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The Differences in the Lignification of Earlywood and Latewood in Larch (*Larix sibirica* Ldb.)

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Abstract

The rate of lignin deposition, its content and composition, and molecular weight distribution at different stages of secondary wall thickening during tracheid development of both earlywood and latewood in Siberian larch (*Larix sibirica* Ldb.) were studied in the stems of 25-year-old trees. The cells of early and late xylem at different stages of secondary wall development were obtained in late June and early in August, respectively. Lignification of the two types of wood was found to involve different dynamics. The intensity of lignin synthesis during earlywood formation increases gradually, reaching the maximum at the last stage of tracheid maturation. In contrast, lignin deposition in the course of latewood development is the highest only in the first stage of lignification and declines by the end of tracheid maturation. There were differences in the composition of alkaline oxidation products of lignin preparations at different developmental stages of early and late xylem. The contents of carbohydrates linked to lignin by ether and ester bonds were the highest in the first stage of lignification and greater in lignifying latewood than in earlywood. Lignins formed at the beginning of lignification of both wood types had higher molecular weights and were more homogeneous than those at the last stage of cell differentiation. The amount of cell wall substances deposited before lignification in earlywood is larger than that in latewood.

**Key Words:** Earlywood and latewood, Carbohydrates, Gel-filtration, Larch, Lignin, Tracheid development stages

Introduction

Lignin, an important component of plant tissues, has been the subject of intensive research. The structural peculiarities of lignin, its concentration in different morphological regions of plant tissue and its deposition during wood formation have been studied by chemical and instrumental methods and in particular by ultraviolet microscopic spectroscopy (Fergus et al. 1969, Fukazawa and Imagawa 1981, He and Terashima 1991, Wu et al. 1992, Yoshinaga et al. 1997 a, b), quantitative interference microscopy (Donaldson 1991, 1992), microautoradiography with a combination of techniques for selective labeling (Terashima et al., 1986, 1988), items detected histochemically with the use of electron microscopy (Kutscha and Schwarzmann 1975), high-resolution transmission electron microscopy in combination with immuno-cytochemistry (Samuels et al. 2002) and fluorescence microscopy (Fukushima and Terashima 1991, Yoshizawa et al. 1991). In the course of these investigations a very complicated picture of the lignification of different tissues and organs of plants was found. Lignin distribution in mature earlywood and latewood of conifers was observed to be also different (Fergus et al. 1969, Wood and Goring 1971, Boutelje 1972, Saka et al. 1982, Donaldson 1985).

Lignification is one of the important processes occurring in woody plant xylem during morphogenesis. Xylem cells, arising due to initial cambium cell division, increase their primary walls during the expansion stage and accumulate secondary wall substances at a later stage. Lignin deposition starts at the last stage following the deposition of some secondary wall substances. According to Siegel (1956, cit. by Biochemistry of phenolic compounds, Ed. Harborne, 1964) the presence of a cell component such as cellulose is necessary for the polymerization process of monolignols. Donaldson (1991) showed that in pine (*Pinus radiata* Don.) secondary wall formation begins in several cells before lignification. A cytochemical study of cell wall formation in poplar trees with potassium permanganate and UV microspectrophotometry showed that the lignification of cell walls was slightly delayed with the deposition of polysaccharides (Grunwald et al. 2002). In Scots pine and Norway spruce (*Picea abies* (L.) Karst) Marjamaa et al. (2003) found lignification begins soon after the thickening of newly formed cell walls, i.e. upon the deposition of cellulose.

Two distinct events are involved in lignin biosynthesis: monolignol biosynthesis and dehydrogenative polymerization (condensation). There are three monolignols (p-coumaryl, coniferyl and sinapyl alcohols) polymerized into lignin. The amount of each of the monolignols, incorporated into lignin, depends on the response of the plant to developmental or environmental events and because of this the relative
abundance of different monolignols residues in lignin varies between species and within species (Whetten and Sederoff 1995). Environmental factors may also influence the polymerization conditions of monolignols, for example, the lignin-matrix interaction (Grabner 2005).

Lignin of conifers consists mainly of guaiacyl subunits derived from coniferyl monolignols. The distinction in lignin distribution in mature earlywood and latewood appears to be determined by the peculiarities of the lignification process of these types of wood that are formed as a result of different water potentials in the tree. Zahner (1963) reported that low internal water potential initiates latewood formation. This distinction due to water availability might be the result of differences in monolignol biosynthesis (because of various activities of enzymes) and the process of dehydrogenative polymerization. It is also possible that different structural organization of polysaccharides and cellulose within cell walls before lignification may play a role. Cell wall polysaccharides are believed to be involved in organizing lignin precursors (Houtman and Atala 1995, Whetten and Sederoff 1995).

The aim of our work was to study the deposition of lignin, its content, composition and molecular weight at consecutive stages of secondary cell wall formation and the amounts of carbohydrate biomass, accumulated before lignification, during the development of earlywood and latewood tracheids in growing larch (Larix sibirica Ldb.) trees.

