Original Paper

Biochemical studies of matured xylem of *Cryptomeria japonica*
Attempts to detect the enzymes involved in the biosyntheses of the heartwood extractives

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Many biochemical actions occur in mature tree xylem after cell differentiation is completed in the differentiating xylem. However, biochemical studies of the mature xylem are scarce. One of the most remarkable actions of mature tree xylem is the process of changing sapwood to heartwood, so-called heartwood formation. Generally, heartwood contains particular organic solvent extractable substances called heartwood extractives. In this study we attempted to investigate the biochemical abilities of mature sugi xylem from the perspective of heartwood extractive formation. First, frozen wood powders were prepared from various parts of the xylem. These powders were subjected to buffer extraction, and crude protein extracts were obtained by ammonium sulfate precipitation. The protein contents were determined colorimetrically, and no increase of protein content corresponding to formation of the heartwood extractives in the intermediate wood was found. Protein constitutions were also examined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and no alteration of the constitutions corresponding to formation of the extractives in the intermediate wood was found. Conversely, the activity of phenylalanine ammonia-lyase, an enzyme that would be involved in the biosyntheses of sugi heartwood extractives, was determined by gas chromatography-mass spectrometry and was significantly increased in the intermediate wood. Consequently, frozen wood powder is useful for biochemical studies of mature tree xylem, and by using the powder detection of the bioability of mature xylem concerning formation of sugi heartwood extractives was achieved.

Keywords: biochemistry, *Cryptomeria japonica*, heartwood extractives, mature xylem, norlignan

1. Introduction

Most tree species have a dark colored zone called heartwood in the central part of their trunk, which is distinguishable from the surrounding pale zone called sapwood. Heartwood usually contains a larger amount of organic solvent extractable substances, namely, heartwood extractives. Heartwood extractives are specific to wood species, and thus can critically influence wood features, such as color, smell, resistance against decay, and so on.

Japanese cedar (*Cryptomeria japonica*, sugi) is one of the most available wood species in Japan. The value of sugi wood is strictly dependent on its color which would be ascribed to the extractives. Main sugi heartwood extractives are classified as diterpenes and norlignans. Especially, the norlignans in sugi heartwood are considered to affect the wood color because it has been revealed that the blackish heartwood or discolored sugi wood contains different kinds of norlignans from those found in the normal sugi wood.

In abnormal sugi wood, altered norlignan biosyntheses should be occurring. However, the biosynthetic pathways of norlignans are not well understood. The biosynthesis of hinokiresinol has only been investigated by both feeding and enzymatic studies using fungi-elicited suspension-cultured asparagus cells (Suzuki *et al.* 2001, 2002), or using suspension-cultured sugi cells (Noguchi *et al.* 1997; Miyoshi *et al.* 2000; Suzuki *et al.* 2004). Main sugi heartwood norlignans are agatharesinol and sequirin C (Ogiyama *et al.* 1983), and studies on the biosynthesis of agatharesinol have just recently been undertaken by a feeding experiment (Imai *et al.* in press). The enzymes or nucleic acids involved in the biosyntheses of sugi heartwood norlignans have not yet been studied.

Because heartwood extractives are formed during the process of sapwood changing to heartwood, the biosyntheses of sugi heartwood norlignans should be carried out by various biochemical functions of the living cells in the mature sapwood. Biochemical studies of mature tree xylem, in which biomacromolecules such as enzymes or nucleic acids are handled, are scarce (Fukazawa and Higuchi 1966a,b; Higuchi *et al.* 1967a,b; Magel *et al.* 1991, 2001) even today when biochemical experimental procedures have become routinely available for general plant materials. The following features of mature tree xylem have hampered execution of the biochemical studies: (1) the amount of living cells, namely,

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ray parenchyma cells and axial parenchyma cells, in the mature xylem are too small for studying their bioabilities, and (2) raw wood materials are considerably moist and the parenchyma cells are surrounded by thick lignified secondary cell walls, which make it difficult to grind such materials for extraction of the biomacromolecules.

