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Enzymatic Formation of Guaiacylglycerol -8-*O*-4′-(Coniferyl Alcohol) Ether from Coniferyl Alcohol with Enzyme Preparations of *Eucommia ulmoides*

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Abstract

Lignans and neolignans are optically active plant secondary metabolites. Research on biosynthesis of lignans has already been advanced especially for the formation of (+) pinoresinol but information on the biosynthesis of 8-*O*-4′- neolignans is still limited. Moreover, the chemical structure (position of substituents on aromatic rings) and stereochemistry of 8-O-4′ neolignans is not clear. Katayama and Kado discovered that incubation of cell-free extracts from *E. ulmoides* with coniferyl alcohol in the presence of hydrogen peroxide gave (+)-*erythro*- and (-)-*threo*- guaiacylglycerol 8-*O*-4′-(coniferyl alcohol) ether (GGCE) (diastereomeric ratio, 3:2) which is the first report on enzymatic formation of optically active -8-O-4′ neolignans from an achiral monolignol. In this aspect, enzymatic formation of guaiacyl 8-O-4′ neolignan is noteworthy to clarify its stereochemistry from incubation of coniferyl alcohol with enzyme prepared from *Eucommia ulmoides*. In this experiment, soluble and insoluble enzymes prepared from *E. ulmoides* were incubated with 30 mM coniferyl alcohol (CA) for 60 min. The enzyme catalyzed GGCE, dehydrodiconiferyl alcohol (DHCA), and pinoresinol identified by reversed phase HPLC. Consequently, diastereomeric compositions of GGCE were determined as *erythro* and *threo* isomer. Enantiomeric composition was determined by the chiral column HPLC. Both enzyme preparations enantioselectively formed (-)-*erythro*, (+)-*erythro* and (+)-*threo*, (-)-*threo*-GGCEs respectively.

Key words: *Eucommia ulmoides*, 8-*O*-4′ neolignan, coniferyl alcohol, diastereomer, enantiomer.

Introduction

Lignans and neolignans are widespread, structurally diverse a group of phenylpropanoid broadly distributed in higher plants. Lignans are connected through the C8-C8′ linkage and neolignans are connected through linkages other than the 8-8′; for example 3-3 (or 5-5), 8-*O*-4, and 8-3 (8-5) linkages (Howorth 1936). The typical lignans and neolignans that have an oxygen atom at 9 and 9′C position are generated by dehydrogenative dimerisation of p-hydroxycinnamyl alcohols termed monolignols (especially coniferyl alcohol and sinapyl alcohol) (Gottlieb 1978). Coupling of mesomeric monolignols radicals leads to several intermonomer linkages: different monolignol radicals give rise to cross-coupling products.

Most of the lignans and neolignans in plants are optically active and display important physiological functions in plant defense and human health. Recently, a "dirigent protein" was discovered to catalyze a regioselective as well as enantioselective coupling of two coniferyl alcohol radicals that yielded (+)-pinerosinol (Gang et al. 1999; Gellerstedt et al. 1995; Greca et al. 1994). In addition, lignins, the cell wall polymers in wood are structurally similar to lignans/neolignans, but they are optically inactive, racemate-like polymers. Lignins are formed through dehydrogenation of monolignols by non-selective peroxidase/H_2O_2 (and laccase/O_2) and subsequent non-enzymatic coupling of the mesomeric radicals (Li et al. 1998), and they linked by 8-*O*-4′ intermonomer linkages (Davin and Lewis 2000).

In contrast to lignans, the biosynthesis of 8-*O*-4′ neolignans has not been studied intensively and awaits clarification. Moreover, information about 8-*O*-4′ neolignans especially GGCE (Lourith et al. 2005) have seldom been described in respect to the identification of its diastereomers (Nakatsubo et al. 1976). There is still confusion about *cis* and *trans* forms of

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benzofuran ring in naturally occurring 8-5′ neolignans because in some papers the 8-5′ neolignans were reported as *cis* form without strong evidence (Tanahashi et al. 1976).

