Subunit composition of hinokiresinol synthase controls enantiomeric selectivity in hinokiresinol formation

Masaomi Yamamura,[a] Shiro Suzuki,[b] Takefumi Hattori[a] and Toshiaki Umezawa*[a,b]

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Asparagus officinalis hinokiresinol synthase (HRS) is composed of two subunits, HRSα and HRSβ. Individually, each subunit forms (*E*)-hinokiresinol (EHR) from 4-coumaryl 4-coumarate, whereas a mixture of both subunits forms (*Z*)-hinokiresinol (ZHR) from the same substrate. In this study, we analyzed the enantiomeric compositions of ZHR and EHR formed after incubation of 4-coumaryl 4-coumarate with recombinant subunit proteins, recHRSα and/or recHRSβ, and with naturally occurring *A. officinalis* ZHR. The enantiomeric composition of ZHR formed by the mixture of recHRSα and recHRSβ was (+)-100% enantiomer excess (e.e.), identical to that of *A. officinalis* ZHR. In contrast, the enantiomeric compositions of EHR formed by recHRSα and recHRSβ, individually, were (–)-20.6 and (–)-9.0% e.e., respectively. These results clearly demonstrate that the subunit composition of *A. officinalis* HRS controls not only *cis*/*trans* isomerism but also enantioselectivity in hinokiresinol formation.

Introduction

Norlignans are found in many coniferous trees (especially in heartwood) and some monocotyledonous plants.[1,2] Norlignans have a diphenylpentane carbon structure and have various biological activities. For example, both (*Z*)- and (*E*)-hinokiresinols, ZHR and EHR, which are phytoalexins of *Asparagus officinalis* and *Cryptomeria japonica*, respectively, have various additional bioactivities; ZHR has angiogenic inhibitor activity,[3] estrogen-like activity,[4] antioxidant activity and antiatherogenic activity,[5] whereas EHR has antifungal activity[6] and estrogen-like activity,[4] and inhibits cyclic AMP phosphodiesterase.[7]

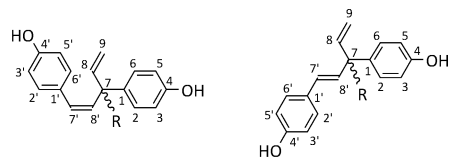

Fig. 1 The structure of hinokiresinols.

Hinokiresinol has the simplest norlignan structure (Fig. 1), suggesting that this is the first compound in the norlignan biosynthetic pathway. Enzymatic conversion of 4-coumaryl 4-coumarate into ZHR and EHR was detected using *A. officinalis* and *C. japonica* cell-suspension cultures, respectively[8,9] (Fig. 2). Recently, Suzuki *et al.* isolated cDNAs encoding hinokiresinol synthase (HRS) from *A. officinalis* cell-suspension cultures.[10] The *A. officinalis* HRS was composed of two subunits, HRSα and HRSβ. The mixture of both recombinant subunit proteins (recHRSα and recHRSβ) catalyzed the formation of ZHR from 4-coumaryl 4-coumarate, whereas incubation of the same substrate with only one type of subunit (recHRSα or recHRSβ) gave rise to EHR (Fig. 3). This result indicates that the subunit composition of *A. officinalis* HRS can control *cis*/*trans* isomerism in hinokiresinol formation. In addition to *cis*/*trans* isomerism, hinokiresinols exhibit enantioisomerism due to the C7 asymmetric carbon atom. ZHR from the rhizome of *Anemarrhena asphodeloides* showed (–)-54% enantiomer excess (e.e.),[4] while EHR from the heartwood of *Chamaecyparis obtusa* was suggested to be an optically pure (–)-isomer by calculating the amplitude of the circular dichroism (CD) spectrum of tetrahydrohinokiresinol (THR) derived from *C. obtusa* EHR.[4] These results strongly suggest that hinokiresinol-producing plants have distinct mechanisms for controlling enantiomeric selectivity in hinokiresinol formation, as well as controlling *cis*/*trans* isomerism.