In this study, we attempted to investigate the biochemical abilities of mature sugi xylem from the perspective of formation of heartwood extractives. First, we prepared frozen wood powders, which were subjected to buffer extraction. Crude protein extracts were then obtained by ammonium sulfate precipitation. The protein contents were determined colorimetrically (Bradford method), and the protein constitutions were also examined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). One of the enzyme activities that would be involved in the biosyntheses of sugi heartwood extractives, namely, phenylalanine ammonia-lyase (PAL), was determined by gas chromatography-mass spectrometry (GC-MS).

2. Materials and Methods

2-1. Wood material

A 32-year old sugi tree was felled in the University Forest (Inabu, Aichi Pref.) of Nagoya University in the middle of June 2004. Wood disks (5 cm in thickness), composed of 16, 6, and 21 years worth of sapwood, intermediate wood and heartwood, respectively, were cut from the log immediately after felling. The wood disks were frozen immediately with liquid nitrogen (liq. N_2), and stored under −80℃ until use.

2-2. Analysis of sugi norlignan

2-2-1. Preparation of wood samples and extraction

The inner part of the sapwood, the intermediate wood and the outer part of the heartwood, were divided according to the annual rings, and wood materials (1 g, fresh weight) were collected from every ring. The samples were cut into small pieces with a cutting knife (5 mm × 5 mm × 5 mm), then extracted thoroughly with methanol (MeOH) for 48 hours using a Soxhlet's extractor. The MeOH solutions were evaporated to give a syrupy material (MeOH extracts). The MeOH extracts were then successively treated with *n*-hexane and ethyl acetate (EtOAc).

2-2-2. Gas chromatography (GC)

A portion of the EtOAc extract was dissolved in pyridine, and an aliquot of the solution was trimethylsilylated (TMSi) using N, O-bis(trimethylsilyl)-trifluoroacetamide (Wako). The TMSi derivative was analyzed by GC on a Shimadzu GC353 model equipped with a TC-1 capillary column (60 m in length × 0.25 mm i.d., film thickness 1 μm) and a flame ionization detector using N_2 as the mobile phase. Oven temperature was programmed to increase from 150℃ to 280℃ at 7℃/minute.

2-3. Enzymatic study

2-3-1. Preparation of wood samples and extraction of proteins

The sapwood was radially divided into 3 years worth of three parts, namely, outer-(including cambial region), middle-, and inner-(intermediate wood side) sapwood. The intermediate wood was divided into two parts, namely, outer-(sapwood side) and inner-(heartwood side) intermediate wood according to the norlignan compositions determined by GC (section 2-2-2, Fig. 1). The heartwood was radially divided into 3 years worth of two parts, namely, outer-(intermediate wood side), and inner heartwood (including pith). From every part, wood sticks (5 mm × 5 mm × 50 mm) were prepared. The wood sticks were frozen with liq. N_2, and shaken vigorously together with stainless steel balls cooled with liq. N_2 in a liq. N_2-cooled stainless steel pot to render frozen wood powders by using a vibration shaker. The frozen wood powders (100 g, fresh weight) were homogenized in a 100 mM potassium buffer (pH 7.5) containing 25 mM 2-mercaptoethanol, 1 mM phenylmethylsulfonyl fluoride, and 6% polyclar VT. The homogenates were then filtered through gauze, and centrifuged for 20 minutes at 8000 g at 4℃. The supernatants were saturated with 70% ammonium sulfate, and the formed materials were collected by centrifugation for 30 minutes at 22000 g at 4℃. The precipitates were resuspended in a 100 mM potassium buffer (pH 7.5, 1.5 ml), and then dialyzed against the same buffer for 24 hours. The dialysates were used as crude protein extracts.

2-3-2. Protein determination

The protein concentrations of the crude protein extracts were determined by the Bio-Rad assay system (Bradford 1977) according to the manufacturer's specifications. Bovine serum albumin was used as the standard.

