Evidence for coniferyl-alcohol oxidase promotion of lignification in developing xylem of conifers

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The literature states that coniferyl-alcohol oxidation to pinoresinol and dehydrodiconiferyl alcohol and continuing oxidation of these dimers into lignin is due to exclusive peroxidase/H_2O_2 participation[1,2]. We propose coniferyl-alcohol oxidase in conifers and report the activity of this enzyme, and not that of peroxidase, to be spatiotemporally correlated with lignification. This appears to be the first evidence for this enzyme in higher plants; hence, aspects of its isolation and purification are noted.

Histochemistry: Histochemical investigations were done on living sections prepared from freshly cut stems of *Abies balsamea* (L.) Mill., *Larix laricina* (Du Roi) K. Koch, *Picea rubens* Sarg., *Pinus banksiana* Lamb., and *Pinus strobus* L. in spring of 1989 and again in spring of 1990. Trees having abundant green foliage, vigourous growth, no obvious symptoms of disease, and between 10-30 yrs old were used. Main-stem material from at least 10 ages (years) of cambium was sampled, felling one tree of each species every two weeks from mid-April to late June. Hand-cut transverse sections were floated on *p*-anisidine HCl (1.0 mM) with or without 10 mM H_2O_2 at room temperature for four hours, rinsed, mounted in water, and examined by bright-field microscopy.

Enzyme extraction and partial purification: Following bark peeling, lignifying xylem scrapings of *P. strobus* were frozen in liquid nitrogen and pulverized to a fine powder with mortar and pestle. The powder (100 g_{fw}) was extracted with 0.05 M phosphate buffer (1000 mL, pH 7.0) for 4 h at ambient temp., then filtered through cheeze cloth (4 layers). The insoluble residue was extracted by continuous stirring with 0.5 M NaCl (1000 mL) for 16 hours at 4°C, filtered through cheeze cloth, and the insoluble tissue transferred to 250 mL centrifuge bottles. After centrifuging (1,380 X g, 4°C, 20 min), the supernatant was decanted and the pellet re-suspended in double-distilled H_2O, repeating this step four times.

The oxidatively active pellet (~50 g) was suspended in double glass-distilled water (1000 mL) to which was added pectinase (*Aspergillus niger*, Sigma P-5146, 50 mg, 6.9 U mg^{-1}), cellulase (*Trichoderma reesi*, Serva 16421, 125 mg, 0.5 U mg^{-1}) and gentamycin (62.5 mg, Flow Lot no. 0050553), and the mixture was stirred for 16 h at room temp. The digested mixture was centrifuged at 1380 X g (20 min, 4°C), the supernatant discarded and the pellet re-suspended in H_2O and re-centrifuged, repeating this washing step 6 times. This final pelleted material (~1 g) is hereafter referred to as 'SL PP' (particulate preparation from lignifying secondary walls).

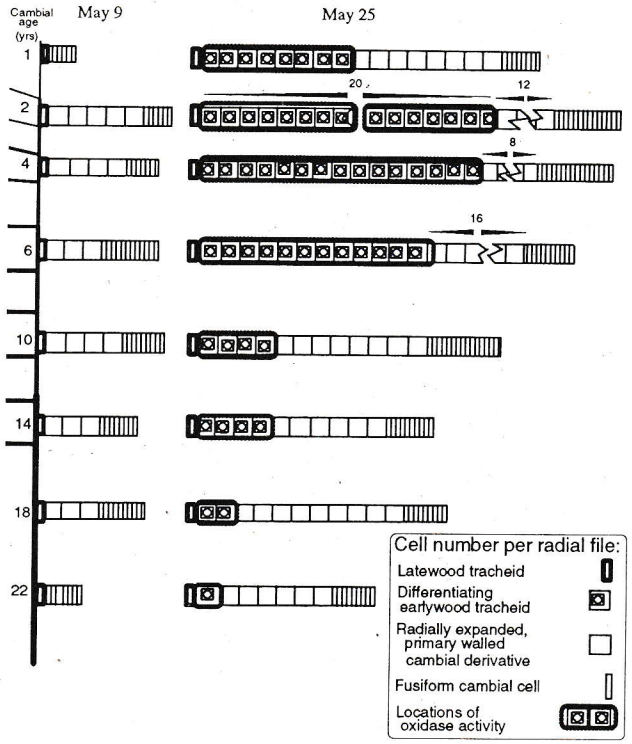
To solublize oxidase activity, SL PP was re-suspended in 20 mL of 0.5 M phosphate buffer (pH 7.0) containing 0.5 M NaCl and gentamycin (50 mg) and left for 16 h (4°C), then centrifuged at 20,000 X g for 0.5 h at 4°C. The supernatant was transferred to a Centriprep 10 (Amicon) and centrifuged at 2455 X g (15 min, 4°C). Water (2.0 mL) was added and the retentate centrifuged again at 2455 X g, repeating this washing step four times and adding gentamycin (5.0 mg) to the final wash. The final retentate (>10,000 M.W. fraction) contained 'solublized SL PP oxidase'.