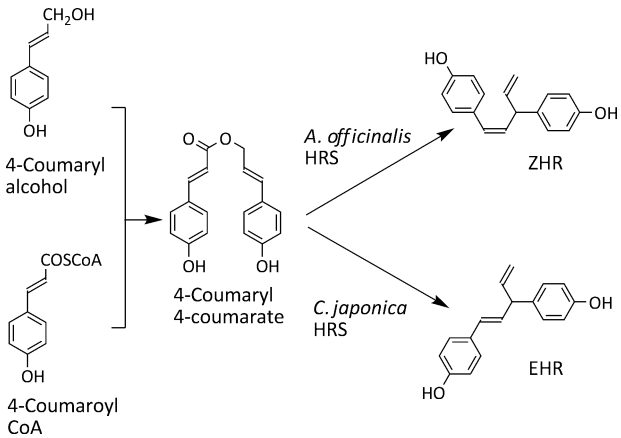

Fig. 2 Biosynthetic pathway of hinokiresinols.

In this study, we show that the subunit composition of *A. officinalis* HRS controls enantioselectivity and *cis*/*trans* isomerism in hinokiresinol formation.

[a]*Research Institute for Sustainable Humanosphere, Kyoto University, Uji, Kyoto 611-0011, Japan*
[b]*Institute of Sustainability Science, Kyoto University, Uji, Kyoto, 611-0011, Japan*

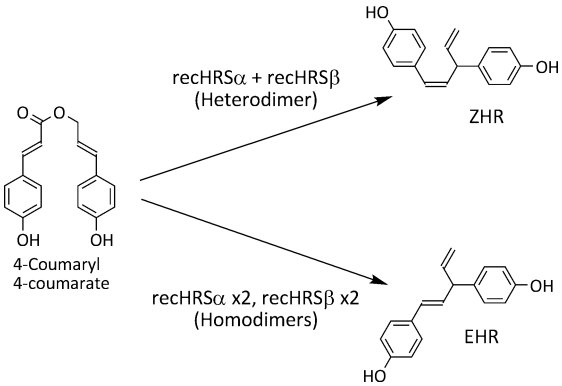

Fig. 3 HRS subunit compositions control *cis*/*trans* selectivity in hinokiresinol formation.

Results

Enantiomeric relationships between (+)- and (−)-hinokiresinols and (+)- and (−)-tetrahydrohinokiresinols

Initially, we synthesized racemic (±)-THRs by catalytic hydrogenation of racemic (±)-EHRs, which were prepared as described by Lassen *et al.*[11] The obtained enantiomers of (±)-THRs were separated on a chiral column. The first peak on the chiral high-performance liquid chromatography (HPLC) chromatogram was the (−)-enantiomer {retention time (t_R) = 10.8 min} and the second peak (t_R = 11.2 min) was the (+)-enantiomer (Fig. 4*A*).

The absolute configuration at C7 in (−)-EHR isolated from heartwood of *C. obtusa* has been assigned as *S* based on its optical rotatory dispersion[12] and the enantioselective synthesis of its di-*O*-methylether.[13] In addition, C7 in (+)-ZHR has an *S* configuration, as determined by CD spectroscopy[4] and vibration CD spectroscopy.[11] On the basis of this information on hinokiresinol enantiomers, we determined the enantiomeric composition of THR derived from *C. obtusa* EHR by chiral HPLC with a UV detector. By hydrogenation, (−)-(7*S*)-EHR isolated from *C. obtusa* heartwood gave optically pure (+)-(7*S*)-THR (Fig. 4*H* and Table 1G). In addition, it was reported that (−)-(7*R*)-ZHR gave (−)-(7*R*)-THR.[4] Together, the stereochemical correlation between (*E*)- and (*Z*)-hinokiresinols and their hydrogenation product, THR, were established as shown in Fig. 5.