2-3-3. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

The crude protein extracts were subjected to separation on 12.5% polyacrylamide gel using a Bio-Rad broad range molecular weight standard. The gel was stained with Coomassie brilliant blue (CBB) according to the manufacturer's specifications (Bio-Rad).

2-3-4. Determination of PAL activity

The crude protein extract (150 μl) was mixed with a 100 mM borate buffer (2 ml) containing 50 mM L-phenylalanine-[ring-2,3,4,5,6-^{2}H], and incubated for 30 hours at 30℃. The reaction mixture was acidified with 3N HCl, and then extracted with EtOAc. *trans*-Cinnamic acid in the extract was identified and quantified by GC-MS to evaluate PAL activity. The GC-MS analysis was carried out on a Shimadzu QP 2010 GC-MS equipped

with a DB-1 capillary column (30 m in length × 0.32 mm i.d., film thickness 1 μm) using helium gas as the mobile phase. Oven temperature was programmed to increase from 150℃ to 280℃ at 7℃/minute, and the ionization energy was 70 eV. In the case of the samples of outer sapwood, outer- and inner intermediate wood, the determination of PAL activity was performed twice.

3. Results and Discussion

3-1. Determination of the norlignan content in sugi wood

Sugi heartwood norlignan consists exclusively of agatharesinol and sequirin C (Ogiyama *et al.* 1983). Figure 1 shows the change of the contents of agatharesinol and sequirin C in the sugi wood. Both norlignans were not present in the sapwood. The content of agatharesinol increased gradually from the outer intermediate wood toward the outer heartwood, then tended to decrease toward the inner heartwood. Conversely, the content of sequirin C increased rapidly from the innermost intermediate wood toward the outer heartwood, then was sustained at relatively high levels in the heartwood. These results suggest that the intermediate wood should be a biosynthesis site of the norlignans and that different kinds of norlignans would be formed in the distinct areas of the intermediate wood.

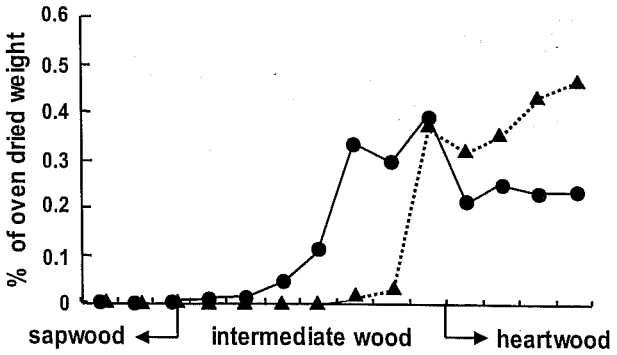

Fig. 1. Change of norlignan contents in sugi wood (% of oven dried wood material)
● : agatharesinol, ▲ : sequirin C

3-2. Determination of protein content in mature sugi xylem

Figure 2 shows the change of the protein contents in the sugi xylem. The protein content was the highest in the outer sapwood. This is because of the fact that the outer part contains the cambial region, where active cell differentiation is occurring. The content lessened greatly in the middle sapwood, then tended to decrease gradually toward the heartwood. Unexpectedly, increase of protein content reflecting the increase of norlignan contents in the intermediate wood was not found.

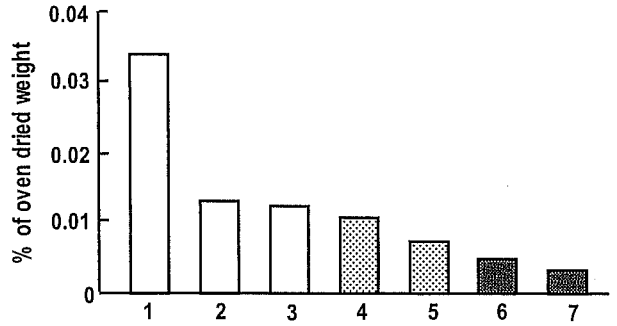

Fig. 2. Change of protein content in sugi mature xylem (% of oven dried wood material)
1: outer sapwood, 2: middle sapwood, 3: inner sapwood, 4: outer intermediate wood, 5: inner intermediate wood, 6: outer heartwood, 7: inner heartwood