Materials and Methods

Plant materials

Xylem, at different stages of tracheid development, was obtained from the stem cuttings of 25-year-old larch trees (height - 9-10 m, diameter at 1.3 m - 8-10 cm). Xylem was collected twice a season: in late June and early in August. In each of these periods all cells of differentiation stages, as well as mature xylem, were either earlywood (June) cells or latewood (August) ones. This corresponds to our studies of wood formation in larch trees growing in Middle Siberia (Antonova and Stasova 1988, Antonova and Stasova 2002). To distinguish earlywood cells from latewood ones we observed that the radial diameter of late tracheids was less than the tangential after completion of expansion.

![Fig. 1. The layers of cells at different stages of secondary wall development of earlywood (a) and latewood (b) tracheids: D-1 before lignification; D-2a, D-2b, D-2c – after the beginning of lignification.](image-url)
The state of annual wood layer development within each stem cutting was identified anatomically, using transverse sections stained by cresyl-violet. It is a metachromatic dye that stains the plant tissue according to its development stage. Due to the availability of chromogen groups and their distribution in tissue the coloration changes from pink in the cambium zone to violet at the next stage and blue after the beginning of lignification (Antonova and Shebeko 1981). The beginning of lignification was also identified by the reaction to phloroglucinol-HCl (reagent Weisner), which gives a red colour to a guaiacyl structure. The beginning of the secondary wall thickening zone was recorded by the appearance of bordered pits. After peeling off the bark the phloem was separated from xylem and the location of the cambium zone was examined at the same time under a microscope because it could be placed in either the phloem or xylem side depending on the internal development. The cells at different stages of development were collected by peeling one cell layer after another with a scalpel. To examine the accuracy of sampling almost every strip was inspected under a microscope after staining by cresyl-violet. In late June earlywood xylem cells with secondary thickening, but without lignification (D-1), the first (D-2a) and the second (D-2b) parts of the lignification zone, and mature xylem (M) were collected (Fig. 1a). In early August latewood cells of D-1, D-2a, D-2b and additionally the third cell layer (D-2c) were isolated (Fig. 1b).

The sampled cell layers were immediately fixed with ethanol to a final concentration not exceeding 80%, weighed, and refrigerated until analyses. Sample weight was estimated taking into account the weight of 96% ethanol employed for fixation. Simultaneously the tissues were sampled for maceration (0.05-0.1 g x 3) and moisture determination (0.5 g x 2).

Tissue maceration was conducted by the Moskaleva (1958) and Odintsov et al. (1967) methods. The number of cells within a suspension after maceration was counted using a Fuchs-Rosenthal hemocytometer and calculated per gram of dry weight of plant tissue.

**Histochemical observation**

To estimate the amount of secondary wall substances deposited before lignification two approaches were used. In one, a cross-section of tracheid walls along radial rows was evaluated. The cell wall cross-section of tracheids situated between the beginning of the wall thickening and lignin deposition were taken as the index of cell wall substances. In the other technique the contents of cellulose, deposited within walls during thickening, were determined (see below).

The cell wall cross-sections were specially obtained from the stem cuttings taken at 1.3 m. Transverse sections (two-thirds of each stem) were stained by cresyl-violet and examined under a microscope at a magnification x1000. Radial and tangential diameters of the tracheids and their lumina were measured at 4 radial rows of each section after the beginning of secondary wall thickening. The cross-sections of tracheid walls were calculated by subtracting lumen areas from tracheid cross-sections (Antonova and Stasova 1997). The standard errors of the means of all measured and calculated parameters were not more than 5%.

**Lignin**

The lignin contents were examined by Venverloo methods (Venverloo 1969) with thioglycolic acid. This method is more suitable than other methods for examining the lignin content and structure in live tissue (Sarkanen 1975).

Tissue suspensions taken for analysis were filtered. Dry residues were homogenized in liquid nitrogen with a mortar and pestle and extracted with 80% ethanol (1:10, v/v) at room temperature with periodic shaking. The solvent was changed every 12 h until a negative reaction to carbohydrates (Dubois et al. 1956). The volumes of extracts were measured. The extracted tissue (120-250 mg x 2) was heated with 7-15 ml thioglycolic acid - HCl (10 g thioglycolic acid to 100 ml HCl 2N) in a water-bath at 100°C. After 1 h heating the mass was stirred and 8-15 ml thioglycolic acid-HCl mixture was added and heating proceeded for 6 h. Then the suspension was cooled and filtered. The residue was thoroughly rinsed with water to pH 7, air dried over night, treated with ethanol for 24 h, filtered and dried again to remove the alcohol. The ethanol-soluble substance obtained was the lignothioglycolic acid fraction I. The ethanol-insoluble residue air-dried for 24 h was placed into 15 ml 2.0% NaOH and stirred. After 24 h the dissolved lignothioglycolic acid was filtered and precipitated with 5N HCl. The next day the precipitate was collected on a previously weighed filter, washed with water to pH 6-7 and then dried for 4 h at 100°C (fraction II).