Enantiomeric compositions of enzymatically formed hinokiresinols and those isolated from plants

Having established the stereochemical correlation between the hinokiresinols, EHR and ZHR, and their tetrahydro derivative, THR, we next determined enantiomeric compositions of ZHR

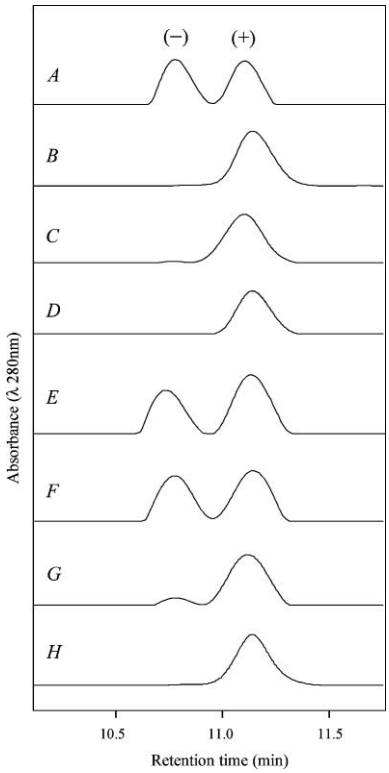

Fig. 4 The chiral HPLC chromatograms of tetrahydrohinokiresinols (THRs). *A*, (±)-THRs derived from (±)-EHRs. *B*, THR derived from ZHR, which was isolated from *A. officinalis* cells. *C*, THR derived from ZHR, which was formed by the enzymatic reaction using *A. officinalis* enzyme preparation. *D*, THR derived from ZHR, which was formed by recHRSα+recHRSβ. *E*, THR derived from EHR, which was formed by recHRSα. *F*, THR derived from EHR, which was formed by recHRSβ. *G*, THR derived from EHR, which was isolated from *C. japonica* cells. *H*, THR derived from EHR, which was isolated from the heartwood of *C. obtusa.*

isolated from *A. officinalis* and ZHR formed by the mixture of recombinant subunit proteins, recHRSα+recHRSβ. THR derived from ZHR, which was isolated from *A. officinalis*, was an optically pure (+)-isomer (Fig. 4*B* and Table 1A), indicating that *A. officinalis* produces enantiomerically pure (7*S*)-ZHR. Similarly, THR derived from ZHR, which was formed by the *A. officinalis* enzyme preparation, was (+)-97.2% e.e. (Fig. 4*C* and Table 1B). THR derived from ZHR, which was formed by the mixture of recHRSα and recHRSβ, was also an optically pure (+)-isomer (Fig. 4*D* and Table 1C). In contrast, the enantiomeric composition of THR derived from EHR, which was formed by the action of

Table 1 Enantiomeric composition of hinokiresinols

		Hinokiresinol	THR	
Entry	Origin	*Z*/*E*	Predominant enantiomer	Enantiomeric composition (% e.e.)
A	*A. officinalis* cells (isolated)	*Z*	(+)-*S*	100
B	*A. officinalis* cells (enzyme)	*Z*	(+)-*S*	97.2
C	recHRSα + recHRSβ	*Z*	(+)-*S*	100
D	recHRSα	*E*	(+)-*S*	20.6
E	recHRSβ	*E*	(+)-*S*	9.0
F	*C. japonica* cells (isolated)	*E*	(+)-*S*	83.3
G	*C. obtusa* heartwood (isolated)	*E*	(+)-*S*	100

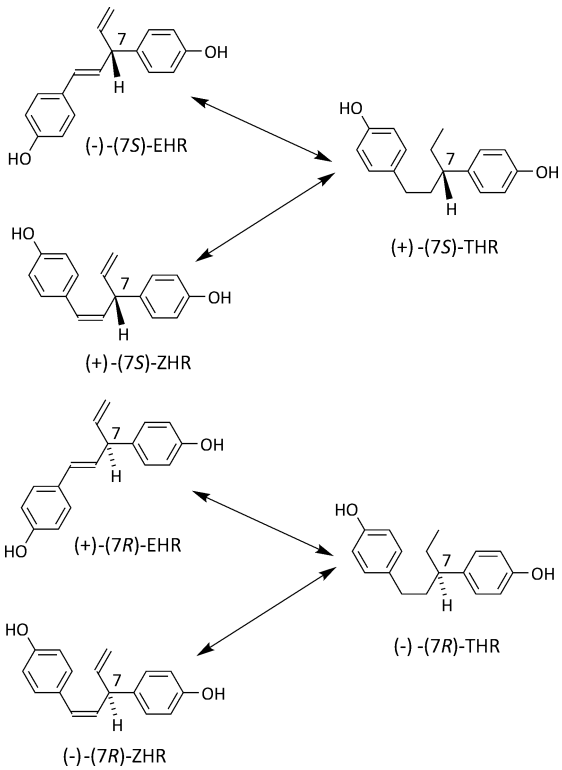

Fig. 5 An enantiomeric correlation between (*E*)- and (*Z*)-hinokiresinols and tetrahydrohinokiresinol.