It is well-known that enzymes involved in the biosyntheses of secondary metabolites, *e.g.*, flavonoid (Latunde-Dada *et al.* 1999; Kaltenbach *et al.* 2001) or terpenoid (Collu *et al.* 1999, 2001) are associated with membranes of organelles. Supposing membrane proteins would participate actively in the formation of norlignan, it is possible their contents in the intermediate wood might increased significantly. In this experiment, only buffer soluble proteins were examined, and further studies are now under way to investigate membrane proteins in sugi xylem.

3-3. Detection of proteins in sugi mature xylem by SDS-PAGE

Figure 3 shows the SDS-PAGE of the crude enzyme extracts prepared from the various parts of sugi xylem. Proteins in the extracts from the sapwood and the outer intermediate wood were detectable by SDS-PAGE using CCB for staining. The staining was faint in the case of the inner intermediate wood, and no

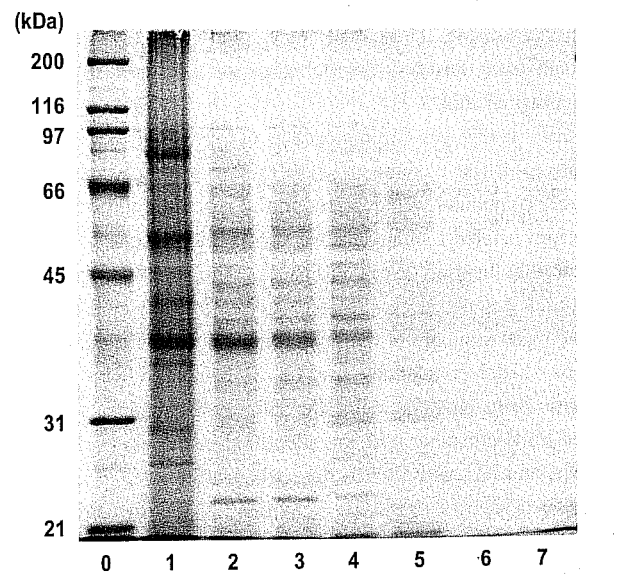

Fig. 3. SDS-PAGE of crude enzyme extracts from mature sugi xylem
0: molecular weight standard, 1: outer sapwood, 2: middle sapwood, 3: inner sapwood, 4: outer intermediate wood, 5: inner intermediate wood, 6: outer heartwood, 7: inner heartwood

staining was observed in the case of the heartwood. These should be due to the low protein contents in the parts. Unexpectedly, remarkable appearances of the proteins that were not present in the sapwood and supposed to be involved in norlignan biosynthesis were not found in the intermediate wood. Again, membrane proteins were not examined in this experiment, and the constitution of the membrane protein might alter greatly in the intermediate wood.

3-4. Determination of PAL activity in mature sugi xylem

PAL is one of the most important enzymes participating in the biosyntheses of secondary metabolites. This enzyme is involved in the phenylpropanoid pathway catalyzing the conversion from L-phenylalanine to *trans*-cinnamic acid. Fukazawa and Higuchi (1966a) attempted to detect the PAL activities in some wood species (*e.g.*, sugi, hinoki (*Chamaecyparis obtsusa*), red pine (*Pinus densiflora*) *etc.*), and found that the enzyme activity was highest at the cambial region, decreasing rapidly toward the sapwood and intermediate wood, and then no activity at all in the heartwood. Magel *et al.* (2001) attempted to detect the activities of PAL and chalcon synthase in *Robinia pseudoacacia*, and found that both enzyme activities were activated in the intermediate wood, and were highest in late autumn. In the previous studies, PAL activity was evaluated frequently by determining the UV absorption or radioactivity of the enzyme reaction mixtures. Since these methods do not detect *trans*-cinnamic acid directly, however, it is unsure whether all the absorption and radioactivity originate only from *trans*-cinnamic acid in the reaction mixture. In this study, we attempted to detect the PAL activity in mature sugi xylem by determining *trans*-cinnamic acid directly in the enzyme reaction mixture by GC-MS. The use of deuterium labeled L-phenylalanine as a substrate ensured the detection of enzymatically formed *trans*-cinnamic acid.