The lignin contents at different steps of secondary cell wall thickening and mature xylem were estimated as total of the fractions of soluble (LTGA-I) and insoluble (LTGA-II) calculated both per dry weight and per cell.

Lignin LTGA-II was also used to examine the structures and molecular weight distribution of lignin formed at different stages of cell wall differentiation.

**Gel-filtration chromatography**

Lignin (15-20 mg) of fraction LTGA-II was dissolved in 1 ml DMSO, filtered and subjected to gel-filtration on a Sephadex G-75 column (0.7 x 34 cm) previously equilibrated with DMSO used also as the eluent. Apart from this the solution of LiCl (1%) in DMSO was applied to suppress possible molecular interaction. The fractions (1 ml) collected were examined by UV spectroscopy at 280 nm. The void volume was determined with Blue Dextran and was 7 ml and the volume of exit of p-coumaric acid was 20 ml.

**Alkaline oxidation**

Alkaline cupric oxide oxidation was done by Hartley’s method (Hartley 1971). This method is less destructive and more suitable for lignin structure investigation than nitro-benzen (Sarkanen 1975).
The preparations of LTGA-II (12-24 mg x 2), CuSO₄·5H₂O (1.7 g per 1 g of LTGA-II) and 3N NaOH (10 ml per 1 g of LTGA-II) were heated at 180 ± 0.2°C for 2.5 h with mixing. The reactive mixture was centrifuged at 7000 g 15 min. The supernatant and washing water (2 x 3 ml H₂O) were combined, extracted by diethyl ether and after acidifying to pH 2.5 with 5N HCl extracted again by Et₂O (3 x 5 ml). These ether extracts were dehydrated by solid sodium sulfate and air-dried. The lignin oxidation products were analyzed by high performance liquid chromatography and TLC.

Alkaline hydrolysis

The lignin of LTGA-II preparations (15-20 mg x 2) was dissolved in 2N NaOH (10-15 ml) in the presence of sodium borohydride. After 20 h at ambient temperature with stirring under N₂, the solutions were acidified to pH 2.5 with 5N HCl. The precipitation recovered by filtration through glass-filters were washed by water to pH 7, then by ethanol and air-dried. The filtrates were acidified to pH 2.5 and extracted by ether (3 x 10 ml). Ether extracts were dried over sodium sulfate and air-evaporated to dryness. Phenolic acids were recovered by TLC and high performance liquid chromatography.

The water layers were combined, evaporated and kept at 5°C overnight. Insoluble residues were recovered by filtration and washed by col. 50% ethanol. These liquids were combined, evaporated under reduced pressure and the residue dissolved in 3 ml of water to analyze (3 x 0.5 ml) by phenol-sulfuric acid (Dubois, 1956) as well as by acid hydrolysis (1N H₂SO₄, 2.5 h, 100°C) to examine the content of carbohydrates and their composition.

Acid hydrolysis

To assay ether linkages in lignin preparations the hydrolysis by a mixture of dioxan-HCl (Scalbert et al. 1985) was used.

Lignin preparations (10-15 mg x 2) were dissolved in a 15 ml dioxan-2 M HCl (9:1 v/v) mixture. After 1 h 25 ml H₂O was added and the precipitate was centrifuged at 7000 g for 20 min. The filtrate was evaporated under reduced pressure to a volume of 7-10 ml and after cooling extracted with ether (3 x 5-7 ml). Ether extracts were dehydrated by sodium sulfate and then air-dried. Phenolic acids were recovered by TLC and HPLC as follows.

The water layers were combined, dried and the residue was dissolved in 5 ml H₂O. In the aliquot samples (2 x 0.5 ml) carbohydrate amount was estimated by Dubois’s method (1956). The residual solution and 4 ml 2N H₂SO₄ was heated at 100°C (2.5 h). Then the solution was neutralized by BaCO₃ and, after removing the precipitate, dried under low pressure. The residue was dissolved into 1 ml acetone and analyzed by TLC to recover the carbohydrates. Alkaline-hydrolyzed preparations of latewood lignin were also subjected to dioxan-HCl hydrolysis.

Carbohydrates contained in lignin preparations were also studied by acid hydrolysis with 1N H₂SO₄ at 100°C 3 h. After treating the solution with BaCO₃ and removing the precipitation the yield of carbohydrates was estimated by phenol-sulfuric acid method. Glucose has been taken as a standard.

TLC analysis

Carbohydrates after hydrolysis were divided on a “Silufol” plate using the mixture of n-butanol-aceton-water (2:7:1, v/v/v). The plate was developed 4 times and air-dried between each chromatography session. The spots were displayed by aniline-phthalat with heating for 5 min at 100°C and identified by authentic sugars.