Enzyme assays: UV/Vis assays (HP8452 2 nm photodiode array spectrophotometer, Hewlett Packard) were done in 2.1 mL phosphate buffer (0.05 M, pH 6.5) at room temp. Tested compounds included coniferyl alcohol (1.0 mg, Fluka), *p*-anisidine HCl (0.1 mMol, Eastman Organic), guaiacol (0.1 - 20 mMol, Sigma), syringaldazine (300 uL of methanolic solution containing 0.078 mg mL^{-1}, Sigma), and L-tyrosine (0.1 mMol, Sigma). Where compounds were not oxidized within 2 h, overnight assays were done. Controls (compound, no enzyme) and blanks (buffer only) were done with each assay.

Gas chromatography - mass spectrometry (GC-MS) of products: For GC-MS, coniferyl alcohol (1.0 mg) was added to 0.1 M phosphate buffer (2.0 mL, pH 7.0) and incubated for 2 h at 25°C with or without solublized SL PP oxidase, stopping the assays by addition of ethyl acetate (EtOAc, 4 X 2.0 mL). After drying the four combined EtOAc extracts under N_2, the residue was derivatized with *N,O -bis-* (trimethylsilyl) trifluoroacetamide (Pierce). Splitless injections (2 µL) were made onto a 0.18 mm (i.d.) x 16 m DB-1 (J&W Scientific, Folsom, CA) open tubular direct-inlet-to-source column at 40°C, purging the injection port after 1 min. Temp. programming was done at 16°C min^{-1} to 300°C with carrier gas (He) flowing at 0.55 mL min^{-1}. Electron impact (70 eV) data were collected using an HP5970B controlled by a HP59970C computer (Hewlett Packard).

In vitro lignin from solubilized SL PP oxidase: Solublized SL PP oxidase (50 µL) was added to a solution of coniferyl alcohol (5.0 mg) in 10 mL phosphate buffer (100 mM, pH 6.5), filter sterilized (0.2 µm, Nalgene 153-0020, Nylon, Lot 736), and incubated at 20 - 25°C in the filter-sterilization flask. The 'control' contained all of the described filter-sterilized ingredients except enzyme. After 48 h the flasks were opened; small volumes were transferred to glass slides and examined using incident light fluorescence microscopy (Reichert-Jung Polyvar, B-1 filter: bandpass 450-495 nm, longpass >520 nm).

