d	a	b	c	d
Vacant space	38	15	22	73	2671	2239	2066	2616	0.014	0.007	0.011	0.28
Amorphous material	134	15	69	72	157	139	322	67	0.85	0.11	0.21	1.07
Vacant space plus amorphous material	38	29	189	60	165	373	510	106	0.23	0.08	0.37	0.57
Vacant space plus microfibrils	5	27	46	227	6	39	44	153	0.83	0.69	1.05	1.48
Amorphous material plus microfibrils	9	–	–	13	–	–	–	4	∞	–	–	3.25
Vacant space, amorphous material plus microfibrils	15	24	103	127	1	10	57	52	15.0	2.4	1.81	2.44

4. DISCUSSION

While studying cell wall synthesis in germinating *Petunia* pollen, ENGELS (1974) identified cellulose by its X-ray diffraction pattern in the residue of a membrane fraction that had been extracted with chloroform and alkali. Using the same source of tissue, HELSPER et al. (1977) were able to demonstrate the presence of newly synthesized β-glucan after incubation of a similar membrane fraction with UDP-[U-^{14}C]glucose. In the present study we have examined the alkali-insoluble residue of this membrane fraction to determine the nature of newly synthesized β-glucan in relation to putative cellulose microfibrils.

Like ENGELS (1974) we did not observe microfibrils in the unextracted membrane fraction. Extraction removed all but 0.1% of the material in this fraction, leaving an alkali-insoluble residue in which microfibrils were evident, also when no *in vitro* synthesized β-glucans were present. These microfibrils occupy an area relatively small compared to that of surrounding amorphous material (*table 2*, column a). On the basis of Engels' X-ray diffraction study of this alkali-insoluble residue, it is probable that the exposed microfibrils are cellulosic. Ultrasound or treatment with H_2O_2/CH_3COOH disaggregated amorphous material, whose composition is unknown, revealing more microfibrils.

Prepaparations of membranes that had been incubated with UDP-[6-^{3}H]glucose prior to extraction for alkali-insoluble material gave labelled residues which were examined by EM-autoradiography preceding and following disaggregation of the amorphous component. Microfibrils were more heavily labelled than amorphous material, which appears more radioactive when seen close to microfibrils. These apparently extend under the amorphous material contribut-

ing to the amount of silver grains. Neither ultrasound nor treatment with H_2O_2/CH_3COOH was noticably effective in dislodging the radioactivity which remained bound to the microfibrils. In control experiments with membranes denatured by heat treatment, incubation with UDP-[6-^{3}H]glucose failed to produce a membrane fraction containing labelled alkali-insoluble material.

In previous work (HELSPER et al. 1977) it was found that the radioactive component in the alkali-insoluble fraction consisted of β-1,3- and β-1,4-glucans. The present results strongly indicate that this material interacted with or attached to pre-existing microfibrils, probably through hydrogen bonding (FREY-WYSSLING 1976, pp. 93–94).

EM-autoradiography provided the means for distinguishing non-labelled microfibrils from those that were labelled. If a microfibril was entirely newly synthesized from UDP-[6-^{3}H]glucose (3.7 Ci/mmole), the silver grain groups/area ratio in EM-autoradiographs could be calculated as follows: 3.7 Ci/mmole means 1 disintegration/3.7 × 10^3 glucose residues in 2 weeks. A 33% efficiency of the applied autoradiographic procedure (VRENSEN 1970) would cause 1 silver grain group/11.1 × 10^3 glucose residues. This corresponds to 1 silver grain group/5.5 μm single β-glucan chain. For a cellulose microfibril, consisting of about 1,000 parallel β-glucan chains (FREY-WYSSLING 1976, p. 18), this means 1,000 silver grain groups/5.5 μm microfibril. The autoradiographs show that this is certainly not the case. A similar treatment of data concerning the *in vitro* synthesis of microfibrils from particulate fractions from *Acetobacter xylinum* (FORGE 1977) would be valuable in establishing the claim of synthesis of cellulose microfibrils. The possibility that labelled microfibrils obtained in the *Acetobacter xylinum* study are aggregates of pre-existing microfibrils to which newly synthesized, radioactive β-glucans have been bound is not excluded. It seems the most reasonable explanation for our results.