Phenolic compounds were dissolved in 1 ml acetone and spotted on a “Silufol” plate. After saturating the plate under a stream of water it was placed one time in a mixture of benzen-acetic acid (9:1, v/v). Spots were visualized with UV-light and by spraying with 2,4-dinitrophenylhidrazine (aldehydes) and a mixture of 0.5% p-nitroaniline in 2N HCl, 5% NaNO₂ and 20% sodium acetate in water (5:20, v/v/v).

The spots identified by cochromatography with authentic compounds and UV-absorption were scrapped and extracted with 0.2% KOH in ethanol (3 ml) (λ = 400 nm). Cinnamic and benzoic acids visualized by UV-absorption were extracted by 3 ml ethanol and examined at 272 nm.

HPLC analysis

Acetonic extracts were evaporated and the residues were redissolved in 1 ml ethanol with the addition of 1 ml 3% acetic acid and centrifuged. Clear solutions were chromatographed on a column (16 x 250 mm) of Diasorb 130 C 16/T (BioChimMak). Separation was obtained with a linear gradient of two solvents A (2.5 % acetic acid) and B (ethanol). A linear gradient was rising for 20 min from 0% B to 5% B at a flow of 0.15 ml/min. The products were detected at 280 nm without quantitative estimations.

Results and Discussion

Cell wall substances

The deposition of lignin always occurs in the perivascular bundle of latewood tracheids before lignification (Stage D-1) was larger than in earlywood ones. The relationship of these areas to the mean maximum values of mature xylem cells of the wall cross-sections before lignification was 22-26% for earlywood and only 9-13% for latewood. Therefore, secondary wall substances synthesized during earlywood formation before lignification were two times greater than in the course of latewood formation.
The other method used to estimate the amount of cellulose at each stage of secondary wall development was to calculate the residue after lignin isolation. The amount of cellulose deposited within earlywood cell walls before lignification was less than in latewood cell walls (9.9% and 12.8%, respectively).

The data, presented as increments of cellulose per cell at each stage of secondary wall thickening (Fig. 3), shows that cellulose synthesis sharply increases at stage D-2a during both earlywood and latewood formation, followed by a decrease in mature xylem. Principal synthesis of cellulose in the first steps of secondary wall development and its gradual decrease in the course of tracheid maturation was previously considered by Antonova and Varaksina (1996), and Antonova (1999) and corresponds to the dynamics of larch tracheid wall development in the secondary wall thickening zone (Antonova and Shebeko 1985, Antonova 1999). The deposition of cellulose, the core of cell walls, occurs within early and late tracheids with similar dynamics.

The differences between the two methods of calculating the deposition of secondary wall substances before lignification appears to be a result of the different spatial distribution of cellulose microfibrils and the amounts of hemicelluloses, deposited at this stage, and their different structures. The role of hemicelluloses in organizing lignin precursors before polymerization, was proposed by Houtman and Atala (1995), Whetten and Sederoff (1995), and Grabner (2005).

The differences in the substances needed to initiate lignification in two wood types can be a result of not only the nature of matrix polysaccharides, but also from the different character of monolignols, the precursors of lignins. Terashima and col. (1988) examined the heterogeneous precursors of lignin and their selective deposition at different stages in pine tracheid formation. The heterogeneity of lignin precursors was also observed during lignification of compression pine wood (Fukushima and Terashima 1991).
Lignin deposition

Even though lignin was not found to be a polymer by histochemical reactions in the early stage of secondary wall formation, small amounts of the substances were isolated by the thioglycolic acid method from D-1 cells (Fig. 4). The total of two isolated fractions (LTGA-I and LTGA-II) was 2.2% and 2.0% per dry weight for earlywood and latewood, respectively. LTGA-II was the dominant substance, 79.5% for earlywood and 72.7% latewood.

The appearance of lignin-like substances, not determined by histochemical reactions, could be the result of the condensation of lignin precursors in acidic conditions during lignin isolation. Such condensation is possible due to the effects of chemical reagents and temperature (Shorigina et al. 1976). The negative influence of an acid medium on lignin obtained in vitro and the appearance of precipitation in such conditions were also reported by Freudenberg and Hubner (1952, cit. by Harborn 1964). However, it is unlikely that the patterns of thioglycolic lignin only resulted from the condensation of precursors during lignin isolation. In this case the drastic differences in molecular weight distribution of these patterns would be improbable (see gel-filtration data).

According to the histochemical reactions, the deposition of lignin starts in the D-2a stage of secondary cell wall development. The amounts (per dry weight) of LTGA-I and LTGA-II increase gradually during secondary wall formation in earlywood and latewood tracheids, but enhancement of LTGA-I occurs considerably faster than LTGA-II (Fig. 4). Starting from the D-2a stage and by the mature xylem stage the content of LTGA-I in the total of fractions drops from 15.2% in the early cells to 7.5%. While in the late tracheids it changes slightly (8.2-6.3%). The total quantity of LTGA-II increases.