Eucommia ulmoides Oliver (Tochu in Japanese, Du-Zhong in Chinese) is a medicinal woody plant whose bark is used as a crude drug in China and its leaves used as tea in Japan. Recently, it has been reported that this plant has pharmacological effects on coronary blood flow, diabetic, hepatic damage, antihistamine, pain relief, diuresis, blood pressure and lipid metabolism, carcinogenesis, and other bioactivities. Many bioactive compounds including lignans are present in *E. ulmoides*. Ho et al. (2005) isolated citrusin B [{guaiacyglycerol-8-*O*-4-(sinapyl alcohol) ether (GGSE)}4-*O*-glucoside] from this plant and subsequently did spectrometric analysis of the resulting (+) GGSE, but its diastereomer, *erythro* or *threo* isomer have not been clarified yet. Citrusin B was first isolated from peels of *Citrus hassaku* Hort. and *Citrus sinensis* Osbeck. with citrusin A [{guaiacylglycerol-8-*O*-4′-(coniferyl alcohol) ether (GGCE)} 4-*O*-glucoside]. However, stereochemistry of neither citrusin A nor B had been identified. To clarify the stereochemistry and enzymatic formation of 8-*O*-4′ neolignans especially GGCE of coniferyl alcohol (CA) moieties, chemical synthesis of CA (both cold and hot) and their related lignans/neolignans such as pinoresinol, dehydrodiconiferyl alcohol (DHCA), and GGCE were done and strictly identified by means of NMR during the chemical synthesis. Additionally, incubation of CA (used as a cold substrate) with cell-free extracts of *E. ulmoides* in the presence of hydrogen peroxide was carried out. Subsequently, insoluble enzyme was also prepared and incubated with CA.

Materials and Methods

Instrumentation and chromatography materials

All reagents and solvents used were reagent grade. Analytical and preparative thin-layer chromatographies (TLC) were accomplished by using plates precoated with Merck silica gel 60 F_{254} (0.25 and 0.50 mm thickness). ^{1}H, ^{13}C, and two-dimensional nuclear magnetic resonance (NMR) spectra (400 MHz) were recorded on a JEOL Alpha 400 FT-NMR spectrometer using TMS as internal standard. Analytical high performance liquid chromatography (HPLC) was carried out on a Jasco PU-2089 equipped with a Jasco UV-2075 plus Intelligent UV/Vis detector and a Shimadzu chromatopac C-R7A plus a reversed phase column (TSK-GEL, ODS-80Ts, column No. E9479). Compounds were separated at a flow rate of 1.0 ml/min using the linear gradient of 30-35% MeOH and 3% AcOH in H2O (v/v). Chiral analysis was performed on a Daicel Chiracel OD column (250 × 46 mm) eluted with EtOH: n-hexane (15:85 v/v) at a flow rate of 1.0 ml/min (for *erythro*-GGCE) and 0.8 ml/min (for *threo*-GGCE) to determine the enantiomeric composition of isolated lignans.

Chemical syntheses of guaiacylglecerol-8-*O*-4′-(coniferyl alcohol) ether (GGCE)

Coniferyl alcohol was synthesized by the method of Tanahashi et al (1976).

Enzymatic dehydrogenation of coniferyl alcohol by peroxidase/H_2O_2

A 1.11 mmol (200.2 mg in 5.5 ml of Milli Q H_2O) solution of coniferyl alcohol (Fig.1) was mixed with 0.1 M K-Pi buffer (20.8 ml, pH 7.0) at room temperature. Peroxidase enzyme solution (60 unit/mg in the same buffer, 0.124 ml) was added slowly to the reaction flask. After the addition of enzyme, 0.5% H_2O_2 solution (10.32 ml of 30% H_2O_2) was mixed drop by drop within 5 min and stirring was continued for ca. 35 min. The reaction mixture was then carefully quenched by the addition of NaCl salt and was then extracted three times with ethyl acetate (CH_3COOC_2H5) and washed once with saturated NaCl solution. The organic layer was dried over anhydrous Na_2SO4 and evaporated to dryness *in vacuo* under nitrogen atmosphere. The residue (140.8 mg, yield 70.3%) was applied onto preparative TLC by 0.50 mm thick three plates [(3% MeOH in CH_2Cl_2) × 3] to isolate pinoresinol (4 mg, yield 2.88%), DHCA (12.5 mg, yield 9.01%) and GGCE (10.9 mg, yield 7.86%).