recHRSα, was (+)-20.6% e.e. (Fig. 4*E* and Table 1D). Similarly, that of THR derived from EHR, which was formed by the action of recHRSβ, was (+)-9.0% e.e. (Fig. 4*F* and Table 1E). The enantiomeric composition of THR derived from *C. japonica* EHR was (+)-83.3% e.e. (Fig. 4*G* and Table 1F).

We further confirmed the enantiomeric compositions of THRs (Fig. 4*C*, *E*, *F*, and *G*) except for optically pure THRs (Fig. 4*B*, *D*, and *H*). This was performed by collecting approximately half of each peak followed by quantification using GC-MS, so that the contamination by the antipodes was eliminated. Based on the quantification, enantiomeric compositions of THRs were estimated as follows: THR derived from ZHR formed by *A. officinalis* enzyme preparation, (+)-95% e.e.; THR derived from EHR formed by recHRSα, (+)-26% e.e.; THR derived from EHR formed by recHRSβ, (+)-13% e.e.; THR derived from EHR isolated from *C. japonica* cultured cells, (+)-80% e.e. Although these enantiomeric compositions are based on the quantification of approximate half of each peak, the % e.e. values are in accordance with those determined by chiral HPLC with the UV detector (Table 1). This confirms that the minor peaks of (−)-enantiomer of THR shown in Fig. 4*C* and *G* are not due to impurities. Thus, each HRS subunit forms both (7*S*)- and (7*R*)-EHRs with the former as a predominant enantiomer, which contrast sharply with the production of optically pure (7*S*)-ZHR with the mixture of recHRSα and recHRSβ.

Discussion

We have previously demonstrated that *A. officinalis* HRS is composed of two subunits (HRSα and HRSβ) and that the subunit composition can control the *cis*/*trans* isomerism of the product.[10] Here, we show that the subunit composition determines the enantiomeric composition of hinokiresinols. Incubation of 4-coumaryl 4-coumarate with the mixture of equal amounts of recHRSα and recHRSβ resulted in an optically pure (7*S*)-isomer of ZHR (Fig. 4*D*, Fig. 6 and Table 1C). This isomer is identical to that isolated from *A. officinalis* cells (Fig. 4*B* and Table 1A). A similar result was obtained with the plant protein from *A. officinalis*; ZHR formed from the same substrate with the plant protein was 97.2% e.e., that is, almost optically pure (Fig. 4*C* and Table 1B). Conversely, when each subunit protein, recHRSα or recHRSβ, was individually incubated with the same substrate, EHR was the sole product.[10] Interestingly, the enantiomeric compositions of EHR thus formed were 20.6 and 9.0% e.e. in favor of the (7*S*)-enantiomer (Fig. 4*E*, *F*, Fig. 6 and Table 1D, E). Taken together, these results clearly indicate that the subunit composition of HRS controls not only *cis*/*trans* selectivity but also enantioselectivity in hinokiresinol formation (Fig. 4 and Fig. 6).