Figure 4 shows gas chromatograms of the EtOAc extract (TMSi derivative) of the enzyme reaction mixtures (outer intermediate wood). A compound was detected in the reaction mixture that the deuterium labeled L-phenylalanine reacts with an enzyme extract denatured by heating (arrow in Fig. 4a). The retention time of the compound was identical to that of authentic *trans*-cinnamic acid, and the mass numbers of the main fragment ions of the compound (Fig. 5a) had increased by five (+ 5) compared with those of authentic *trans*-cinnamic acid (Fig. 5b). Consequently, deuterium labeled *trans*-cinnamic acid was identified in the enzyme reaction mixture, and PAL activity was detected definitely.

Figure 6 shows the PAL activities in mature sugi xylem evaluated by quantifying *trans*-cinnamic acid in the enzyme reaction mixtures by GC-MS. PAL activity was highest in the outer sapwood due to the cambial region contained in this part. In the middle- and inner- sapwood much lower PAL activities were found. Conversely, the activity increased significantly in the

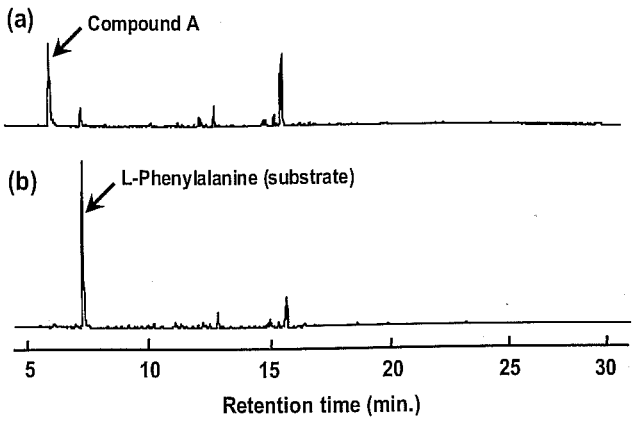

Fig. 4. Gas chromatograms of EtOAc extracts of enzyme reaction mixtures (outer intermediate wood)
(a) the reaction using crude enzyme extract, (b) the reaction using crude enzyme extract denatured by heating

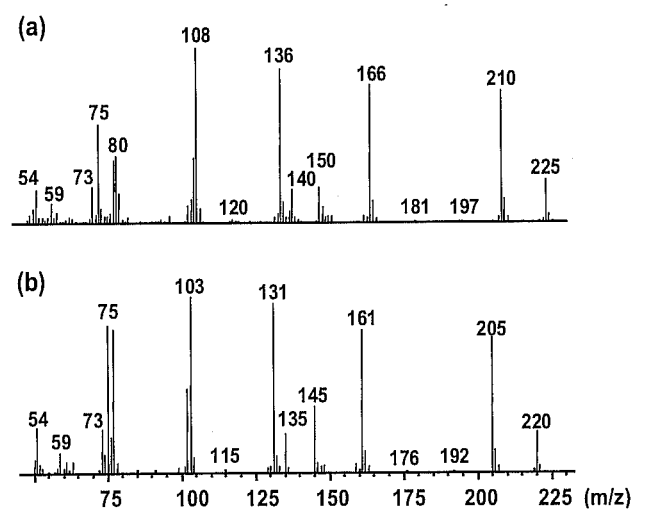

Fig. 5. Mass spectra of (a) the enzymatically formed compound A and (b) authentic *trans*-cinnamic acid

