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台灣八角蓮 Pinoresinol lariciresinol reductase 與 Secoisolariciresinol dehydrogenase 之基因選殖與表現

Gene Cloning and Heterologous Expression of Pinoresinol Lariciresinol Reductase and Secoisolariciresinol Dehydrogenase Gene from *Podophyllum pleianthum* Hance

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本論文係盧佩君君（R95B47101）在國立臺灣大學微生物與生化學研究所完成之碩士學位論文，於民國九十七年六月十九日承下列考試委員審查通過及口試及格，特此證明

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中文摘要

鬼臼素 （Podophyllotoxin） 對癌細胞具強效毒殺作用，其化學衍生物已應用於臨床抗癌藥物之使用。本研究從台灣八角蓮 （*Podophyllum pleianthum* Hance）中選殖出鬼臼素生合成路徑中之 pinoresinol lariciresinol reductase 基因 PLR （將 pinoresinol 先轉化為 lariciresinol，再轉化為 secoisolariciresinol） 以及 secoisolariciresinol dehydrogenase 基因 SDH （可將 secoisolariciresinol 轉化為 matairesinol）。選殖 *sdh* 是以台灣八角蓮 RNA 為材料，經反轉錄-聚合酶連鎖反應 （RT-PCR） 得到 cDNA 序列，此序列與美洲八角蓮 *Podophyllum peltatum* 之 *sdh* cDNA 序列進行比對後，達到 98.1% 的一致性，其胺基酸序列的一致性亦達 98.2%。選定在 25 ^{o}C、0.01 mM isopropyl-β-D-1-thiogalactopyranoside （IPTG） 誘導 9 小時為重組蛋白 SDH 於大腸桿菌表現之最適條件。變性膠體電泳（Sodium dodecyl sulfate polyacrylamide gel electrophoresis, SDS-PAGE） 分析可以清楚看到與 SDH-His tag 蛋白質 （34 kDa） 分子量大小相同之重組蛋白之生成。重組蛋白轉化的結果以高效能液相層析 （High performance liquid chromatography, HPLC）系統進行分析，比較其反應產物 matairesinol 之滯留時間 (23.97 分鐘) 及其 UV 全波長吸收光譜，皆和標準品相吻合。故可確認台灣八角蓮 *sdh* 之 cDNA 序列已成功選殖，並可在大腸桿菌內進行具有酵素活性之表現。此外，由台灣八角蓮經由 cDNA 末端快速擴增技術（Rapid amplification of cDNA ends, RACE）PCR 與 RT-PCR 得到之 *plr* 序列，與美國金鐘連翹 （*Forsythia intermedia*） 之 *plr* cDNA 序列比對後，達到 68.9% 的一致性，其胺基酸序列的一致性亦達 75.2% 一致性與 85% 的相似度。西方墨點結果顯示目標蛋白質已表現於大腸桿菌，可以清楚看到與 PLR-His tag 蛋白質 （39 kDa） 分子量大小相同之重組蛋白之生成。

關鍵字：鬼臼素；台灣八角蓮；美洲八角蓮；大腸桿菌；美國金鐘連翹

Abstract

Podophyllotoxin possesses strong tumor-specific cytotoxicity, and its chemical derivatives have been employed in clinical cancer treatment. From *Podophyllum pleianthum* Hance, pinoresinol lariciresinol reductase (PLR) gene was cloned, which can convert pinoresinol to lariciresinol and consequently to secoisolariciresinol, and secoisolariciresinol dehydrogenase (SDH) gene, which can convert secoisolariciresinol to matairesinol. The total RNA of *Podophyllum pleianthum* Hance was used as the material for *sdh* cloning by using reverse transcriptase PCR (RT-PCR). The cDNA sequence of *sdh* which got from *Podophyllum pleianthum* Hance aligned with *sdh* cDNA sequence from *Podophyllum peltatum*. It reached to 98.1% identity. The alignment of amino acid sequence reached to 98.2%. The optimum conditions was used 0.01 mM IPTG inducting 9 hours at 25^{o}C for expression of SDH in *E. coli*. The result showed a 34 kDa of protein in SDS-PAGE, the same size for *sdh* with a SDH-Histag design. The conversion of recombinant enzyme reaction was further analyzed by HPLC, the retention time (23.97 min) and the UV absorption spectrum matched with the characters of authentic matairesinol. It indicated that the cDNA sequence of *sdh* was cloned from *Podophyllum pleianthum* Hance and expressed in *E.coli* functionally. Meanwhile, cDNA of *plr* got from *Podophyllum pleianthum* Hance by using rapid amplification of cDNA ends PCR (RACE PCR) and RT-PCR, was aligned with *plr*

cDNA sequence from *Forsythia intermedia*, and it reached to 68.9% identity. Alignment of amino acid sequence also reached to 75.2% identity and 85% similarity. Western blotting proved the expression of target protein PLR in *E. coli*. The results showed a 39 kDa of recombinant protein expressed in SDS-PAGE, the same size for *sdh* with a PLR-His tag design.

Keywords: Podophyllotoxin; *Podophyllum pleianthum* Hance; *Podophyllum peltatum*; *Escherichis coli*; *Forsythia intermedia*

Abbreviation

cDNA	complementary DNA
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic acid
HRP	horseradish peroxidase
HPLC	high performance liquid chromatography
IPTG	isopropyl-β-D-1-thiogalactopyranoside
kDa	kilodaltons
MWCO	molecular weight cut off
dNTP	deoxynucleotide
OD	optical density
PBS	phosphate buffer saline
PCR	polymerase chain reaction
PLR	pinoresinol lariciresinol reductase
PVDR	polyvinylidene difluoride
RT-PCR	reverse transcription-PCR
RACE PCR	rapid amplification of cDNA ends PCR
SDH	secoisolariciresinol dehydrogenase
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside

中英文專有名詞對照表

Escherichia coli	大腸桿菌
Electrophoresis	電泳
Forsythia intermedia	美國金鐘連翹
HPLC	高效能液相層析儀
Lignan	木酚素
PCR	聚合酶連鎖反應
Plasmid	質體
Podophyllum peltatum L.	美洲八角蓮
Podophyllum pleianthum Hance	台灣八角蓮
Primer	引子
Promoter	啟動子
Restriction enzyme	限制酶
Western blot	西方墨點法

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Chapter I

Introduction

1.1 Lignan

The lignans are group of structurally diverse chemical compounds with a broad range of medicinal/health protective roles in addition to important physiological functions *in planta.* They have been found in a large number of species belonging to more than 60 families of vascular plants and have been isolated from different plant parts: roots and rhizomes, woody parts, stems, leaves, fruits and seeds, and, in some other cases, from exudates and resins (Castro et al., 1996). Lignans are one of the major classes of phytoestrogens, which are estrogen-like chemicals and also act as antioxidants. Plant lignans are polyphenolic substances derived from phenylalanine via dimerization of substituted cinnamic alcohols, known as monolignols, to a dibenzylbutane skeleton (Fig. 2).

1.2 Podophyllotoxin

Podophyllotoxin occupies a unique position among the lignan natural products, such as strong antiviral agents and as antineoplastic drugs, since its glucopyranoside derivative was recognized as a potent antitumor factor (Cragg and Newman, 2005). This discovery entails a particularly fascinating account, involving a multitude of investigations conducted over a period of more than a century. It is the most abundant lignan isolated from Podophyllin, a resin produced by species of the genera

Podophyllum (Ayres and Loike, 1990; Imbert, 1998). *Podophyllum peltatum* L., *Podophyllum emodi* Wall. and *Podophyllum pleianthum* Hance (Fig. 3) (syn. *P. hexandrum* Royle), are not the only natural sources of podophyllotoxin. This and the related cyclolignans have been described in other genera such as *Jeffersonia*, *Diphylleia* and *Dysosma* (Berberidaceae), *Catharanthus* (Apocynaceae), *Polygala* (Polygalaceae), *Anthriscus* (Apiaceae), *Linun* (Linaceae), *Hyptis* (Verbenaceae), *Teucrium*, *Nepeta* and *Thymus* (Labiaceae), *Thuja*, *Juniperus*, *Callitris* and *Thujopsis* (Cupressaceae), *Cassia* (Fabaceae), *Haplophyllum* (Rutaceae), *Commiphora* (Burseraceae), and *Hernandia* (Hernandiaceae) (Miyata et al., 1998; Lim et al., 1999; Udino et al., 1999; Walton and Brown, 1999; Gordaliza et al., 2000; Petersen and Alfermann, 2001; Dekebo et al., 2002; Gu et al., 2002; Suzuki et al., 2002).