Reasons for the absence of new cellulose microfibril formation following *in vitro* synthesis of β-glucan by the membrane fraction from *Petunia* pollen tubes include: 1. The possibility that such β-glucan molecules are precursors to other polysaccharides such as xyloglucans, a constituent of the amorphous matrix of the cell wall (ALBERSHEIM 1978), 2. Need for a linear arrangement of enzyme particles (BROWN & MONTEZINOS 1976; FORGE 1977) that has been disrupted during homogenization of the pollen tube, or 3. The presence of a large population of pre-existing microfibrils in the membrane fraction that compete with few newly synthesized β-glucans to associate with other *in vitro* synthesized β-glucan chains.

ACKNOWLEDGEMENTS

Author thanks Dr. M. M. A. Sassen and Dr. M. Kroh for fruitful suggestions and critical discussions, Drs. Ph. van Elteren and Drs. A. Dinkhuijsen for their help in the statistical analysis and Dr. G. Barendse for reading the manuscript.

REFERENCES

ALBERSHEIM, P. (1978): Concerning the structure and biosynthesis of the primary cell wall of plants. In: *International Review of Biochemistry, Biochemistry of Carbohydrates II* (ed. D. J. MANNERS), Vol. **16**: 127–150. University Park Press, Baltimore.

BROWN, R. M. & D. MONTEZINOS (1976): Cellulose microfibrils: Visualization of biosynthetic and orienting complexes in association with the plasma membrane, *Proc. Nat. Acad. Sci. USA* **73**: 143–147.

COOPER, D. & R. St. J. MAINLEY (1975): Cellulose synthesis by Acetobacter xylinum. II Investigations into the relation between cellulose synthesis and cell envelope components. *Biochim. Biophys. Acta* **381**: 97–108.

ELBEIN, A. D.,G. A. BARBER & W. Z. HASSID (1964): The synthesis of cellulose by an enzyme system from a higher plant. *J. Am. Chem. Soc.* **86**: 309–310.

ENGELS, F. M. (1974): Function of Golgi vesicles in relation to cell wall synthesis in germinating Petunia pollen. IV. Identification of cellulose in pollen tube walls and Golgi vesicles by X-ray diffraction. *Acta Bot. Neerl.* **23**: 209–215.

FORGE, A. A. (1977): The synthesis of cellulose microfibrils by particulate fractions released from Acetobacter xylinum by washing. *Ann. Bot.* **41**: 447–454.

FREY-WYSSLING, A. (1976): *The Plant Cell Wall.* 3rd edition. Gebr. Borntraeger. Berlin, Stuttgart.

GLASER, L. (1958): The biosynthesis of cellulose in cell-free extracts of Acetobacter xylinum *J. Biol. Chem.* **232**: 627–636.

HELSPER, J. P. F. G., J. H. VEERKAMP & M. M. A. SASSEN (1977): β-Glucan synthetase activity in Golgi vesicles of Petunia hybrida. *Planta* **133**: 303–308.

KROH, M. & C. H. J. VAN BAKEL (1973): Incorporation of label into the intercellular substance of stylar transmitting tissue from Petunia pistils labeled with tritiated myo-inositol. An electronmicroscopic autoradiographic study. *Acta Bot. Neerl.* **22**: 106–111.

LOWRY, O. H., N. J. ROSENBROUGH, A. L. FARR & R. J. RANDALL (1951): Protein measurement with the Folin reagent. *J. Biol. Chem.* **193**: 265–275.

POTTER, J. L. & R. A. WEISMAN (1971): Differentiation in Acanthamoeba: β-glucan synthesis during encystment. *Biochim. Biophys. Acta* **237**: 65–74.

VAN DER WOUDE, W. J., C. A. LEMBI, D. J. MORRÉ, J. I. KINDINGER & L. ORDIN (1974): β-Glucan synthetases of plasma membranes and Golgi apparatus from onion stem. *Plant Physiol.* **54**: 333–340.

VRENSEN, G. F. J. M. (1970): Some new aspects concerning the efficiency of electron microscopic autoradiography with tritium. *J. Histochem. Cytochem.* **18**: 278–290.

WISSE, E. & A. D. TATES (1968): A gold latensification-elon-ascorbic acid developer for Ilford L 4 emulsion. In: *Electron Microscopy* (ed. D. S. BOCCIARELLI) **II**: 465–466. Tipografia Poliglot a Vaticana, Roma.