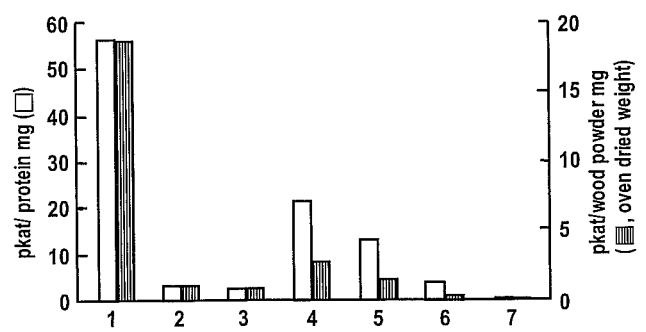

Fig. 6. Change of PAL activity in mature sugi xylem
1: outer sapwood, 2: middle sapwood, 3: inner sapwood, 4: outer intermediate wood, 5: inner intermediate wood, 6: outer heartwood, 7: inner heartwood
□ : The activity on protein mg, ▥ : The activity on wood powder mg (oven dried weight)

intermediate wood. In the outer heartwood, PAL activity lessened, and no activity was found in the inner heartwood.

In the previous studies on heartwood formation, the role of intermediate wood has often been discussed. Some studies have suggested that drastic changes from sapwood to heartwood should be occurring in intermediate wood, but others have not found increased bioabilities that could cause such changes. Fukazawa and Higuchi (1966a) and Higuchi *et al.* (1967a) did not find increased activities of the two enzymes, namely, PAL and phenol oxidase, which would be involved in the biosyntheses of heartwood extractives in the intermediate wood. Their findings contrasted with our study that PAL activity increased in the intermediate wood (Fig. 6). The amounts of the samples used in the previous studies (2 g) were much smaller than that in the present study (100 g). The enzyme activities were detected colorimetrically in the previous studies, while a more sensitive and distinctive method, namely, GC-MS was applied in the present study. Because of these differences, increased enzyme activities were able to be detected successfully in the intermediate wood in the present study.

As mentioned above (Section 3-1, Fig. 1), the contents of sugi norlignans increased in the intermediate wood toward the heartwood (Fig. 1), and PAL activity also increased in this region (Fig. 6). Recently, it was demonstrated that agatharesinol, a sugi heartwood norlignan, was biosynthesized through a phenylpropanoid pathway (Imai *et al.*, in press). Consequently, activation of the bioability reflecting the formation of sugi norlignan was observed in the intermediate wood, and intermediate wood was characterized biochemically as a particular region concerning the biosynthesis of heartwood extractives in sugi.

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スギ成熟木部の生化学的研究
心材抽出成分の生合成に関与する酵素の検出の試み

今井貴規・伊藤恵理子・福島和彦

これまで樹木成熟木部の生化学的な研究は少ない。成熟木部が行う重要な生理活動の一つとして心材形成が挙げられる。一般に心材には，辺材には存在しない特徴的な抽出成分が含まれる。本研究では，心材成分生合成の観点からスギ成熟木部の生理的活性を生化学的に調査した。まず，辺材，移行材および心材から凍結木粉を調製した。これら木粉を緩衝液抽出し，その抽出液からタンパク質を硫安沈澱させた。タンパク質量を比色

定量した結果，移行材において抽出成分の生成を反映するタンパク質量の増加は認められなかった。タンパク質組成を電気泳動によって調査した結果，移行材において抽出成分の生成を反映するタンパク質の質的変化は認められなかった。一方，スギ心材成分の生合成に関与すると考えられるフェニルアラニンアンモニアリアーゼの活性をガスクロマトグラフ質量分析法によって測定した結果，この酵素が移行材において活性化されることが確認された。したがって，凍結木粉が成熟木部の生化学的な調査に有用であることが示され，これを使用することによって心材成分生合成に関与するスギ成熟木部の生理的活性を検出することができた。

キーワード：生化学，*Cryptomeria japonica*，心材成分，成熟木部，ノルリグナン