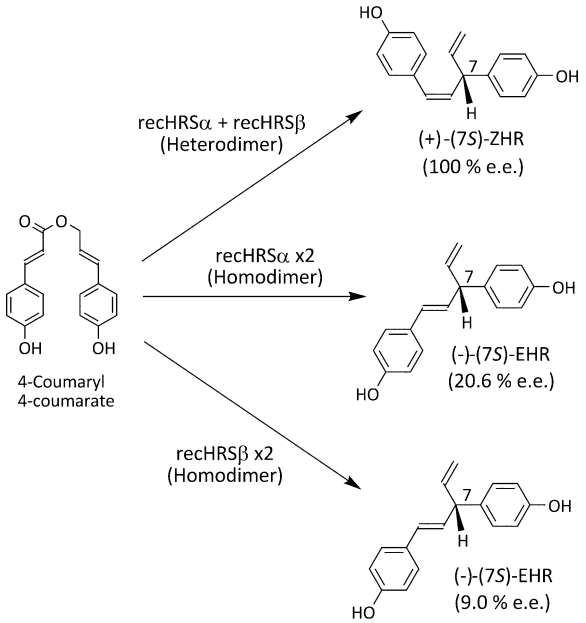

Fig. 6 HRS subunit compositions control enantioselectivity in hinokiresinol formation.

The optically pure ZHR was isolated from *A. officinalis* cultured cells. However, the enantiomeric composition of ZHR isolated from the rhizome of *A. asphodeloides* was not optically pure; (7*R*)-54% e.e. Similarly, EHR isolated from *C. obtusa* heartwood was the optically pure (7*S*)-isomer, whereas EHR obtained from *C. japonica* cultured cells was not optically pure; (7*S*)-83.3% e.e. (Fig. 4*G* and Table 1F). These results indicate that the enantiomeric compositions of hinokiresinols vary among plant species. This may result from the different subunit compositions of HRS of each plant species.

Together, our results show a novel example of enantiomeric control in the biosynthesis of natural products. This differs from the enantioselectivity in the biosynthesis of lignans, which represent another class of phenylpropanoid compounds closely related to norlignan in terms of structure and biosynthesis.

Lignans with 9(9′)-oxygen also show a diversity of enantiomeric compositions.[2,14,15] First, dibenzylbutyrolactone lignans are optically pure, while furofuran and furan lignans are mixtures of both enantiomers and exhibit various enantiomeric compositions. Second, dibenzylbutyrolactone lignans are levorotatory. Third, predominant enantiomers of furofuran, furan, and dibenzylbutane lignans vary among plant species. Fourth, the absolute configurations of the predominant enantiomers of various lignans isolated from a single plant species sometimes differ.[14,15] The biosynthesis of lignans with 9(9′)-oxygen is initiated by the enantioselective dimerization of two coniferyl alcohol units with the aid of a unique and effective asymmetric inducer, dirigent protein (DP), to yield optically active pinoresinol (furofuran).[16–21] However, the enantiomeric composition of pinoresinol varies widely among plant species,[2,14,15] suggesting that enantiomeric control by DP is not strong enough to yield optically pure pinoresinol *in planta*. Additionally, the enantiomeric compositions of lignans are determined by the first step mediated by DP, and also subsequent metabolic steps mediated by pinoresinol/lariciresinol reductase and secoisolariciresinol dehydrogenase.[14] Recently, Nakatsubo *et al.* demonstrated that pinoresinol reductases (PrR, a variation of PLR in *Arabidopsis thaliana*) together with a dirigent protein(s) are involved in enantiomeric control in lignan biosynthesis in *A. thaliana*.[22] This study demonstrated conclusively that differential expression of PrR isoforms with distinct selectivities for substrate enantiomers can determine enantiomeric composition of the product, lariciresinol.[22]

The basic carbon skeleton of hinokiresinols consists of phenylpropane (C_6C_3) and phenylethane (C_6C_2) units that are linked *via* a C7–C8′ bond. This bond can not be formed *via* the coupling of two *p*-coumaryl-alcohol-derived phenoxy radicals as in the case of enantioselective lignan formation, which inevitably leads to C8–C8′ bonds. Hence, an enantioselective mechanism, which is totally different from that in lignan biosynthesis, was expected for norlignan biosynthesis. In this study, we show that this mechanism relies on appropriate subunit composition of HRS. That is, combining the subunits of HRS in an appropriate manner can form ZHR with an identical enantiomeric composition to that of the ZHR from *A. officinalis* (Fig. 4, Fig. 6 and Table 1).