The figure shows how *in situ* oxidase activity was discovered in live sections incubated on *p*-anisidine solution. On May 9, no oxidation of *p*-anisidine occurred, but when H_2O_2 was added peroxidase activity became ubiquitously evident in the oxidase-free tissues. On May 25, oxidation occurred in the absence of added H_2O_2, red product arising at cell corners and around developing bordered pits, the locations where



Pattern of development of oxidase activity in the cambial region of *Pinus strobus* L. on two dates in spring. On May 9th no oxidase activity was detected although the cambium was actively dividing and had produced numerous radially expanded primary walled derivatives. On May 25th, oxidase activity was restricted to actively differentiating tracheids and exhibited a basipetal progression of development.

lignification was underway (toluidine blue O staining). Again, by addition of H_2O_2, peroxidase was determined to be present in both lignifying and non-lignifying regions. The findings, including the development of oxidase activity beginning in the upper tree crown and its progression basipetally as diagrammed here for *P. strobus*, were corroborated with all five species in spring of 1989 and again in 1990.

SL PP readily oxidized *p*-anisidine, *o*-dianisidine, and *p*-phenylenediamine but could not oxidize guaiacol, syringaldazine, nor tyrosine. Following incubation of coniferyl alcohol with solublized SL PP oxidase for 2 h at room temp., GC-MS yielded two major metabolites in addition to coniferyl alcohol (R_t 12.13 min). The first, R_t 19.84 min, yielded the mass spectrum 574 (M^+, 2.6%), 559 ($M\text{-}15^+$, 0.3%), 484 (20.0%), 454 (2.7%), 209 (4.0%), 179 (1.9%), 129 (1.7%), 115 (1.1%), 103 (11.3%), 89 (2.3%), 75 (8.3%), 73 (100%), being the spectrum of **dehydrodiconiferyl alcohol** (TMSi X 3). The second, R_t 20.54 min, yielded the mass spectrum 502 (M^+, 23.7%), 487 ($M\text{-}15^+$, 7.7%), 277 (8.4%), 263 (5.9%), 252 (6.3%), 247 (6.7%), 235 (37.2%), 223 (49.3%), 219 (5.0%), 209 (34.8%), 205 (7.6%), 194 (29.5%), 179 (14.3%), 166 (11.6%), 131 (9.6%), 103 (5.6%), 89 (7.6%), 81 (6.1%), 75 (10.0%), 73 (100%), being the spectrum of **pinoresinol** (TMSi X 2).

After 48 h of incubation, the clear coniferyl-alcohol solution incubated at room temp. with solublized SL PP oxidase was cloudy. By microscopy the cloudiness was found to be due to the presence of hydrophobic globules having green-yellow fluorescence, the fluorescence being identical to that of lignin in mature conifer xylem.

The inability of SL PP oxidase to oxidize guaiacol, tyrosine or syringaldazine makes it unlikely that the enzyme is a peroxidase, polyphenoloxidase, or laccase. By GC-MS we have established that SL PP consumes dioxygen during oxidation, and UV/VIS spectrophotometry measurements have shown that it cannot utilize H_2O_2 in catalysis. Electrophoretic investigations have suggested there to be more than one oxidative enzyme in solublized SL PP oxidase preparations, the enzyme most active on coniferyl alcohol having pI ~9. We consider that coniferyl-alcohol oxidase, in addition to polymerizing coniferyl alcohol directly into guaiacyl lignin, may enable peroxidase participation in lignification through generation of H_2O_2.

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References:

1) Higuchi, T. (1990) *Wood Sci. Technol.* **24**, 23-63.

2) Harkin, J. M. and Obst. J. R. (1973) *Science* **180**, 296-297.

The use of specific antivasopressin antisera to detect immunoreactive vasopressin in human skeletal muscle.

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The presence of arginine vasopressin (AVP) in pituitary, pineal, adrenals and thymus, is now well documented and generally accepted (1-4). Our more recent finding of an immunoreactive (IR) AVP substance in human foetal and neonatal skeletal muscle (5) is novel and has stimulated further research to establish this substance as AVP. Here we describe further charecterization of the substance using high performance liquid chromatographic (HPLC) separation of crude acid extracts followed by analysis of eluates with a series of different anti-AVP antisera of defined specificity.
Skeletal muscles were examined from a16 week foetus after prostaglandin termination of pregnancy and from a25 week and 40 week (term) stillbirth and neonatal death at autopsy. After dissection, the tissues were snap frozen in dry-ice and stored at -70 C until extracted and separated by HPLC as previously described (5).
Four anti-AVP antisera of differing specificity (Table 1) were used to produce IR profiles from the HPLC elutions of the 3 muscle acid extracts. Two of the antisera (R3 and 312-5) were donated by Dr V. Ang , St Georges Hospital Medical School, London and Dr P. Baylis, the Royal Victoria Infirmary, Newcastle, respectively. Details of these assays are reported elsewhere (6,7).