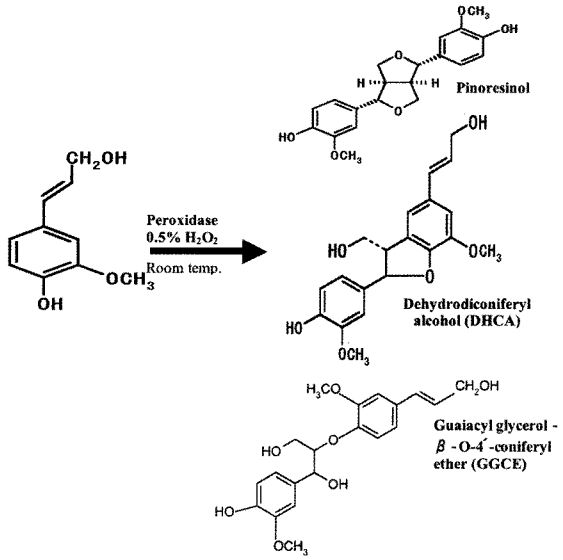

Fig. 1. GGCE produced by dehydrogenation of CA with peroxidase

Pinoresinol

^{1}H NMR delta ($CDCl_3$): 6.90 (2H, d, J = 1.95, H-2,2′), 3.91 (6H, s, OCH_3-3,3′), 5.587 (1H, s, OH-4,4′), 6.895 (2H, d, J = 8.05, H- 5, 5′), 6.814 (2H, dd, J = 1.70, 8.29, H-6,6′), 4.732 (2H, d, J = 4.39, H-7,7′), 3.102 (2H, dd, J = 4.63, J = 6.43, H-8,8′), 4.245 (2H, dd, J = 7.07, J = 9.02, H-9_a,$9'_a$), and 3.871 (2H, dd, J = 3.78, J = 9.14, H-9_b,$9'_b$).

DHCA

^{1}H NMR delta ($CDCl_3$): 3.81 (3H, s, OCH3-3), 3.86 (3H, s, OCH3-3′), 5.64 (1H, s, OH-4), 5.85 (1H, d, J = 7.3, H- 7), 6.54 (1H, d, J = 15.61, H-7′), 3.62(1H, m, H-8), 6.24 (1H, dt, J = 15.86, J = 5.97,H-8′), 3.90-3.93 (1H, m, H-9$_a$), 3.987 (2H, dd, J = 13.47, J = 6.01, H-9$_b$,9′$_b$), 4.30 (1H, d, J = 5.85, H-9′), and 6.86-6.91 (2H, m, H-5, Ar-H).

(±)Guaiacylglycerol-8-*O*-4′-coniferyl alcohol ether (GGCE)

^{1}H NMR delta (acetone-d_6 +TMS): 7.10 (H-2), 7.11 (H-2′), 3.82 (s, OCH_3-3), 3.90 (s, OCH3-3′), 5.64 (s, ArOH-4), 6.77 (H-5), 6.93 (H-5′), 6.90 (H-6), 6.88 (H-6′), 4.90 (OH- 7), 6.52 (H-7′), 4.27 (H-8), 6.28 (H-8′), 3.50 (9H$_a$), 3.85 (9H$_b$), and 4.20 (9′H).