The control of enantioselectivity is very important from a pharmaceutical standpoint. It is well known that each enantiomer has different biochemical properties. Thus, the selective synthesis of only one enantiomer is a challenging subject in the production of chiral pharmaceuticals. In this context, the results presented herein could provide a new approach in enantioselective organic synthesis. Further research should be carried out to elucidate the mechanisms that control *cis*/*trans* and enantiomeric selectivities by HRSs. X-ray crystallographic analysis of *A. officinalis* HRS may play a critical role in such research.

Experimental

Plant materials

A cell-suspension culture of *A. officinalis* cv. Akuseru was initiated and elicited with autoclaved *Fusarium solani* IFO4542 mycelia for 18 h as described previously.[8] The cells were collected using a strainer and washed with distilled water, and the excess water was blotted with paper towel. The cells were weighed and stored in liquid nitrogen until use. A cell-suspension culture of *C. japonica* cv. Kumotooshi was initiated and maintained as described by Suzuki *et al.*[9] The cells were collected and stored in liquid nitrogen until use.

Instrumentation

Nuclear magnetic resonance (NMR) spectra were obtained with a JNM-LA400MK FT-NMR system (JEOL Co., Ltd.) Chemical shifts and coupling constants (J) are expressed in δ and Hz, respectively.

GC-MS was carried out with a Shimadzu QP-5050A GC-MS system (Shimadzu Co., Ltd.) [electron impact mode (70 eV); column, Shimadzu Hicap CBP10-M25-025 column (Shimadzu Co., Ltd.; 20 m × 0.22 mm); carrier gas, helium; injection temperature, 240 °C; column temperature (40 °C at t = 0 to 2 min, then to 230 °C at 25 °C min^{-1})]. The samples for GC-MS were dissolved in *N*,*O*-bis(trimethylsilyl)acetamide (BSA) and left standing at 60 °C for 45 min; then an aliquot of the BSA solution was subjected to GC-MS analysis.

Reversed-phase HPLC was performed with a Shimadzu LC-6A liquid chromatograph (Shimadzu Co., Ltd.), detection being at λ = 280 nm. The reversed-phase columns used were a Waters Novapak C_{18} (Waters Co., Ltd.; 3.9 mm × 150 mm, 4 μm) and a YMC-Pack Pro C_{18} RS column (YMC Co., Ltd.; 4.6 cm × 150 mm, 3 μm). The mobile phase used for both columns was with CH_3CN–H_2O (40 : 60) at a flow-rate of 1 ml min^{-1}.

Chiral HPLC was performed with an HPLC system comprising a Waters 600E system controller (Waters Co., Ltd.), a Waters 60F fluid pump (Waters Co., Ltd.), and a Waters 2487 dual λ absorbance detector (Waters Co., Ltd.), detection being at λ = 280 nm. The chiral column used was a Chiralpak AD column (Daicel Chemical Co., Ltd.; 4.6 mm × 150 mm, 3 μm) and the mobile phase was EtOH at a flow-rate of 0.5 ml min^{-1}. The sign for optical rotation of THR enantiomers was determined using a JASCO OR-990 chiral detector (JASCO Co., Ltd.). Silica gel column chromatography and silica gel thin-layer chromatography (TLC) employed Kieselgel 60 (Merck Co., Ltd.; 70–230 mesh) and Kieselgel 60 F_{254} (Merck Co., Ltd.; 20 × 20 cm, 0.5 or 0.25 mm), respectively.

Chemicals

4-Coumaryl 4-coumarate was synthesized as described previously.[8] (±)-THRs were prepared as follows. (±)-EHRs, which were synthesized exactly by the method of Lassen *et al.*,[11] were dissolved in methanol and hydrogenated under hydrogen with 5% palladium on carbon as a catalyst for 1.5 h at room temperature. The mixture was filtered and concentrated to dryness under reduced pressure. (±)-THRs: *m*/*z* [EI, THR TMS ether] 400 (M^+, 19%), 385 (2), 207 (63), 179 (100); ^{1}H-NMR (400 MHz; $CDCl_3$) δ: 0.73 (3H, t, J = 7.3), 1.49 (1H, m), 1.64 (1H, m), 1.78 (1H, m), 1.89 (1H, m), 2.36 (3H, m), 6.71 (2H, d, J = 8.4), 6.77 (2H, d, J = 8.6), 6.95 (2H, d, J = 8.4), 7.01 (2H, d, J = 8.4). The ^{1}H-NMR spectrum was consistent with published data.[4,23,24] All other commercial reagents were obtained from Nacalaitesque Co., Ltd. or Wako Pure Chemicals Co., Ltd., unless otherwise noted.