Extracts of *Podophyllum* species have been used by diverse cultures since remote times as antidotes against poisons, or as cathartic, purgative, antihelminthic, vesicant, and suicidal agents (Ayres and Loike, 1990). Podophyllin was included in the US Pharmacopoeia in 1820 and the use of this resin was prescribed for the treatment of venereal warts, attributing this action to podophyllotoxin. The destructive effect of this resin on experimental cancer cells in animals was also described. The antiviral activity of an aqueous extract of *Podophyllum peltatum* was investigated (Bedows and Hatfield, 1982). From this extract, podophyllotoxin was found to be the most active component in

inhibiting the replication of measles and herpes simplex type I virus (Hammonds et al., 1996; Sudo et al., 1998). In fact, podophyllotoxin is included in many Pharmacopoeias and used as an antiviral agent in the treatment of condyloma acuminatum caused by human papilloma virus (HPV) (Syed et al., 1995) and other venereal and perianal warts (Lassus, 1987; Ayres and Loike, 1990; Wantke et al., 1993; Beutner, 1996). The application of podophyllotoxin cured almost all the warts completely in less time than other strategies and with fewer side effects. Podophyllotoxin and analog compounds are also active against cytomegalovirus and Sindbis virus (MacRae et al., 1989). Podophyllotoxin is also effective in the treatment of anogenital warts in children and against molluscum contagiosum that is generally a self-limiting benign skin disease that affects mostly children, young adults and HIV patients (Markos, 2001). They either inhibit these viruses at an essential early step in the replication cycle after entry of the virus into the cells or reduce the capacity of infected cells to release viruses, a property also shown by other antimitotic agents such as colchicine or vincristine. There are several reports regarding the formulations of podophyllotoxin (Syed et al., 1994; Beutner, 1996; Claesson et al., 1996). Podophyllotoxin has other uses in dermatology: it is also a useful agent in psoriasis vulgaris (Truedsson et al., 1985; Schwartz and Norton, 2002).

1.3 Etoposide, Teniposide, Etopophos and new cytotoxic derivatives

Three semisynthetic derivatives of podophyllotoxin, etoposide, teniposide and etopophos (etoposide phosphate) (Fig. 4), are widely used as anticancer drugs and show good clinical effects against several types of neoplasms including testicular and small-cell lung cancers, lymphoma, leukaemia, Kaposi's sarcoma, etc (Ayres and Loike, 1990; Schacter, 1996). Among the plethora of physiological activities and potential medicinal and agricultural applications, the antineoplastic and antiviral properties of podophyllotoxin congeners and their derivatives are arguably the most eminent from a pharmacological perspective. Semisynthetic derivatives of epipodophyllotoxin, e.g. etoposide (Allevi et al., 1993), etopophos and teniposide induce a premitotic block in late S or early G2 stage (Hande, 1998). These results from binding of etoposide to topoisomerase II, an enzyme required for the unwinding of DNA during replication. Topoisomerase II forms a transient, covalent DNA-protein link, the cleavable complex, which allows one double strand of DNA to pass through a temporary break in another double strand. Etoposide binds to and stabilizes the cleavable complex preventing repair of the double-strand breaks. Etopophos, launched in 1996 by Bristol-Myers Squib (Schacter, 1996; Sudo et al., 1998), is a water-soluble phosphate ester prodrug of etoposide. The prodrug can be administered in higher doses than etoposide as a short intravenous injection, where after it is rapidly converted to the parent compound by plasma phosphatases, and thus constitutes an improved formulation of etoposide.

1.4 Biosynthesis of podophyllotoxin

The full biosynthetic route of cyclolignans has not been elucidated yet, but several studies in different species of *Forsythia* (Oleaceae), *Linun* and *Podophyllum* led to the propositon of a pathway (Fig. 5) (Petersen and Alfermann, 2001). In the initial steps, coniferyl alcohol was synthesized from phenylalanine by phenylpropanoid enzymes (Van Uden et al., 1997). Instead of an alternative route to give the polymeric product lignin, lignans are obtained by dimerization of coniferyl alcohol to yield pinoresinol (Davin et al., 1997). After several steps this compound is transformed into matairesinol, which through a yet unknown way involving yatein yields deoxypodophyllotoxin (Broomhead et al., 1991; Xia et al., 2001).

Podophyllotoxin has traditionally been isolated from podophyllin, resin of *Podophyllum* rhizome. *Podophyllum emodi* (Indian Podophyllum) is preferred to *Podophyllum peltatum* (American *Podophyllum*) because the first one gives more resin and this is richer in podophyllotoxin than the resin of the second one (Giri and Narasu, 2000). The content in podophyllotoxin is about 4.3% of dry weight in *P. emodi* against 0.25% in *P. peltatum* (Jackson and Dewick, 1984). Recently, a new extraction process was described based in rehydration of powdered tissues of *P. peltatum* prior to extraction with organic solvent. This allows endogenous β-glucosidases to hydrolyze lignans thus increasing the yield of podophyllotoxin of rhizomes and leaves to about

5.2% of dry weight (Canel et al., 2001). The finding that leaves of *P. peltatum* are rich source of podophyllotoxin is interesting since leaves are renewable organs that store lignans as glucopyranosides (Moraes et al., 2001). After that, the new extraction protocol was applied to other genera: *Linum*, *Juniperus*, *Hyptis*, *Teucrium*, *Nepeta*, *Dysosma*, *Jeffersonia*, *Thymus* and *Thuja* (Bedir et al., 2002), resulting in another alternative source of podophyllotoxin: needles from *Juniperus virginiana* L. showed 4.7% of dry weight of podophyllotoxin. However, the collection of known plants that are natural sources of podophyllotoxin is limited and insufficient to supply the increasing demand of this compound as starting material for the synthesis of etoposide and the semisynthesis of new derivatives. Actually, problems such as the availability of the endangered *P. emodi* and the difficulties in its cultivation (Dhar et al., 2002) must be solved. In this sense, the possibility of *in vitro* propagation and optimization of the cultivation of *Podophyllum* species have been studied (Moraes-Cerdeira et al., 1998).

These are the reasons that underline the need to find alternative sources of podophyllotoxin. Actually, full chemical synthesis of the podophyllotoxin skeleton, with its four chiral positions and the trans-g-lactonic ring, is not an option from a commercial point of view. Canel et al. (2000) and Botta et al. (2001) reviewed the four general approaches to the chemical synthesis of podophyllotoxin derivatives that have been developed: the oxo-ester route, the dihydroxy acid route, the tandem conjugate

addition route or the use of Diels–Alder reaction. Recent alternatives are being explored, usually starting with the preparation of four coplanar rings and the late introduction of the E ring, as enzyme-catalyzed asymmetric of Berkowitz et al. (2000a) or dearomatizing cyclization of Clayden et al. (2003) and others (Masunari et al., 2001; Galland et al., 2001; Charruault et al., 2002). Alternatively to the isolation from natural sources and to the chemical synthesis, several strategies based in the use of biotechnology led to the *in vitro* production of cyclolignans (Giri and Narasu, 2000). Some of them are the biotransformations carried out by Kutney (1999) with whole cell fermentations. The peroxidase enzyme of a *Nicotiana sylvestris* cell culture in a bioreactor catalysed the oxidative cyclization of a dibenzylbutanolide towards the cyclolignan derivative. In a similar way cyclolignans have been obtained with different stereochemistries in the C ring with cell line of *Podophyllum peltatum*, *Catharanthus roseus* and *Cassia didymobotrya* (Walton and Brown, 1999). Use of plant cell and organ cultures is one of the strategies being developed presently as reviewed by Petersen and Alfermann (2001).

Podophyllotoxin and its derivative 6-methoxypodophyllotoxin have been obtained by *in vitro* production of differentiated organ cultures, mainly roots, undifferentiated calli and suspension cell cultures of different species of *Podophyllum*, *Linum*, *Juniperus* and *Callitris* (Empt et al., 2000; Chattopadhyay et al., 2001). Transgenic hairy roots

produced by infection of plants with *Agrobacterium rhizogenes*, are valuable source of root derived phytochemicals (Giri and Narasu, 2000) and have been considered as 'the best experimental system for production of secondary metabolites' (Giri and Narasu, 2000). With this technique, Oostdam et al. (1993) reported a 5–10 fold higher production of 6-methoxypodophyllotoxin than in untransformed cell suspension cultures. Finally, genetic engineering on metabolic pathways of lignanic branch and other alternative biosynthetic branches towards the polymeric compound lignin, could lead to a reduction of lignin synthesis thus channelling precursors towards the synthesis of lignans.

1.5 Secoisolariciresinol and matairesinol

Furthermore, secoisolariciresinol and matairesinol confer dietary protection to humans, particularly against the onset of breast and prostate cancers (Adlercreutz and Mazur, 1997). Both compounds secoisolariciresinol and matairesinol are present to different extents in various whole-grain cereal foods, seeds and berries, and are converted by intestinal microflora (Borriello et al., 1985) during digestion to form the mammalian lignans, enterolactone and enterodiol. For about 2 decades only secoisolariciresinol and matairesinol were known to be precursors of enterolignans, but recently it was shown that also pinoresinol and lariciresinol are efficiently converted

into enterolignans (Heinonen et al., 2001). Enterolignans possess several biological activities by which they may reduce the risk of cancer and cardiovascular diseases. Enterolignans have weak estrogen-like activity (Mousavi and Adlercreutz, 1992), may inhibit enzymes such as aromatase and 5a-reductase, and stimulate the production of sex hormone-binding globulin (Basly and Lavier, 2005). In addition, plant lignans, and to a lesser extent also enterolignans, have antioxidant activity (Niemeyer, 2003). In epidemiological studies, some evidence for protection of lignans against hormone-related cancers and cardiovascular diseases was found, but results were not consistent (Arts and Hollman, 2005). Enterolignan concentrations in biological fluids have been used as a biomarker for lignan intake in several of these studies.