Plant materials

E. ulmoides plants were obtained from Kagawa University farm, Miki-cho, Kagawa, Japan.

Enzyme preparation

Soluble enzyme

Soluble enzyme was prepared by the method of Katayama and Kado (1998). All manipulations were carried out at 4 °C unless otherwise stated. Defoliated young shoot of *E. ulmoides* (17-32 cm height, 19.06 g) were washed by tap and distilled water, wiped, sectioned (1-2 mm), frozen in liquid nitrogen, and reduced to a powder by means of a mortar and pestle. The resulting powder was homogenized with PVPP (4 g), acid washed sea sand (20 g), and K-Pi buffer containing 10 mM DTT (48 ml). The homogenate was applied onto a PD-10 column (Pharmacia, Sephadex G-25 M) equilibrated with K-Pi buffer (25 ml). The excluded fraction (3.5 ml) was collected and used as crude enzyme.

Insoluble enzyme

Insoluble enzyme was prepared by the method of Davin and Lewis (2005). All enzyme preparations were carried out at 4 °C unless otherwise stated. Defoliated young shoots of *E. ulmoides* (20-30 cm long, 15.8 g) were washed with tap and distilled water, sectioned (1-2 mm), frozen in liquid nitrogen, and reduced to a powder by means of a mortar and pestle. The powder was transferred to a 1 L beaker containing K-Pi buffer (50 mM, pH 7, 158 ml) and triton X-100 (1%). After 4 h stirring, the homogenate was filtered through one layer of nylon cloth, and the insoluble residue was ringed with cold distilled water (475 ml) and squeezed to remove excess fluid. The insoluble residue was then extracted with 0.5 M NaCl (158 ml) for 16 h, filtered through one layer of nylon cloth, ringed with 632 ml cold distilled water, and squeezed as before. The moist residue was reground with a mortar and pestle to afford an insoluble stem residue (11.8 g). This insoluble enzyme preparation (free of soluble and ionically bound enzymes) was assayed immediately.

Enzyme assay

Soluble enzyme

Each assay mixture consisted of soluble enzyme (200 ml) and the mixed solution of 30 mM coniferyl alcohol (previously dissolved in K-Pi buffer 0.1 M, pH 7, 20 ml). The enzymatic reaction was initiated by the addition of H_2O_2 (10 mM, 10 ml): assays were conducted in ten subsequent series. Following normal incubation at 30 °C for 60 min, glacial AcOH (20 ml) was added. The assay mixture was then extracted with EtOAc (500 ml). The aqueous layer was further extracted twice with EtOAc (500 ml). The EtOAc solutions were combined, washed twice with saturated brine, dried over anhydrous Na_2SO_4, and evaporated under reduced pressure. Subsequently, control #1 (without H_2O_2) and control #2 (cell-free extract boiled at 90 ° C for 10 min.) were also carried out by the same reaction procedure. The resulting EtOAc extract was then reconstituted in MeOH (100 ml) and filtered. An aliquot (10 ml) of the filtrate was applied to C_{18} column reversed-phase HPLC analysis with gradient elution; fraction containing GGCE, DHCA and pinoresinol were collected, evaporated, and reconstituted in MeOH with aliquots subjected to Chiralcel OD for chiral column HPLC resolution.

Insoluble enzyme

Each assay consisted of insoluble cell wall residue (100 mg) suspended in K-Pi buffer (50 mM, pH 7, 342 ml). The enzymatic reaction was initiated by the addition of the solution of 30 mM coniferyl alcohol (previously dissolved in K-Pi buffer 50 mM, pH 7, 20 ml), assays were repeated three times. Following normal incubation at 30 °C for 3 h, glacial AcOH (20 ml) was added. The assay mixture was then extracted with EtOAc (500 ml). The aqueous layer was further extracted twice with EtOAc