Table1.Antisera specificity (% cross-reaction)

Cross-reactants Antiserum	AVP	LVP	AVT	OT	DDAVP
TG1	100	0.02	0.8	<0.01	15.0
R3	100	-	<0.01	<0.01	-
312-5	100	5.0	-	<0.01	0.05
S278	100	49.0	<0.01	<0.01	

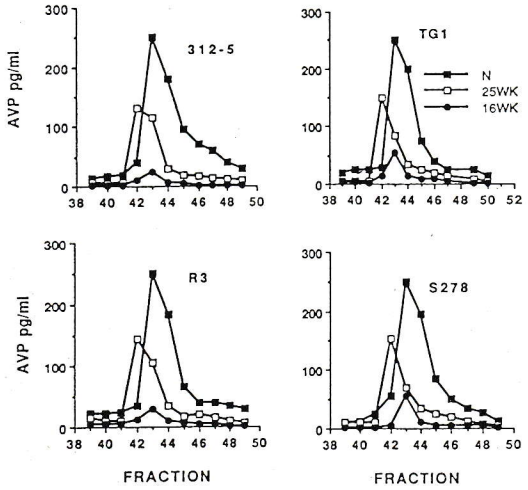
(LVP=lysine vasopressin, AVT=arginine vasotocin, OT=oxytocin DDAVP=desamino-D arginine vasopressin).

Table 2 gives the IR-AVP concentrations obtained from the direct assay of the 25 week foetal muscle preparation with the 4 antisera. IR-AVP concentrations were detected at approximately the same level by each antiserum.

Table 2. IR-AVP concentration in a 25 week foetal muscle extract.

Antiserum	AVP pg/ml
TG1	2300
R3	2000
312-5	3400
S278	2600

Figure 1 shows the IR profiles obtained after HPLC separation of crude acid extracts of 16, 25 and 40 week skeletal muscle. The profiles obtained ere identical for each antiserum and the major IR-AVP peak found inthe same position as the counts peak obtained from the elution of tritiated AVP.



We have previuosly identified an IR-AVP substance of low molecular weight (<3000mw) eluting in an identical position to standard AVP (following reverse-phase HPLC), in a selection of human skeletal muscle samples from 15 weeks gestation to term (5). Results from this study shows that the IR-AVP detected previously is not peculiar to the antiserum used (TG1), and is detected by at least 2 other well documented RIAs for AVP. The data presented constitutes, therefore, further evidence for the presence of AVP in human skeletal muscle. The question of production or sequestration remains open.

1. Nicholson H.D., Swann R.W., Burford G.D., Waithes C.D., Porter D.G., Pickering B.T. (1984). Reg. Pept. 8, 141-146.
2. McIntosh N., Smith A., Carter N.D. (1984). Biochem. Soc. Trans. 12, 252-253.
3. Geenen V., Legros J.J., Franchimont P., Defresne M.P., Boniver J., Ivell R., Richter D. (1987). Ann. NY Acad. Sci. 496, 56-66.
4. Liu B., Poulter L., Neascu C., Burbach H. (1988). J. Biol. Chem. 263, 72-75.
5. McIntosh N., Stephen R.I., Arkley M.M., Smith A. (1991). Biochem. Soc. Trans. 19, 175(S).
6. Jenkins J>S., Mather H.M., Ang V.T.Y. (1980). J.Clin. Endocr. 50, 364-367.
7. Baylis P.H. and Heath D.A. (1977). Clin. Endocr. 7, 91-102.