1.6 Research background

Isolated and cloned a cDNA encoding a dirigent protein was studied intensively during the last 15 years. Dinkova-Kostova et al. (1996) cloned a cDNA encoding this enzyme from *Forsythia. intermedia* (PLR-Fi1). The heterologously expressed protein is specific for (+)-pinoresinol and forms enantiomerically pure (+)-laciresinol and (-)-secoisolariciresinol. The presence of cDNAs corresponding to two stereochemically distinct PLRs in a single plant species, *Thuja plicata*, has been demonstrated by Fujita et al. (1999). Four cDNAs with high homologies to the PLR from *F. intermedia* were

grouped two (1 and 3) by two (2 and 4). Heterologously expressed PLR-Tp1 reduces (+)-pinoresinol to (-)-secoisolariciresinol, PLR-Tp2 reduces (-)-pinoresinol to (+)-secoisolariciresinol. The authors did not determine the relationship between the expression of the different PLRs and the enantiomeric composition of lignans in *T. plicata*. However, in other plant species not only the sign of optical rotation of particular lignans but also the enantiomeric composition can vary (Umezawa, 2003). The stereochemistry of lignans can also vary in different parts of a plant. Petioles of *Arctium lappa* accumulate mainly (+)-secoisolariciresinol, whereas seeds of the same plant species accumulate predominantly lignans with opposite stereochemistry (i.e., (-)-matairesinol, (-)-arctigenin and (-)-secoisolariciresinol) (Suzuki et al., 2002). Enzyme preparations of petioles catalyze the formation of mainly the (+)-enantiomers of pinoresinol-secoisolariciresinol, extracts from seeds catalyze the formation of an enantiomeric excess of the lignans with opposite optical rotation. The authors explain these results by the presence of two PLR isoforms in *A. lappa* which show different enantioselectivity and are expressed differentially. Unfortunately, no PLR cDNAs were cloned from *A. lappa* to prove this assumption. Seeds of *Linum usitatissimum* contain almost (97–99%) pure (+)-secoisolariciresinoldiglucoside derived from (+)-secoisolariciresinol, the opposite enantiomer of the one found in *Forsythia* (Ford et al., 2001; Sicilia et al., 2003). A heterologously expressed cDNA encoding PLR-Lu

converts (-)-secoisolariciresinol into (-)-lacireisnol and seems to be inhibited by (+)-pinoresinol (Davin and Lewis, 2003). von Heimendahl et al. (2005) cloned a cDNA encoding this enzyme from *Linum album* (PLR-La1). The heterologously expressed protein is specific for (+)-pinoresinol and forms enantiomerically pure (+)-laciresinol and (-)-secoisolariciresinol, the same enantiomers as in *Forsythia* spp. plants. The research of recombinant SDH only published by Xia et al (2001). They cloned *sdh* cDNA from *Podophyllum peltatum* and *Forsythia intermedia*, and expressed in recombinant host. Until now, only few *plr* and *sdh* was cloned and confirmed activity by recombinant expression.

Matairesinol is believed to be the precursor of bioactive molecules such as (-)-podophyllotoxin in *Podophyllum peltatum* (Broomhead et al., 1991; Xia et al., 2000), (+)-trachelogenin in *Ipomoea carica* (Schroder et al., 1990), and plicatic acid in *T. plicata*. In this study, the enzymology of formation of matairesinol from pinoresinol in *P. pleianthum* Hance was investigated. This resulted in the the cloning of the corresponding cDNAs, the expression of functional recombinant proteins in *Escherichia coli* and the purification to apparent homogeneity of pinoresinol lariciresinol reductase and secoisolariciresinol dehydrogenase.

Chapter II

Materials and Methods

2.1. Plants, microorganisms and vectors

Escherichia coli JM109 was chosen for vector preservation, *E. coli* M15 was chosen as a bacterial expression host. yT&A vector was used as a preservation vector for constructing the expression sequence. The expression vector for *E. coli* was pQE-30 Xa supplier. Leaves of *Podophyllum pleianthum* Hance were kindly provided by Dr. Tse-Hwie Liu (Development Center for Biotechnology, Taipei, Taiwan) as a gift.

2.2. Construction of plasmids

2.2.1 Construction of *sdh* expression pQE-30 Xa

pQE30-Xa plasmid, a *E. coli* expression vector, was purchased from Qiagen (Germany). A vector map of pQE-30 Xa was provided by Qiagen and shown in Fig.6.

Total RNA was extracted from *P. pleianthum* by using Cetyl Trimethyl Ammonium Bromide (CTAB) method as previously reported (Liao et al., 2004). Tissues of *P. pleianthum* were collected, frozen immediately in liquid nitrogen, and stored at -80°C prior to total RNA extraction. (The tubes and bottles were treated with 0.1% diethypyrocarbonate (DEPC) solution at 37°C overnight, autoclaved twice at 121°C for 20 min, and then dried at 100 °C before use. The tips used in RNA extraction were RNase-free and DNase free.) The extraction buffer was 3% (W/V) CTAB containing 3% (W/V) PVP (Mw 40,000), 25 mM EDTA, 2.0 M NaCl, 100 mM Tris–HCl (pH 8.0),

0.5 g/L spermidine, and 0.1% DEPC (V/V) at pH 8.0. Additionally, 0.5% SDS (W/V) solution with 0.1% DEPC, 10 M LiCl with 0.1% DEPC, and the mixture of chloroform and isoamyl alcohol (24 : 1, V/V) were also prepared. (All the buffers and solutions were incubated at 37°C overnight before autoclaving (121°C, 20 min) twice.) A 4% β-mercaptoethanol was added into the extraction buffer when used. Plant tissues, including fresh leaves, stems, and roots were used for total RNA extraction. Two grams of plant tissues were placed in a clean mortar and liquid nitrogen was added to keep the materials frozen and brittle. The materials were then ground to very fine powder with a pestle and the latter was transferred to a 50 mL polypropylene tube containing 20 mL of preheated (65°C) extraction buffer. The tube was treated in a water bath at 65°C for 10 min with occasional shaking to mix the contents. One volume of chloroform and isoamyl alcohol (24: 1, V/V) was subsequently added to the mixture, and gently mixed, for about 10 min and centrifuged at 12,000xg at 4°C for 10 min. The supernatant was transferred to a new tube, extracted with chloroform and isoamyl alcohol (24: 1, V/V) for 10 min, and then centrifuged at 12,000xg at 10°C for 10 min. This step was repeated once more and the supernatant was transferred to a new tube containing 1/4 volume 10 M LiCl. The contents were mixed thoroughly, stored at 4°C overnight (12 h), and RNA was pelleted at 12,000xg at 4°C for 30 min. The RNA pellet was dissolved very gently in 500 μL 0.5% SDS, extracted with chloroform and isoamyl alcohol (24: 1, V/V), and

then centrifuged at 12,000xg at 4°C for 10 min. The supernatant was again transferred to a new tube, and a 2-fold volume of ethanol was added and mixed thoroughly for precipitating total RNA at -20°C for 2 h. RNA was pelleted at 12,000xg at 4°C for 30 min, washed in 75% ethanol twice, dried in a vacuum, re-dissolved in 200 μL DEPC-treated MiniQuantum water, and stored in -80°C for further use. The quantity and quality of total RNA were detected at 260 nm and 280 nm, respectively (HITACHI) and 10 μL sample was run on 1.0% agarose gel, followed by stained with Ethidium bromide.

Forward primer SDHF：5'-atgggatccacttctacacc-3'and reverse primer SDHR：5'-agccaatccatgtttcaatg-3' were designed based on GenBank accession number AF352735 to amplify the cDNA fragment encoding *sdh* by using One-Step RT-PCR kit purchased from GeneMark Technology. (Tainan, Taiwan) Amplification was carried out in a thermo Px2 thermal cycler machine using the conditions of 30 min of reverse transcription at 50°C, 2 min of denaturation at 95°C, 35 cycles for 30 seconds at 94°C and 30 seconds at 45°C, and a final extension phase at 72°C for 1 min and 30 seconds. The PCR mixture as following: 1 μL of 5x GeneMark One-Step RT-PCR reaction mix, 10 μL of enhancer, 0.6 μM of each primer and 1 μL of GeneMark One-Step RT-PCR Enzyme Mix in a final volume of 50 μL. According to the molecular cloning sites of pQE-30 Xa, the forward primers added a *Kpn*I site and the reverse primer added a *Pst*I

site to the sequence. The primers were designed as following: Forward primer kSDHF : 5'-acggtaccatgggatccacttctacacc-3'and reverse primer pSDHR : 5'-gcctgcagagccaatccatgtttcaatg-3', The PCR mixture as following: 5 μL of 10X PCR buffer Buffer, 400 μM of each dNTP, 0.6 μM of each primer and 1 μL of DNA polymerase in a final volume of 50 μL. The thermalcycling condition was as mentioned above. The PCR products were confirmed by electrophoresis using 1.2% agarose gel. Product of the appropriate size was recovered by Gel/PCR DNA Fragments Extraction kit. This *sdh* gene coding sequence obtained from RT-PCR is immediately ligated with yT&A cloning vector, the ligation products were transformed into *E. coli* strain JM109 for preservation. The transformants were grown on LBA plates and selected by blue/white screening. (Sambrook et al., 2001) Colonies grown were first checked by colony PCR and then sequence was confirmed by sending transformants to Tri-I Biotech (Taipei, Taiwan) for sequencing service.

yT&A vector containing *sdh* gene was then processed with *Kpn*I and *Pst*I endonucleases. Empty pQE-30 Xa vector was processed with the corresponding restriction enzymes as well. The end product was confirmed by 1.2% agarose gel electrophoresis. Products with the appropriate size were recovered by Nucleospin kit. pQE30-Xa vector and tailored *sdh* gene with or without the signal sequence was then ligated, and then transformed into 100 μL JM109 competent cell by heat shock under

42°C for 45 seconds. Transformants were transferred into 1 mL SOC medium for recovery at 37°C for 90 min, then spreaded on LBA plates. Colonies grown were first checked by colony PCR and then sequence was confirmed by sending transformant colony to Mission Biotech (Taipei, Taiwan) for sequencing service. pQE-30 Xa vector containing *sdh* was named pQESDH (Fig. 6).