If to evaluate the amount of lignin per cell, which can be considered as absolute in xylem tissue at each stage of tracheid differentiation, one can observe a more intensive increase of lignin from one stage to another (Fig. 5). Comparing the integral curves of xylem lignification shows that the rates of lignin deposition at consecutive stages of tracheid maturation of two wood types are different. The increments in the amount of lignin are calculated as the difference between the quantity in earlier and later stages reflected by real changes in the lignin deposition rate during early and latewood formation (Fig. 6). The calculations per dry weight (Fig. 6a) and per cell (Fig. 6b) show a similar lignification dynamics. According to these data, in the course of earlywood development the lignification of tissue increased gradually reaching a maximum intensity at the late stage of cell maturation. In this

Fig. 5. The content of fractions LTGA-I (1) and LTGA-II (2) in lignin isolated from larch xylem at successive lignification stages during earlywood and latewood formation (calculated per cell, mg · 10⁻⁶).

Fig. 6. Increment of lignin (a – per dry weight, b – per cell) at successive stages of lignification during earlywood and latewood development, % of total lignin in mature xylem.
period 47% of the lignin is synthesized (Fig. 6a). Terashima et al. (1988) observed similar peculiarities in pine by microautoradiography method and according to this study principal lignification occurred after the start of S3 formation. Earlywood appears to form during the experiment. In terms of our data the deposition of lignin during latwood formation occurs with the most intensity just after the start of lignification (Fig. 6). In layer D-2a 46% of all lignin is formed. In the course of secondary wall development and maturation of tracheids the intensity of the process declined. The contrast in lignification dynamics between earlywood and latwood can be a result of both the heterogeneity of lignin precursors and the carbohydrate gel in whose medium the polymerisation of phenylpropanoid units occurs.

The differences in the availability of phenolic acids for dehydrogenative polymerization also appears to be one of the reasons in the distinctions of lignin deposition rates in the course of earlywood and latwood formation (Antonova et al. 2005). One of compounds controlling the number of cinnamic acids accessible to peroxidase and regulating peroxidase-dependent polymerization of phenolic compound is believed to be ascorbic acid (Takahama 1993). As we showed the content of ascorbate and dehydroascorbate reflects the activities of oxidation-reduction metabolism of ascorbate and is different at successive stages of secondary wall thickening of both early and late xylem in larch (Antonova et al. 2005).

Alkaline oxidation

In the composition of alkaline oxidation products of LTGA-II (Table 1) isolated from the D-1 layer of earlywood aldehydes are absent. At the same time the presence of ferulic acid giving guaiacyl structures of lignin and synapic, the precursor of syringylpropane units of polymer, are observed by TLC (Table 1) and HPLC analysis.

The alkaline oxidation products of LTGA-II from D-1 during latwood lignification contain vanillin with trace amounts of syringylaldehyde and p-hydroxybenzaldehyde that originate from p-hydroxycinnamic alcohol (Table 1). The presence of aldehydes in the lignin at this stage during latwood formation indicates some condensation of phenylpropanoids. This means that LTGA-II can be regarded as lignin polymer. A small amount of ferulic acid was revealed in the alkaline oxidation products while sinapic acid is absent (Table 1).

The presence of an appreciable amount of sinapic acid at the D-1 stage in developing earlywood (Table 1) may account for some the delay in lignin deposition during earlywood formation. First, syringyl units have less reactivity than guaiacyl ones (Sarkanen 1975). Secondly, according to Grabner (2005) sinapyl-p-coumarate impairs the copolymerization of ferulate with monolignols by accelerating the inactivation of wall-bound peroxidase. Lu and Ralph (1999) suggest that sinapyl p-coumarate esters can appear before lignin polymerization due to enzymatical acylation with p-coumarate. The differences in sinapic acid before polymerization in earlywood and latwood may be the result of the different activities of ferulate 5-hydroxylase, which is an essential step in the formation of sinapyl alcohol that occurs as a result of the hydroxylation of ferulate (Whetten and Sederoff 1995). Greater mass in secondary walls, deposited before the start of lignification in development of earlywood in compared to latwood, appears to be the consequence in the delay of lignin deposition.

Starting with the D-2a stage of secondary wall development of both earlywood and latwood the lignin preparations differed in the composition and amounts of the compounds (Table 1). The principal aldehyde of the

Table 1. The contents of alkaline oxidation products of LTGA, isolated from xylem at different secondary cell wall development stages during earlywood and latwood formation.