Isolation of ZHR from *A. officinalis*

We isolated ZHR from *A. officinalis* cells as described previously.[8,25] Freeze-dried *A. officinalis* cells were extracted with hot methanol. After β-glucosidase treatment, the extracts were purified with silica gel TLC and reversed-phase HPLC using a Waters Novapak C_{18} column, giving rise to ZHR: *m/z* [EI, ZHR TMS ether] 396 (M^+, 100%), 381 (27), 230 (91), 217 (47) and 179 (39).

Isolation of EHR from *C. japonica*

Freeze-dried *C. japonica* cells were extracted with hot methanol and then treated with β-glucosidase. The extracts were purified using silica gel TLC and reversed-phase HPLC using a Waters Novapak C_{18} column, yielding EHR: *m/z* [EI, EHR TMS ether] 396 (M^+, 100%), 381 (25), 230 (89), 217 (36) and 179 (31).

Preparation of ZHR with the *A. officinalis* enzyme

Enzyme preparation; a crude enzyme preparation from *A. officinalis* cells[8] was incubated with 4-coumaryl 4-coumarate (final conc. 0.6 mM) as a substrate at 30 °C for 3 h as described previously.[10] The reaction mixture was then extracted with three times EtOAc, the organic layer was then washed with saturated aqueous NaCl, dried over Na_2SO_4, and concentrated *in vacuo*. ZHR was isolated and purified by TLC and reversed-phase HPLC using a Waters Novapak C_{18} column.

Preparation of EHR and ZHR with recombinant HRS proteins

recHRSα and recHRSβ were prepared and incubated with 4-coumaryl 4-coumarate as described previously.[10]

Thus, the mixture containing equal amounts of recHRSα and recHRSβ (recHRSα : recHRSβ = 1 : 1) was incubated with 4-coumaryl 4-coumarate (final conc. 2 μM) at 30 °C for 1 h. The reaction mixture was extracted three times with EtOAc. The organic layer was washed with saturated aqueous NaCl, dried over Na_2SO_4, and concentrated *in vacuo*. This enzyme reaction was repeated five times and the reaction products were combined to obtain sufficient quantities of ZHR for enantiomeric analysis. ZHR thus obtained was purified by reversed-phase HPLC using a YMC-Pack Pro C_{18} RS column.

recHRSα was incubated with the same substrate at 30 °C for 1 h. The reaction mixture was extracted three times with EtOAc. The organic layer was washed with saturated aqueous NaCl, dried over Na_2SO_4, and concentrated *in vacuo*. The reaction was repeated twice, and the products were combined and purified to afford EHR. Incubation of the same substrate with recHRSβ giving rise to EHR was carried out exactly as described above.

Determination of enantiomer excess of hinokiresinols

Isolated and enzymatically prepared hinokiresinols were individually hydrogenated to THR. Each of the obtained THRs was subjected to the chiral HPLC analysis, and the enantiomeric composition of THR was determined by calculating the peak area of each enantiomer recorded by the UV detector. In addition, the enantiomeric compositions of THRs were confirmed by GC-MS analysis. To avoid contamination by antipodes, the former half of the peak of (–)-enantiomer (t_R = 10.6–10.8 min: from the start to the top of the first peak) and the letter half of the peak of (+)-enantiomer (t_R = 11.2–11.4 min: from the top to the end of the second peak) were recovered. The solvent was evaporated off. Each enantiomer thus obtained was mixed with 0.1 μg of acetosyringone, and subjected to GC-MS measurement. Enantiomeric compositions of THRs were estimated by using the amounts of each enantiomer quantified by comparing chromatographic peak areas of molecular ions between THR and acetosyringone.

Conclusion

We have demonstrated that the combination of *A. officinalis* HRS subunits controls enantioselectivity as well as *cis*/*trans* isomerism in hinokiresinol formation.

Acknowledgements

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