2.2.2 Construction of *plr* expression pQE-30 Xa

Total RNA was extracted from *P. pleianthum* Hance by using the TRIZOL® reagent. Degenerated primers used to clone a first fragment of a *plr* cDNA from *P. pleianthum* Hance were designed based on a multiple sequence alignment of the following amino acid sequences: PLR-Fi1 (GenBank accession number U81158), PLR-Tp1 (GenBank accession number AF242503), PLR-Tp2 (GenBank accession number AF242504), PLR-La1 (GenBank accession number AJ849358), PLR-Lu1 (GenBank accession number AJ849359) (Fig. 7). The sequences of the resulting primers are PLRFOR1: 5'-carctnaarctngtngargcnathaargargcn-3' and oligo dT. Amplification was carried out in a thermo Px2 thermal cycler machine using the conditions of 30 min of reverse transcription at 50°C, 30 min of denaturation at 95°C, 30 cycles for 1 min at 94°C and 1 min at 50°C, and a final extension phase at 72 °C for 1 min and 40 s. The PCR mixture as following: 1 μL of 5x QIAGEN OneStep RT-PCR Buffer, 400 μM of

each dNTP, 10 μL of 5x Q-Solution, 0.6 μM of each prime and 2 μL of QIAGEN OneStep RT-PCR Enzyme Mix in a final volume of 50 μL. And another degenerated primers were designed to amplify the first PCR products. The resulting primers were designed as following: PLRFOR2: 5'-ccntcngarttyggnayngaycc-3' and PLRREV2: 5'-abrtanatngtyttrttnagngt-3'. The resulting amplicon was ligated into yT&A cloning vector and transformed into *E. coli* JM109 and sequenced. RACE experiments were used for rapid amplification of the 5' and 3' end of the obtained cDNA fragment (Fig. 8 and Fig. 9). To acquire the 5' terminus of the cDNA, 5 μg of the sample RNA was mixed with 2.5 pmol gene specific primer as follows: PLR-GSP1-5': 5'-GAGGATCGTCGATGGT-3'. cDNA was synthesized and purified using the 5'-RACE sysntem version 2.0 (Invitrogen) according to the manufacturer's instruction. Five microliters of the dC-tailed cDNA was next used as the template in a 50 μL PCR reaction by using Super-Therm DNA polymerase with the gene specific primer PLR-GSP2-5': 5'- TGCCATCTCCGCTTAGAGCACACTT-3' and the adaptor primer: 5'-ggccacgcgtcgactagtacgGGIIGGGIIGGGIIG-3'. PCR amplification was performed as follows: 2μL of the synthesized cDNA, 25 pmol of each degenerated primer, 1.5 mM $MgCl_2$, 0.2 mM dNTPs and 1 μL Super-Therm DNA polymerase per 50 μL reactions. PCR conditions were 94°C, 3 min; denaturation 94 °C 30 s; annealing 46°C 30 s; elongation 72°C 1 min in 35 cycles; final extension 72°C 3 min. A nested PCR was

performed with LpPLRGSP3-5': 5'-CCCCCCAGAAAGTATCCAGCAAAACA-3' and UAP: 5'-CUACUACUACUAGGCCACGCGTCGACTAGTAC-3' as gene specific primers. The reverse transcription for the 3'- end of the cDNA was the same as 5'RACE with the primer AP: 5'-GGCCACGCGTCGACTAGTACTTTTTTTTTTTTTTTT-3'. The cDNA was amplified with the LpPLR-GSP1-3' primer: 5'-GAACCTGGAAGAGCAACATTTGACGAA-3' and UAP as mentioned above. The full length open reading frame of the cDNA was amplified by RT-PCR with the primers sacPLRF : 5'-gagctcATGGCTAAGAGTAGAGTTCTCATTGTTG-3' and pstPLRR : 5'-ctgcagCAAATATCGTTTGAGGTATTCGGAC-3', introducing an *Sac*I restriction site at the start codon and a *Pst*I site behind the stop codon. The resulting cloning steps were carried out as described in Section 2.3.1 except for an annealing temperature at 55°C. pQE-30 Xa vector containing *plr* was named pQEPLR (Fig. 10).

2.3 Sequence alignment

To compare the cDNA sequence of *plr* and *sdh*, and the amino acid sequence of PLR and SDH was aligned by SIM-Alignment tool and Genious 2.0.6.

2.4 Construction of *E. coli* expression hosts

For construction of M15 expression hosts, pQESDH expression plasmids were obtained by High-Speed Plasmid Mini Kit (Geneaid, Taiwan). M15 competent cells were prepared according the following procedures. M15 stocks were refreshed in 5 mL LB medium containing 100 μg/mL ampicillin and 25 μg/mL kanamycin (LBAK) under 37^{o}C overnight. Then 1 mL of overnight incubation medium was added into 100 mL LBAK, incubated at 37^{o}C for 2~3 h until OD_{600} reached 0.6. Collect the cells by centrifuge under 1,000xg for 15 min, then gently washed the cells two times with 0.1 M $CaCl_2$, once with 25 mL and the second time with 8 mL. The cells were collected by centrifuge under 1,000xg for 15 min, finally resuspended in 2 mL 0.1 M $CaCl_2$. One mL of prepared competent cell was mixed with 2 μL of expression plasmid, heat shock at 42 °C for 45 seconds and then spread on LBAK plates. Plates were incubated at 37 °C overnight and colonies were checked by colony PCR.

2.5 Expression of *sdh* and *plr* gene in *E. coli*

E. coli strain M15 transformants were induced and inducted for expression under the same following conditions. The strains were first incubated in 3 mL LBA medium, 37^{o}C shaking under 130 rpm for 6 h. One mL of this incubation medium was then transferred into 100 mL of LBA medium and incubated at 37^{o}C shaking under 130 rpm

overnight. Next day, 5 mL of culture medium was transferred into 100 mL LBAK medium, incubated at 37^{o}C shaking under 130 rpm for about 60~90 min, until OD_{600} reached 0.6. The induction temperature was 25^{o}C and the shaking condition was 130 rpm. The concentration of IPTG used for inducing M15 transformants were 0 mM, 0.01 mM, 0.05 mM and 0.1 mM, respectively. Cells were collected at 0 h, 3 h, 6 h, 9 h, 12 h and 15 h, respectively, after IPTG induction, and each sample was centrifuged at 4,000xg for 10 min to collect the cells for further analysis. To divide samples into soluble protein fraction and insoluble protein fraction, cells were resuspended in 1/10 incubation volume of break buffer. Lysozyme with a final concentration of 1 mg/ml was used and the reaction was kept on ice bath for 30 min. Then the cell suspensions were sonicated on ice bath, 10 sec with 10 sec interval for 6 rounds. After sonication, the solution should become more clarified. The samples were then centrifuged under 10,000xg for 30 min. The soluble protein fractions were in supernatant. The insoluble protein fraction (pellets) was resuspended in equal volume of break buffer for sonication.

2.6. Purification of recombinant protein

Purification of recombinant SDH was followed the protocol of The QIA*expressionist*™. The column was assembled according to the manufacturer's

instructions. First of all, the top adapter of the column and cap the bottom outlet were removed. Secoond, completely resuspend a 50% Ni-NTA Superflow slurry and pour the slurry into the column. Then the resin was allowed to settle, inserted top adapter and adjust to top of bed. Elution step first equilibrated column with 5-fold column volumes of lysis buffer (containing 10 mM imidazole). Then the lysate was aplied to column and washed with lysis buffer (containing 20 mM imidazole) until the A_{280} is stable. Finally, the column was washed with wash buffer (containing 250 mM imidazole) until the A_{280} is stable. Elute the protein with elution buffer.