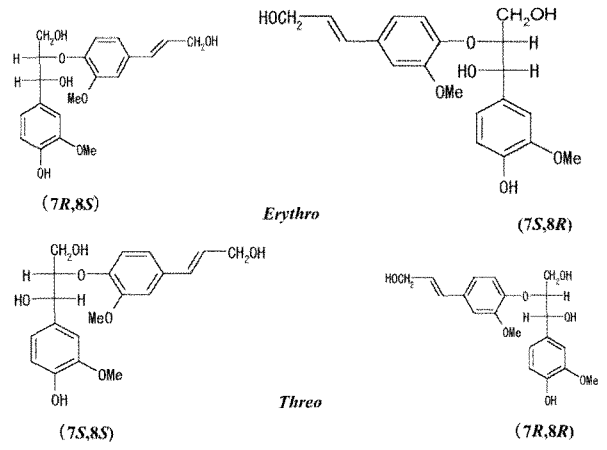

Fig. 2. Four stereo isomers of guaiacylglycerol-8-*O*-4′-coniferyl ether (GGCE)

(500 ml). The EtOAc solutions were combined, washed twice with saturated brine, dried over anhydrous Na_2SO_4, and evaporated under reduced pressure. Subsequently, control #1 (insoluble residue was boiled at 90 °C for 10 min.) was also carried out by the same reaction procedure. The resulting EtOAc extract was then reconstituted in MeOH (100 ml) and filtered. An aliquot (10 ml) of the filtrate was applied to C_{18} column reversed-phase HPLC analysis with gradient elution; fraction containing GGCE, DHCA and Pinoresinol were collected, evaporated and reconstituted in MeOH with aliquots subjected to Chiralcel OD for chiral column HPLC resolution. Chiral analysis was performed on a Daicel Chiracel OD column (250 × 46 mm) eluted with EtOH:n-hexane = 15:85 (v/v) at a flow rate of 1.0 ml/min (for *erythro*-GGCE) and 0.8 ml/min (for *threo*-GGCE) to determine the enantiomeric composition of isolated lignans.

Results and Discussion

Biosynthesis of GGCE

Enzymatic formation of GGCE catalyzed by a soluble enzyme preparation with coniferyl alcohol:

Incubation of soluble enzyme preparation from defoliated young shoots of *E. ulmoides* with coniferyl alcohol in the presence of hydrogen peroxide at 30 °C for 60 min gave GGCE in preference to DHCA and Pinoresinol. Fig. 3 shows the identical peak area with authentic sample. When soluble enzyme preparation denatured by heating in boiling water for 10 min was incubated with the substrate for 60 min or at the same time the cell-free extract was incubated with the substrate without hydrogen peroxide, no significant formation was observed. Compounds were separated as well as identified by reversed-phase HPLC at a flow rate of 1.0 ml/min using the following linear gradient solvent system: 30% MeOH-3% AcOH in H2O (v/v), and preparative TLC with different solvent system; [benzene:acetone = 2:1 (×3)], [EtOH: n-hexane = 3:1 (×1)], [4% MeOH in CH_2Cl_2 (×2)] for GGCE with the authentic samples obtained by dehydrogenation of CA with horseradish peroxidase and H_2O_2.

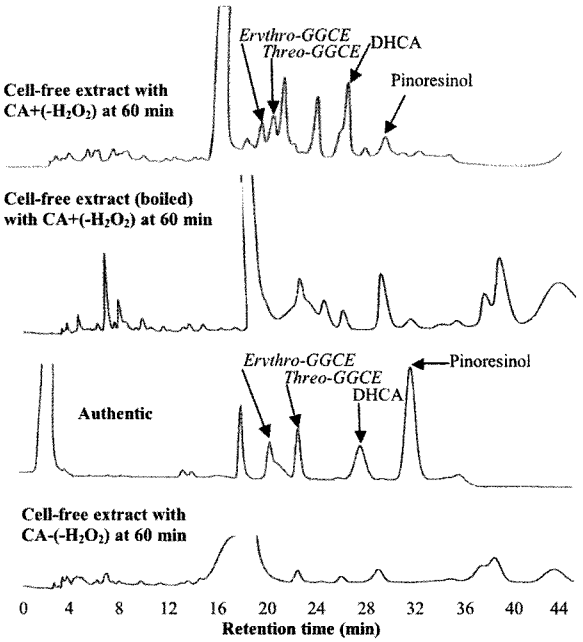

Fig. 3. Soluble enzyme catalyzed guaiacylglycerol-8-*O*-4'-(coniferyl alcohol) ether (GGCE), DHCA and pinoresinol were identified by reversed phase HPLC