<table>
<thead>
<tr>
<th>Stages of secondary cell wall development</th>
<th>The contents of alkaline oxidation products, % LTGA</th>
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<tr>
<td></td>
<td>Vanillin</td>
</tr>
<tr>
<td>Earlywood</td>
<td></td>
</tr>
<tr>
<td>D-1</td>
<td>0</td>
</tr>
<tr>
<td>D-2a</td>
<td>10.7</td>
</tr>
<tr>
<td>D-2b</td>
<td>3.9</td>
</tr>
<tr>
<td>M</td>
<td>1.5</td>
</tr>
<tr>
<td>Latwood</td>
<td></td>
</tr>
<tr>
<td>D-1</td>
<td>0.95</td>
</tr>
<tr>
<td>D-2a</td>
<td>3.48</td>
</tr>
<tr>
<td>D-2b</td>
<td>2.5</td>
</tr>
<tr>
<td>D-2c</td>
<td>2.5</td>
</tr>
<tr>
<td>M</td>
<td>2.8</td>
</tr>
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</table>

Note: Average error of means was 5 - 6.5 %
Alkaline oxidation products of LTGA-II is vanillin. The content decreases considerably at successive stages of secondary wall development of earlywood. During latewood formation the content is low at the D-2a stage and changes slightly at later stages. It displays a gradual increase of dehydrophenylpropanoid condensation in the lignin of earlywood and the highest degree of condensation at the beginning of lignification in latewood, which corresponds with the different dynamics of lignin deposition in both types of wood (Fig. 6).

Apart from vanillin, the alkaline oxidation products of LTGA from lignifying early xylem also contain syringaldehyde and p-hydroxybenzaldehyde, the amounts of which decline during tracheid maturation (Table 1). Additionally ferulic and synapic acids were revealed and their amounts also decrease in mature xylem. Trace amounts of P-coumaric acid were only found in mature xylem lignin.

Lignin preparations of developing latewood contain traces of syringaldehydes, but only at the D-2a stage (Table 1). The amount of p-hydroxybenzaldehyde gradually increases in mature xylem, which coincides with a decrease in the lignin deposition rate during latewood cell maturation (Fig. 6). The presence of p-hydroxyphenyl units is favourable to the condensation of the guaiacyl structure (Terashima and Fukushima 1988).

The phenolic acids, the alkaline oxidation products of latewood lignin preparations, contain only ferulic acid and in amounts substantially less than in the course of earlywood development. Therefore, both the distinct dynamics of lignification and the difference in the mass of secondary wall carbohydrates, deposited before this process in early and late xylem development, are evidently accounted for by the different composition of lignin precursors and in particular, by different amounts of p-hydroxyphenylpropanoid units taking part in the polymerization process and by the presence of different sinapyl units.

The differences in structural units of lignin at early stages of its formation are fixed by histochemical reactions. The Mäule’s reagent (for syringyl structure) shows a specific colouration of the middle lamella and cell walls at the very outset of earlywood lignification and disappears rapidly in the course of lignin formation. Mäule’s reaction is negative even at the early stage of lignification in latewood.

**Ether and ester bonds**

The linkages between lignin and carbohydrates are very strong and survive in the course of both acid and alkaline hydrolysis (Elkin and Lubavina 1980). According to the results of acidolysis (Fig. 7a) the amounts of ether-linked carbohydrates are the greatest at early stages of lignification and decrease at stages of both earlywood and latewood development but increase again considerably in mature xylem. At all stages ether-linked carbohydrates per lignin weight were greater in latewood than in earlywood. In the composition of carbohydrates linked with lignin by the ether bonds trace amounts of arabinose, glucose and xylose were observed at all differentiation stages of both early and late xylem (Table 2). Galactose and glucuronic acid also appear at the late stage (M). Benzoic and cinnamic acids, from the phenolic components were found to be linked with carbohydrate moiety.

These results appear to demonstrate that lignin may be ether-linked to branched hemicelluloses such as arabinoglucuronoxylan, xyloglucan or arabinogalactan (or may be arabinogalactan proteins) at the beginning and as galactoglucomannan at the end of lignification. Structural studies of chemical bonds between lignin and carbohydrates in spruce wood showed that lignin is linked to all types of sugar units of hemicelluloses and benzyl ether bonds are possible (Eriksson et al. 1980).

Lignification has been known to start in the middle lamella and then in cell walls (Kutsch and Schwarzmann 1975, Grunwald et al. 2002). Arabinogalactan proteins are present at different stages of tracheid development in larch but mainly in the cambium zone and in the primary cell wall before secondary wall formation and lignification (Antonova et al. 1988). It is possible that arabinogalactan proteins attract and “anchor” the lignin precursors.
The quantity of ester-linked carbohydrates (alkaline hydrolysis) in the lignin preparations decreases in the course of cell differentiation up to the end of tracheid maturation (Fig. 7b). As in the case with ether bonds the content of ester-linked carbohydrates in latewood lignin preparations is greater than in lignins from earlywood. Carbohydrate components included in such linkages contain the residues of xylose, arabinose and glucose (Table 2). Bound phenolic acids have been found to be ferulic and p-coumaric acids. Acid hydrolysis of LTGA-II before dioxan-HCl and alkaline treatments, showed the presence of mainly arabinose in the preparations from earlywood and latewood.