2.7. Protein expression analysis

2.7.1. SDS-polyacylamide gel electrophoresis (SDS-PAGE)

The molecular mass of heterologous expression protein was determined by SDS-polyacylamide gel electrophoresis (SDS-PAGE) (12.5% polyacrylamide). Molecular mass standards solution was treated the same way as other samples: for each 10 μL of protein sample, same volume of 2X SDS sample buffer were added together with 2 μL of tracking dye and incubated in boiling water for 5 min. The electrophoresis was carried out at 150 V for 1 h 20 min. The gel was stained in 0.27% Coomassie Brilliant Blue R-250 solution and destained in a mixture of acetic acid, ethanol and water (10:20:70, v/v/v).

2.7.2 Western blot analysis of His-tag

For Western blot analysis, prestained marker was used while performing SDS-PAGE to separate the proteins by their molecular mass. Prestained marker was directly added for electrophoresis without further processing. The PVDF membrane (Hybond-P PVDF Membrane) was rinsed once in 100% methanol before establishing the transfer sandwich and kept wet at all times. While establishing the transfer sandwich, the sponges and filter papers were soaked in the transfer buffer and air bubbles were prevented. The whole cassette was established with the membrane one the side connected to the positive charge and the SDS-PAGE on the side connected to the negative charge. The proteins were transferred onto polyvinylidene difluoride (PVDF) membrane under 100 voltages 1 h at 4^{o}C. After checking whether the prestained marker was successfully transferred onto the membrane, the membrane was washed twice with TBS buffer for 10 min each time, with the side attached to the gel facing up. This side stayed facing up for the following washing procedures. The membrane was then blocked for 1 h with blocking buffer provided by Penta-His HRP Conjugate Kit (Qiagen, Germany), then washed with TBS-Tween/Triton buffer 10 min twice, and washed once again with TBS buffer for 10 min. Anti-His HRP Conjugate was added into a new blocking buffer with a ratio of 1:2,000, then the anti-His HRP conjugate solution was added onto the membrane for hybridization for 1 h. The membrane was then washed

with TBS-Tween/Triton buffer for 10 min twice and TBS buffer for 10 min once. Chemiluminescence was used for detection. Oxidizing reagent and enhanced luminal reagent was mixed 1:1 ratio prior to adding on the membrane for detection. Detection was performed by Auto-chemi (UVP).

2.7.3. High performance liquid chromatography (HPLC)

The HPLC system used was the Shimadzu VP series, consisted of a SCL-10Avp system control, SPD-M10Avp diode array detector, SIL-10ADvp auto injector and two LC-10ATvp liquid chromatograph pumps. The analysis was performed at room temperature using a Hypersil-Keystone HyPurity C18 column (250 x 4.6 mm particle size 5 μm, Thermo, Japan) equipped with guard column. Two buffers were used to create a mobile phase gradient during detection. Buffer A consisted of 98% water and 0.2% glacial acetic acid. Buffer B consisted of 10% water, 90% acetonitrile. Buffer A and B were degassed using Transsonic Digital S (Elma, Germany) under 100% Ultrasound power for 30 min. The mobile phase gradient was as following: An initial ratio of 70A:30B was followed by a linear gradient to 50A:50B, over 55 min, then back to 70A:30B, for equilibration of the system over 5 min. Matairesinol and secoisolariciresinol have the maximum spectrum absorption at 280 nm, therefore 280 nm was chosen for retention time detection, and full spectrum were used for

identification.

2.7.4. Biotransformation of rSDH *in vitro*

SDH activity was assayed as previously reported (Xia et al., 2000). Soluble protein fractions of *E. coli* cells were analyzed for enzyme reaction by the same following procedures. The enzyme reaction mixture was as following: first, via incubation of the purified enzyme (in Tris-HCl buffer Ph 8.8 with 5 mM DTT, 500 μL) with secoisolariciresinol (2.8 mM, in 100% ethanol, 50 μL) in the presence of 40 mM NAD (in KPi buffer, pH 7.5, 50 μL) for 2 h. The purified recombinant SDH which was boiled 5 minutes was used as the control test. The resulting assay mixture was extracted with EtOAc for products matairesinol and secoisolariciresinol recovery. The EtOAc soluble fractions were combined, evaporated to dryness in vacuum, then reconstituted in MeOH-3% HOAc in H_2O (1:1, 200 μL), subjected to reversed-phase HPLC analysis.

2.7.5 Kinetic parameters of rSDH

Initial velocity studies were using 20 mM Tris-HCl buffer, pH 8.8 containing the purified secoisolariciresinol dehydrogenase (0.4 mg) at six different secoisolariciresinol concentrations (15 μM, 45 μM, 75 μM, 105 μM, 135 μM and 165 μM) with a constant NAD concentration (100 μM). Incubations were carried out at 20°C for 4 min for secoisolariciresinol. Kinetic parameters were determined from Lineweaver-Burk plots.

Chapter III

Results

3.1 Gene cloning of *sdh*

The *P. pleianthum* was chosen for this *sdh* gene cloning study because it is an endemic and rare herb in Taiwan. *sdh* was amplified from the total RNA of *P. pleianthum* by RT-PCR. The size of this encoding sequence was 834 base pairs. (Fig.12). The SDH contained 278 amino acids, with a calculated molecular weight of 30 kDa. Alignment of the nucleotide sequence with the NCBI database, the nucleotide sequence of *sdh* showed 98.1% identity with the gene of *Podophyllum peltatum* (Fig. 13). The deduced amino acid sequence of SDH was used to compare with other amino acid sequences deposed in the NCBI database. The SDH also exhibits 98.2% identity and 99% similarity with *P. peltatum* (Fig.14). SDH from *P. pleianthum* was found to contain conserved sequence "GxGGxG" in the NAD binding domain (Fig.14).

3.2 Expression of *sdh* in M15

To investigate the biochemical properties of SDH, we expressed the six-histidine N terminal-tagged proteins in *E. coli* M15. pQESDH was successfully constructed and transferred into the M15 strain. Nothing was added at induction time point for uninducted control. The apparent molecular weight, determined by SDS-PAGE (12.5% gel), was about 34 kDa (Fig.15). Using the pQE30-Xa expression system, the N-terminal of the expression protein contains a six-histidine tag and a factor

Xa recognition site, resulting in a 4 kDa increase in the molecular weight of the expressed recombinant protein. Thus, the actual molecular weight of SDH was 30 kDa, corresponding to the expected value. The band was very weak at soluble protein fraction initially, but after 3h of induction the expression level all raised to a high level among the three IPTG concentrations. M15 transformant strains inducted with the three IPTG concentrations had a similar expression pattern. Induction with 0.01 mM IPTG had a maximum expression in the insoluble protein fraction after 12h of induction, while induction with 0.1 mM reached to the high level at 9 h. That showed induction with 0.1 mM IPTG had a faster accumulation rate in the insoluble protein fraction. There was no band for both soluble and insoluble protein fractions of uninducted control. Therefore, we chose the minimum IPTG concentration (0.01mM) for 9 h of induction as the expression conditions.

3.3 Purification of recombinant SDH

E. coli M15 was transformed with the expression vector which expressed the histidine-tagged protein and induced with IPTG. After treatment of the His-binding column, the quantification of recombinant SDH was measured by Bradford protein assay (Bradford, 1976). The protein concentration of unpurified cell lysate was 5190 μg/mL, and the protein concentration of purified recombinant SDH was 3348 μg/mL.

The ratio of recombinant SDH in the total soluble protein fraction of cell lysate suspensions was 15.05%. Fig.16 shows the Western blotting analysis of un-purified and purified recombinant SDH proteins. The lane of purified recombinant SDH is a major band, but a minor band at about 78 kDa was also observed, it is suspected that the formation of dimer of recombinant SDH.

3.4 Biotransformation of rSDH *in vitro*

Fig. 17 shows HPLC assay results of authentic secoisolaricireisnol and authentic matairesinol. Fig. 17B and 17C are their UV absorption spectrum, respectively. Both they showed the maximum absorbance at 280 nm. Fig.18B shows the HPLC analysis after 2 h of rSDH incubated with secoisolariciresinol. Compared with Fig.18A (the negative control), the products appeared at 23.97 min as the arrow indicated. In addition to the same retention time, the photo diode array analysis revealed the maximum absorbance at 280 nm, which was consistent to the analysis of authentic matairesinol. It proved that we got the product after incubation of secoisolariciresinol with the recombinant secoisolariciresinol dehydrogenase from *Podophyllum pleianthum* Hance. According to the results, before the reaction there was 27 μg of substrate (secoisolariciresinol), and then 13.5 μg of product (matairesinol) was produced. The conversion rate was 50%.

3.5 Kinetic parameters of recombinant SDH

Kinetic properties using racemic secoisolariciresinol (that is mixture of + and -) as a substrate were examined, with initial velocity studies using substrate concentrations ranging between 15 and 165 μM, while keeping the NAD concentration constant (100 mM). Apparent *Km* values ~ 231.48 μM with apparent maximum velocities ~13.25 (expressed as μmol/min/mg protein) were obtained from Lineweaver-Burk plots. At high substrate concentrations (≧ 75 μM), the reaction followed typical Michaelis-Menten kinetics. However, at lower substrate concentrations, there was a deviation from classical Michaelis-Menten kinetics. The basis of which need be further investigated.