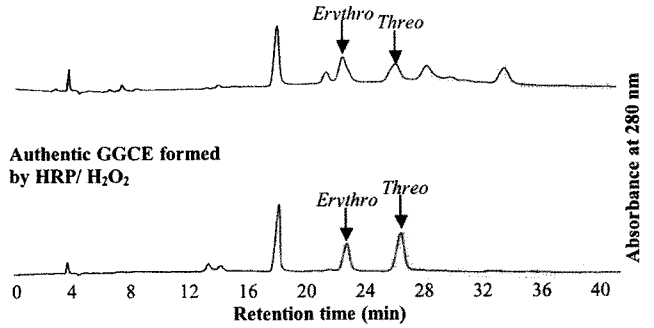

Fig. 4. Reversed phase HPLC of GGCE formed by incubation of coniferyl alcohol with cell free extracts of *E. ulmoides* in the presence of hydrogen peroxide

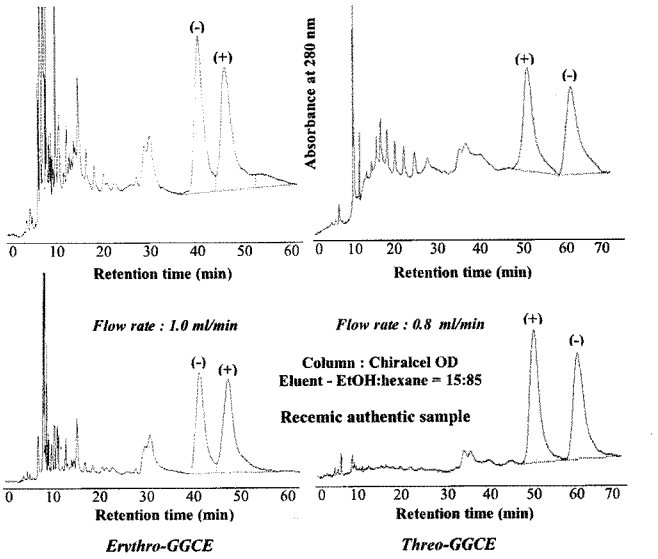

Fig. 5. Chiral HPLC analysis of *erythro*- and threo-GGCE formed following incubation of coniferyl alcohol with cell-free extracts from *E. ulmoides* in the presence of hydrogen peroxide

Fig. 4 shows the diastereomeric composition of *erythro*-and *threo*-GGCEs with the authentic *erythro*-and *threo*-GGCEs. Both peak area corresponding to authentic compound.

Soluble enzyme preparation enantioselectively produced (-)-*erythro*, (+)-*erythro* and (+)-*threo*, (-)-*threo*-GGCEs respectively (Fig. 5). Lourith et al. (2005) found that chiral HPLC analysis of *erythro*- and *threo*-GGCE formed following incubation of coniferyl alcohol with cell-free extracts from *E. ulmoides* in the presence of hydrogen peroxide. This result supports current

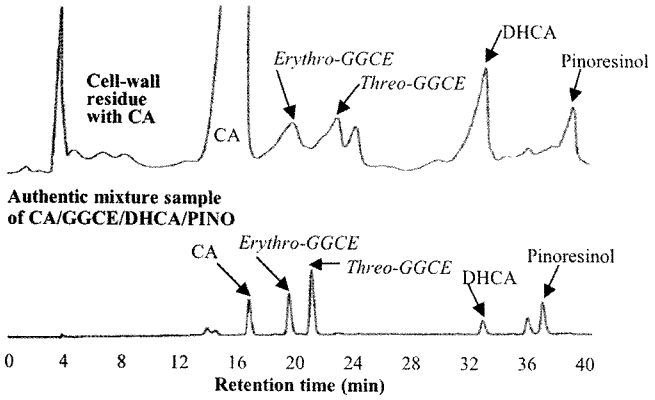

Fig. 6. Insoluble enzyme catalyzed guaiacylglycerol-8-*O*-4'-(coniferyl alcohol) ether (GGCE), DHCA and Pinoresinol were identified by reverse phase HPLC

findings which were obtained by without radio labeled precursor of radical species of coniferyl alcohol.