According to all the data, covalent bonds between forming lignin particles and carbohydrates appear only during the polymerisation of monolignols. In the course of the growth of lignin globules, the quantity of linkages between them and carbohydrates decreases but increases again due to the ether bonds that arise with the appearance of galactoglucomannan within the S2 layer. Eriksson et al. (1980) observed that galactose carbohydrate residues are involved in lignin-carbohydrate complex in spruce wood. Samuels et al. (2002) showed that mannsans are localized in the secondary cell wall during xylogenesis in pine.

The presence of cinnamic acids in alkaline oxidation products, ester-linked ferulic and p-coumaric acids, and ether-linked acids with carbohydrate residues in lignin preparations suggests that there is a link between polysaccharides and lignin globules that may be created at the beginning of lignification through phenolic bridges to both ester and ether linkages. Iiyama et al. (1990) discovered similar linkages for lignin in wheat internodes. At the late stage of larch wood lignification this connection is likely occurred mainly due to ether linkages. However, all these linkages should also be investigated.

**Table 2. The composition of sugars in hydrolyzates of the residues after dioxide-HCl and alkaline treatments of LTGA-II fractions isolated from earlywood and latewood at different stages of tracheid differentiation.**

<table>
<thead>
<tr>
<th>Stages of secondary wall development</th>
<th>The composition of sugars</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Dioxan-HCl treatment</td>
</tr>
<tr>
<td></td>
<td>Gal</td>
</tr>
<tr>
<td>Earlywood</td>
<td></td>
</tr>
<tr>
<td>D-2a</td>
<td>-</td>
</tr>
<tr>
<td>D-2b</td>
<td>-</td>
</tr>
<tr>
<td>M</td>
<td>+</td>
</tr>
<tr>
<td>Latewood</td>
<td></td>
</tr>
<tr>
<td>D-2a</td>
<td>-</td>
</tr>
<tr>
<td>D-2b</td>
<td>-</td>
</tr>
<tr>
<td>D-2c</td>
<td>-</td>
</tr>
<tr>
<td>M</td>
<td>trace</td>
</tr>
</tbody>
</table>

Note: nd – not determined: the symbols ",-" and "+" mean the absence or presence of spots of sugars on the "Silufol" plates (the differences between + and ++ were evaluated according to the intensity and the area

**Gel-filtration chromatography**

The gel-filtration results showed very low molecular weights of the principal parts of LTGA-II from the D-1 stage of earlywood (Fig. 8a), which corresponded to the exit volume of mono- and dimer compounds, and a slightly high molecular weight component. Alkaline oxidation of this preparation showed the presence of phenolic acids only (see above).

In contrast, LTGA-II from the D-1 stage of latewood had high molecular weights (Fig. 8b) and produced aldehydes after alkaline oxidation, which (Table 1) indicates the polymerization of monolignol because it can be considered lignin in spite of the absence of a specific colour with histochemical reagents. Of course, some “soiling” of this cell development stage can be assumed because some cells could already start lignin deposition, but this preparation can still be considered initial lignin of the middle lamella.

Molecular weight distribution of LTGA preparations at other developmental stages shows some common peculiarities of the lignification process in both types of wood. The preparation from D-2a had a high molecular weight and was rather homogeneous (Fig. 8a, b). However, during lignification the dispersity of polymer increased and its molecular weight declined.

To suppress the possible cooperation between different parts of macromolecules and macromolecules themselves a solution of 1% LiCl in DMSO was used for chromatography of LTGA-II fractions obtained from developing latewood. The data demonstrated almost identical molecular weight distributions of the lignin patterns, high molecular weight at the beginning of lignin deposition and its decrease in the course of secondary wall formation (Fig. 8c). This means the high molecular weight of lignin patterns at early stages of lignification is the result of polymerisation of monolignols rather than internal or external interactions in macromolecules.
The decrease in lignin molecular weight during tracheid differentiation appears to be the result of a difference in the nature of hemicelluloses that accumulate in cell walls at successive developmental stages. For example, Terashima and Seguchi (1987) reported that pectin gel is more effective for dehydrogenative polymerisation of monolignols than mannan. According to our unpublished data the amount and composition of pectin substances in larch xylem changed with the degree of tracheid development.

The data suggests that at early stages lignin forms as bulk-polymer. One possible mechanism of bulk-dehydropolymer formation is a reaction-limited aggregation by the type of cluster-cluster (RLA C-C) (Witten and Sander 1981, 1983). The formation of bulk-polymer occurs in the presence of a high initial concentration of phenoxyl radicals (Lai Yau-Zon and Sarkanen 1975, Whetten and Sederoff 1995). According to our unpublished data the high amounts of phenolic acids were found only at the D-1 stage, i.e. before lignification, during both earlywood and latewood development. The availability to the action of enzymes, leading to the formation of monolignols and to phenoxyl radicals, can be distracted by the activities of these enzymes and the presence of ascorbate, which interfere in this process. But a part of the monolignols can already be in “reactionary volume” (middle lamella and then S 1 layer), where they will combine in clusters. The isolation of a lignin-like preparation from D-1 of early xylem appears to prove this. The limiting factor in

![Fig. 8. Gel-filtration curves of LTGA-II isolated from larch xylem with different degree of secondary cell wall development (D-1 before lignification; D-2a, D-2b, D-2c - after the beginning of lignification, M - mature xylem) during earlywood (a) and latewood (b and c) formation with DMSO (a, b) and DMSO + LiCl (c) as the eluent. The arrows show the volumes of the exit of Dex Blu and p-coumaric acid.](image)
this case appears to be not diffusion, but the reactions of cluster combinations.