3.6 Gene cloning of *plr*

One fragment of *sdh* was amplified from the total RNA of *Podophyllum pleianthum* Hance by using degenerated primers. The size of this fragment was 320 base pairs. (Fig. 19). Alignment of the deduced amino acid sequence with the NCBI database, the fragment of PLR showed the highly conserved sequence with *Forsythia intermedia* and *Linum perenne.* (Fig. 20). Then we designed the gene specific primer base on this fragment of *plr* for 5' and 3' RACE PCR. The products of 5'- and 3'-RACE PCR were about 662 and 614 base pairs, respectively. A full length cDNA with

933 bp encoding a putative PLR was cloned by an RT-PCR with gene specific primers followed by 5'- and 3'-RACE (Fig. 21). The PLR contained 311 amino acids, with a calculated molecular weight of 35 KDa. Alignment of the nucleotide sequence with the NCBI database, the nucleotide sequence of *plr* showed 68.9% identity with the gene of *Forsythia intermedi* (Fig. 22). The deduced amino acid sequence of PLR was used to compare with other amino acid sequences deposed in the NCBI database. The PLR also exhibits 75.2% identity and 85% similarity with *Forsythia intermedi* (Fig. 23). For existence of NADPH binding domain in PLR had been reported, we used the SIM-Alignment tool to compare the amino acid sequence of the putative NADPH binding domain with PLR from *Forsythia intermedi*. PLR from *Podophyllum pleianthum* Hance was found to contain the conserved sequence "GxxGxxG" in the NADPH binding domain (Fig. 23).

3.7 Expression of *plr* in M15

pQEPLR was also successfully constructed and transferred into M15 cells. The same conduction conditions were tested as mentioned at **2.5**, and three IPTG concentrations (0.01 mM, 0.05 mM, and 0.10 mM) were tested. Nothing was added at induction time point for uninduced control. SDS-PAGE of soluble protein fractions did not show difference between uninduced samples and the induced samples (Fig. 25). It only reveals the higher IPTG concentration used, the less soluble proteins was obtained.

Meanwhile, SDS-PAGE analysis of insoluble protein fractions showed an obvious band at 39 KDa, as well the recombinant SDH, because of a six-histidine tag and a factor Xa recognition site resulting in a 4 kDa increase in the molecular weight of the expressed recombinant protein. Thus, the actual molecular weight of PLR was 35 kDa, corresponding to the expected value. It suggests that recombinant PLR was produced in M15 in the form of inclusion bodies. We further used Western blotting to examine the expression of recombinant PLR in *E. coli*. At the soluble protein fraction, it was surprising to find that rPLR was expressed even before IPTG induction. The signal in the SDS PAGE was very low at the beginning, but after 6 h of induction the expression level raised to a higher level and then kept constantly. It was also observed for both soluble and insoluble protein fractions of uninduced control. Meanwhile, M15 transformants inducted with 0.01 mM IPTG also had an expression pattern similar to the uninduced control in Western blotting. For both soluble and insoluble protein fractions, the expression level obviously increased from 6 h to 15 h of induction. In addition, M15 transformants inducted with 0.01 mM, 0.05 mM and 0.1 mM IPTG showed the similar expression pattern in Western blotting assays, no matter in the soluble or insoluble protein fraction (Fig. 26).

Western blotting showed that the insoluble protein fractions had the similar expression patterns with soluble protein fractions. And obvious smear bands appeared at

the later stage (12^{th} and 15^{th} h). This phenomenon reveals that degradation of recombinant PLR might happen. Although the expression in the insoluble protein fraction was higher than in the soluble protein fraction, to avoid to get degraded and non-function proteins, we still chose the soluble protein fraction as the materials for enzyme reaction. The expression conditions of a minimum IPTG concentration (0.01mM) and 9-h induction were decided.

Chapter IV

Disscussion

Two genes (*plr* and *sdh*) were cloned from *Podophyllum pleianthum* Hance and were expressed in an *E. coli* system for enzyme's characterization. SDH of *Podophyllum pleianthum* Hance showed a high nucleotide sequence similarity and amino acid sequence identity with the relative gene of *Podophyllum peltatum* (Fig. 13 and 14). Meanwhile, PLR of *Podophyllum pleianthum* Hance showed high nucleotide sequence similarity and amino acid sequence identity with the relative gene of *Forsythia intermedia* (Fig. 22 and 23). The conserved sequence "GxGGxG" of NAD binding domain of SDH and "GxxGxxG" of the NADPH binding domain of PLR indicate they can bind with the cofactor NAD and NADPH, respectively. PLR and SDH are the corresponding enzymes involved in oxidation-reduction reaction.

In expression of rSDH, the conduction of three IPTG concentrations didn't show obvious difference in expression efficiency. We finally chose the minimum IPTG concentration because of the economy and to lower the toxicity to hosts (Fig. 15). *In vitro* reaction revealed that the recombinant SDH catalyzed secoisolarciresinol to matairesinol (Fig. 18), and the conversion rate was 50% after 2 h of incubation. According to the previous report, rSDH showed stereospecificity to its substrate (Xia et al., 2001). In this study racemic secoisolariciresinol was chosen as the substrate, and our results suggest the rSDH also show a stereospecificity to the substrate. We speculate that the conversion rate could reach to 100% if we use the right substrate (+) - or

(-)-secoisolariciresinol). Kinetic parameters of rSDH were also measured. The rSDH showed *Km* values ~ 231.48 μM with apparent maximum velocities ~13.25 (expressed as μmol/min/mg protein) obtained from Lineweaver-Burk plots. Compared with SDH's gene from *Podophyllum peltatum* (Xia et al., 2001), the *Km* values of the SDH was 160.2 μM, and the maximum velocities was 7.1 (expressed as μmol/min/mg protein). The values of *Km* and the maximum velocities didn't express a big variation.

In expression of rPLR, it was found that PLR was to be expressed even without IPTG induction (Fig. 24 and 25), which showed the phenomenon of leakage. And the amount of expressed recombinant PLR was detected in the fraction of insoluble proteins. M15 cells had a higher intracellular *Lac*I protein concentration due to possessing pREP4 vector. This reduces the T5 promoter activity more aggressively than the JM109 transgenic strain. There was still a difference in induction ability between 0.01 mM and 0.1 mM IPTG. Induction with 0.1 mM IPTG had a faster accumulation rate of rPLR in the insoluble protein fraction, while induction with 0.01 mM IPTG could accumulate to a higher level at 9 h of induction. M15 transgenic cells were incubated at 37^{o}C to raise cell number and inducted at 25^{o}C for foreign protein expression. Because of the leakage in expression, whether 37^{o}C was still an optimum incubation temperature of increasing cell number therefore was doubted. The expression of rPLR in the insoluble protein fraction in *E.coli* suggests that the expressed rPLR was major in the form of inclusion

bodied. This phenomenon would be resolved by lowering temperature during IPTG induction. As previous reports mentioned, PLR from *Linum album* (PLR-La1), *Forsythia intermedia* (PLR-Fi1), *Thuja plicata* 2 (PLRTp2) and *Linum corymbulosum* Reichenb were specific for (+)-pinoresinol (Dinkova-Kostova et al., 1996; Fujita et al., 1999; von Heimendahl et al., 2005; Bayindir et al., 2008; Nakatsubo et al., 2008), and PLR from *Linum usitatissimum* (PLR-Lu1), *Thuja plicata* 1 (PLR-Tp1), *Arabidopsis thaliana* (AtPrR2) were specific to (-)-pinoresinol (Dinkova-Kostova et al., 1996; Fujita et al., 1999; von Heimendahl et al., 2005; Bayindir et al., 2008; Nakatsubo et al., 2008). To prove the rPLR's function of *P. pleianthum*, *in vitro* reaction of bioconversion should be investigated further.

Chapter V

Conclusion and Perspective

5.1 Conclusion

We cloned the cDNA sequence of *plr* from *Podophyllum pleianthum* Hance, which reached 68.9% identity with *plr* cDNA of *Forsythia intermedia,* and amino acid sequence reached 75.2% identity and 85% similarity. And cDNA sequence of *sdh* reached 98.1% identity with *sdh* cDNA of *Podophyllum peltatum,* and amino acid sequence reached to 98.2% identity.

The conversion of rSDH reaction was further analyzed by HPLC, the retention time (23.97 min) and the UV absorption spectrum of the product matched with the characters of authentic matairesinol.

Western blotting proved the expression of target protein *plr* in *E. coli*. That shows we have cloned *sdh* and *plr* from *Podophyllum pleianthum* Hance, and they could be expressed functionally in recombinant *E. coli.*

5.2 Perspective

The isolation and characterization of the pinoresinol lariciresinol reductase gene and secoisolariciresinol dehydrogenase gene from *Podophyllum pleianthum* Hance, involved in the formation of matairesinol and secoisolariciresinol, was of considerable interest because this represents the key step in the biochemical pathway to the phytoestrogenic and health-protecting lignan, matairesinol and secoisolariciresinol.