Formation of GGCE catalyzed by an insoluble enzyme preparation

Cell wall residue of *E. ulmoides* incubated with coniferyl alcohol at 30 °C for 180 min also generate GGCE, DHCA, and pinoresinol respectively. Fig. 6 shows the identical peak area with authentic sample whereas; no significant formation was observed when insoluble enzyme preparation denatured by heating in boiling water for 10 min incubated with the substrate for the same time. Compounds were separated as well as identified by (C_{18} column) reversed-phase HPLC at a flow rate of 1.0 ml/min using the following linear gradient solvent system: 35% MeOH-3% AcOH in H_2O (v/v) and preparative TLC with different solvent system; [benzene: acetone = 2:1 (×3)] , [EtOH: n-Hexane = 3:1 (×1)], [4% MeOH in CH_2Cl_2 (×2)] for GGCE with the authentic samples obtained by dehydrogenation of CA

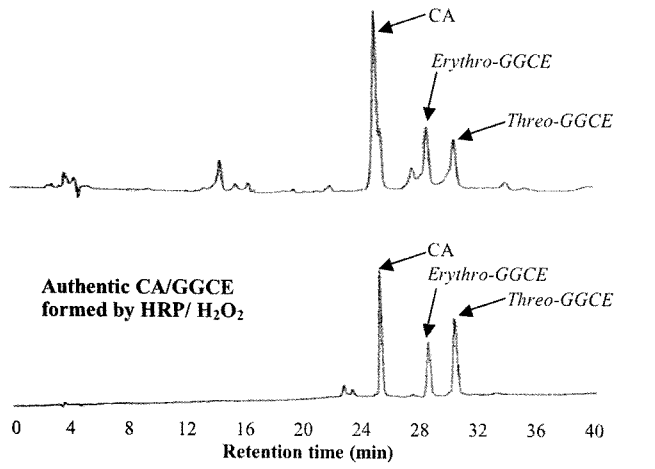

Fig. 7. Reversed phase HPLC of GGCE formed by incubation of coniferyl alcohol with cell-wall residue of *E. ulmoides*

with horseradish peroxidase and H_2O_2. Radical species of coniferyl alcohol was easily generated with higher proportion than sinapyl alcohol radical due to the higher oxidation potential of coniferyl alcohol and the coupling of radical species with the similar oxidation potentials was more favored (Katayama and Sogo 1989). This result demonstrated that the couplings with the same oxidation potentials species were easily occurred to confer GGCE.

Fig. 7 shows the diastereomeric composition of *erythro*-and *threo*-GGCEs with the authentic *erythro*-and *threo*-GGCEs. Both peak areas corresponding to authentic compounds. Insoluble enzyme preparation enantioselectively formed (-)-*erythro*, (+)-*erythro* and (+)-*threo*, (-)-*threo*-GGCEs, respectively with the authentic compound (data not shown).

Guaiacylglycerol-8-*O*-4'-(coniferyl alcohol) ether (GGCE), contains two guaiacyl rings (Fig. 2) with two methoxy groups (Katayama et al, 2004). On the other hand, radical species of coniferyl alcohol (Fig. 1.) bears one guaiacyl ring with one methoxy group. It was found that both enzyme preparations formed *erythro* isomer composition of GGCE with the *threo* isomer composition of GGCE. It can be assumed that both isomers were formed by the coupling with the same oxidation potential species (coniferyl alcohol). So, this study clarifies that two moles of radical species give one mole of neolignan.

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