At the next stages of lignification, especially at the end of tracheid maturation (Fig. 8), the polymer appears to grow as an end-wise polymer, with branching due to the inclusion of low molecular weight fragments. The low lignification rate at the first stage could be due to the presence of both guaiacylpropanoid and syringylpropanoid monolignols and the lower reactive capacity of the latter. The presence of an essential quantity of ascorbate before lignification (Antonova et al. 2005) can also interfere with the transformation of cinnamic acids in respective alcohols and their coupling because of the using of oxidizing agents by ascorbate to be oxidized. Dehydrogenative polymerisation of monolignols is catalyzed via hydrogen peroxide-dependent peroxidase(s), O2-requiring laccase (Dean and Eriksson 1992) or in conjunction (Freudenberg 1959). Laccase doesn’t require toxic hydrogen peroxide and can be included in lignification at the first stage of the process (Sterjiades et al. 1993). Laccase were isolated from differentiating xylem of pine, which coincide in time and place with lignin formation (Bao et al. 1993). Savidge and Udagama-Radeniya (1992) reported the physico-chemical properties of a laccase-like wall-bound enzyme, a copper containing coniferyl alcohol oxidase, and its spatio-temporally correlation with lignification in conifers. One type of this enzyme had relatively low in vitro activity corresponding to very slowly lignification in vivo (Udagama-Radeniya and Savidge 1995). This peculiarity is in agreement with the low rate of lignin deposition in early xylem at the D-2a stage. It suggests that the increasing intensity of lignification in the following stages occurs because of the activation of hydrogen peroxide-dependent peroxidase. One of the pathways of hydrogen peroxide generation may be the oxidation of ascorbate within cell walls (Forti and Ellis 1995).

In lignification of latewood the peroxidase + H 2O 2 system seems to play a more important role than laccase+O 2 because the rate of lignin deposition is very high only at the first stage. This shows there is a correlation with very low ascorbate content and high amounts of dehydroascorbate at this stage of late xylem lignification in larch (Antonova et al. 2005). Increasing peroxidase activity can be the result of water stress in a tree. Tsutsumi and Sakai (1993) reported that water stress stimulates wall-bound types of peroxidase and increases the accumulation of lignin with guaiacyl units in poplar cullus. Moreover, water stress causes a decrease in the ascorbate level because of a decline in biosynthesis and an increase in ascorbate oxidase activity (Mukherjee and Choudhuri 1983, Smirnoff and Colombe 1988). As mentioned previously an internal water deficit causes latewood formation in conifers (Zahner 1963, Antonova 1999).

There were other differences in the structure of lignin macromolecules from earlywood and latewood. The average molecular weight of mature earlywood lignin was lower and dispersity was less than the main core of latewood lignin (Fig. 8). The latter consisted of two fractions and the content of the low molecular weight fraction increased during the maturation of xylem.

Macromolecular heterogeneity of lignins that arise during earlywood and latewood development in larch can be the reason for the polyhronic nature of the delignification process in wood (Pen and Pen 1998).

**Conclusion**

The amount of carbohydrate substances that accumulate in larch tissue before lignin deposition is greater in earlywood than in latewood, while there is more cellulose deposited in latewood than in earlywood. This means that the distribution and macromolecular organization of polysaccharides in cell walls before the polymerization of lignin precursors is different during earlywood and latewood formation.

Lignin deposition during tracheid differentiation of earlywood and latewood occurs at different rates. The intensity of lignin synthesis in the course of earlywood development gradually increases and reaches its maximum at the end of tracheid maturation. During latewood tracheid development the synthesis of lignin is greatest at the outset of lignification and decreases by the end of xylem differentiation.

The early stages of lignification and the period before lignin deposition in earlywood and latewood are characterized by different contents, composition and degree of condensation of phenylpropanoid units.

The contents of carbohydrates connected to lignin by ether and ester bonds decreases during lignification of both earlywood and latewood with some increase of ether linked carbohydrates in mature xylem.

At the beginning of lignification of earlywood and latewood the lignin formed has high molecular weight. Molecular weight distribution of lignin gradually changes and the average molecular weight decreases during cell differentiation because of the deposition of molecules with less molecular weight in the S2 layer. At the late stage of cell differentiation the lignin of earlywood is more homogeneous than latewood. All data suggest that the polymerization of monolignols occurs by the type of bulk-polymer at the beginning of lignification and subsequently as end-wise process. The formation of lignin by an end-wise polymer type appears to occur more in earlywood than in latewood.

**References**