Related studies will be directed toward several different ways, one is introducing the *sdh* and *plr* into other organisms for co-expression in order to establish a lignan bioconversion system. And behind matairesinol the pathway step still didn't figure out, we may try to investigate the pathway continually.

Figures

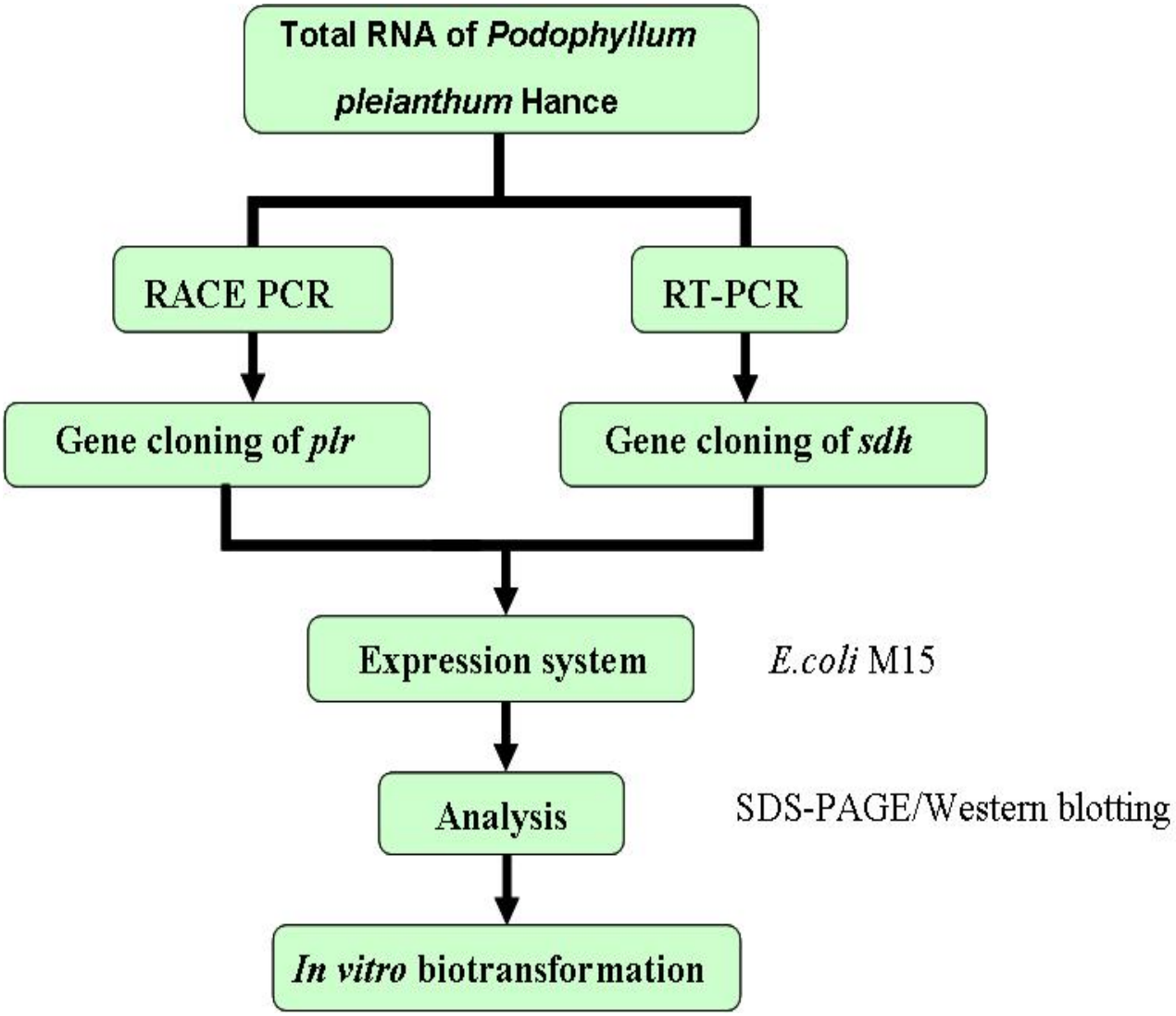


Fig. 1 The outline of this study

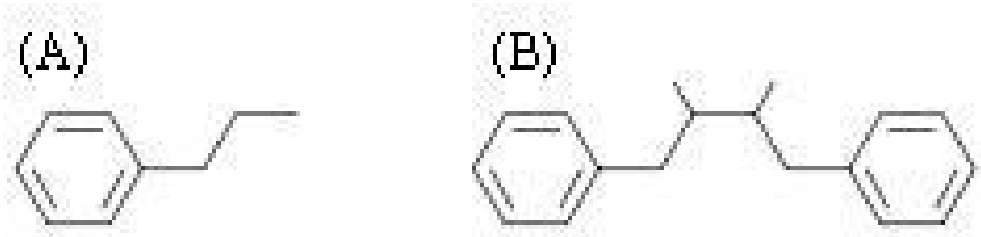


Fig. 2 (A) Phenylpropanoids, are built up of C6C3 units. (B) Dibenzylbutane skeleton. Pictures obtained from the internet by the following website: http://en.wikipedia.org/wiki/lignans

Fig. 3 *Podophyllum pleianthum* Hance. Photo obtained from the internet by the following website: http://www.botanic.jp/plants-ha/hakkak_4.jpg

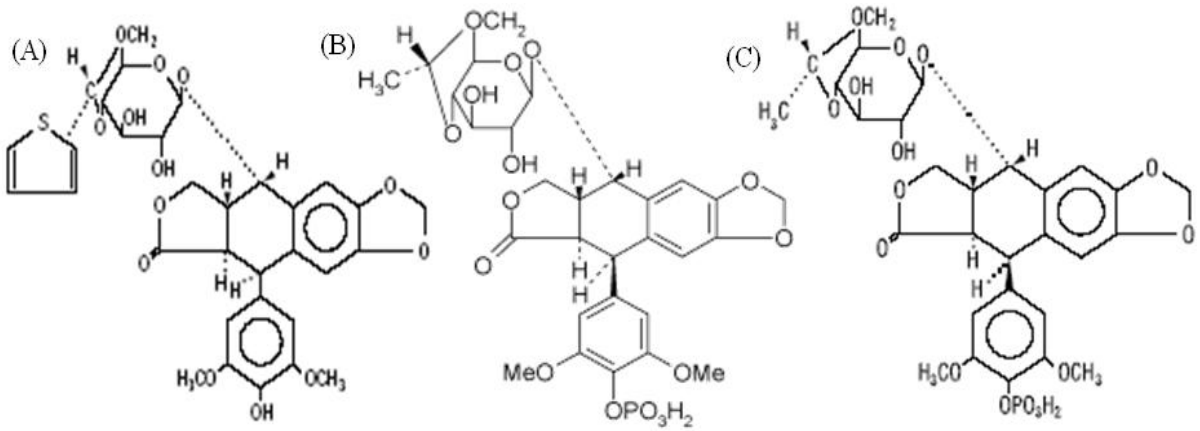


Fig. 4 (A) Teniposide. (B) Etoposide. (C) Etopophos. Pictures obtained from the internet by the following website: http://www.rxlist.com/script/main/hp.asp

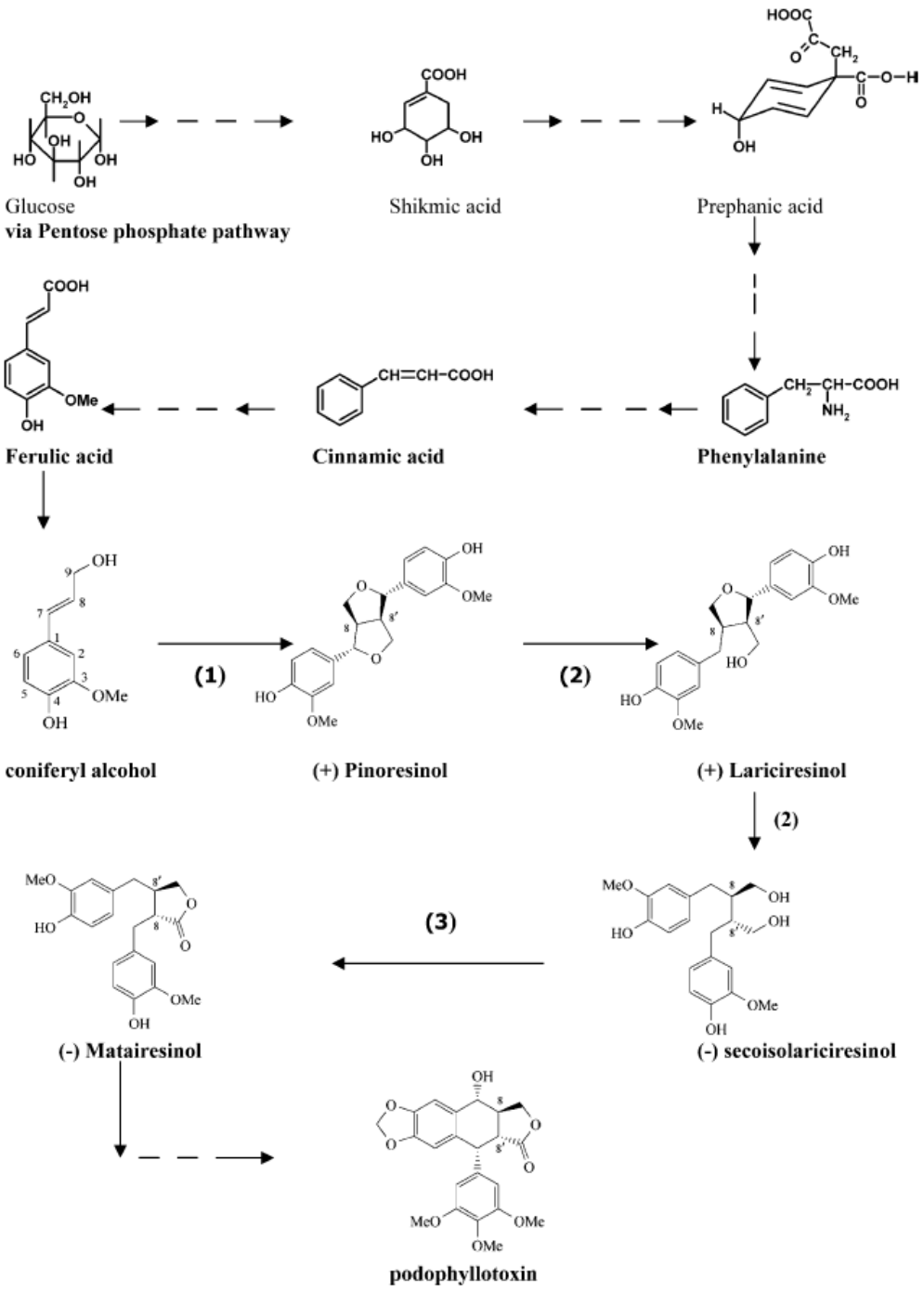


Fig. 5 A hypothetical biogenetic scheme for podophyllotoxin production. (1): Pinoresinol synthase, (2): pinoresinol lariciresinol reductase, (3): secoisolariciresinol dehydrogenase. (Farkya et al., 2004)

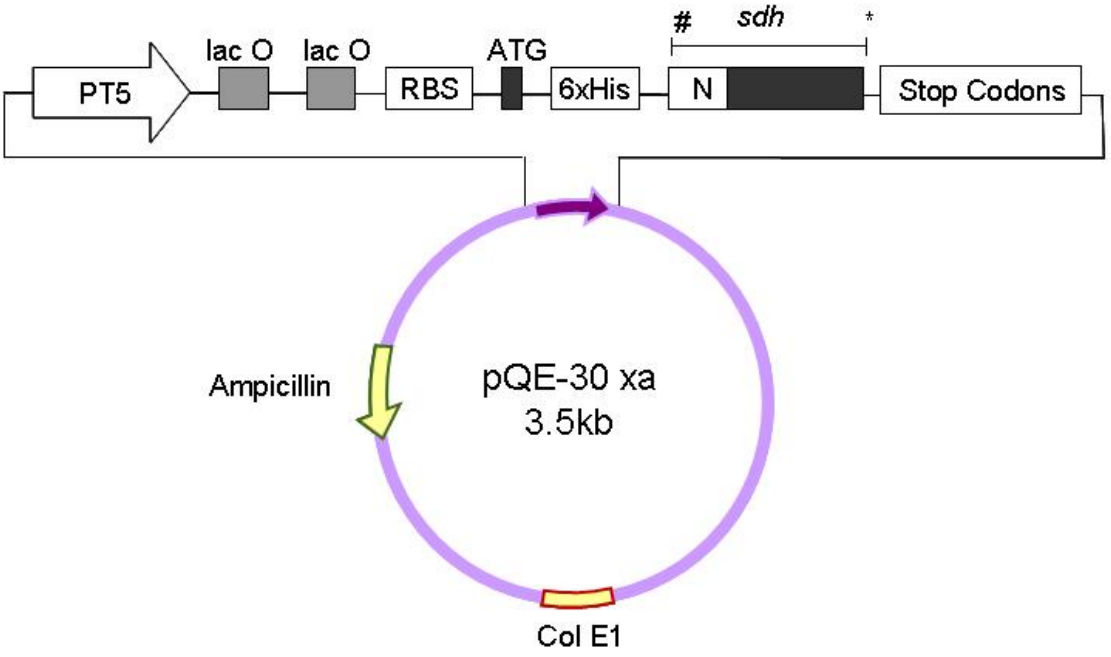


Fig. 6 The gene map of pQESDH construct. PT5: phage T5 promoter, lac O: lac operator, RBS: ribosome binding site, ATG: translation start site, H: 6 x His-tag, N: N-terminal membrane anchor, *sdh*: secoisolariciresinol dehydrogenase encoding sequence, #: *Kpn*I restriction site, *: *Pst*I restriction site.

Fig. 7 Amino acid sequence alignment among PLRs from *Linum album* (PLR-La1, Accession No. AJ849358), *Forsythia intermedia* (PLR-Fi1, Accession No. U81158), Thuja plicata (PLR-Tp2, Accession No. AF242504), *Linum usitatissimum* (PLR-Lu1, Accession No. AJ849359) and *Thuja plicata* (PLR-Tp1, Accession No. AF242503). PLRFOR1、PLRFOR2 and PLRREV2 were the degenerated primers for *plr* cloning.

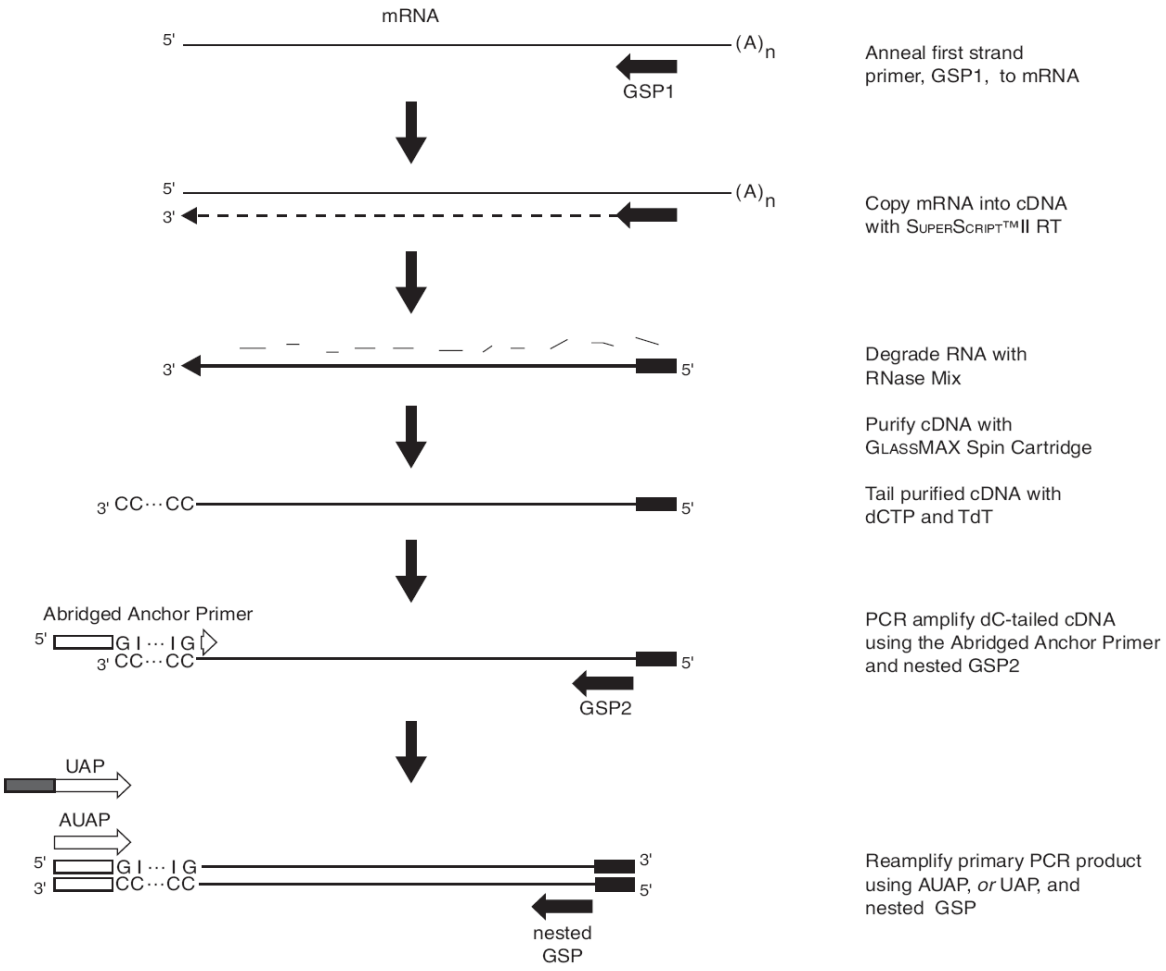


Fig. 8 Overview of the 5' RACE System procedure. The figure is available for downloading from Invitrogen company's World Wide Web site (http://www.invitrogen.com/site/us/en/home.html).

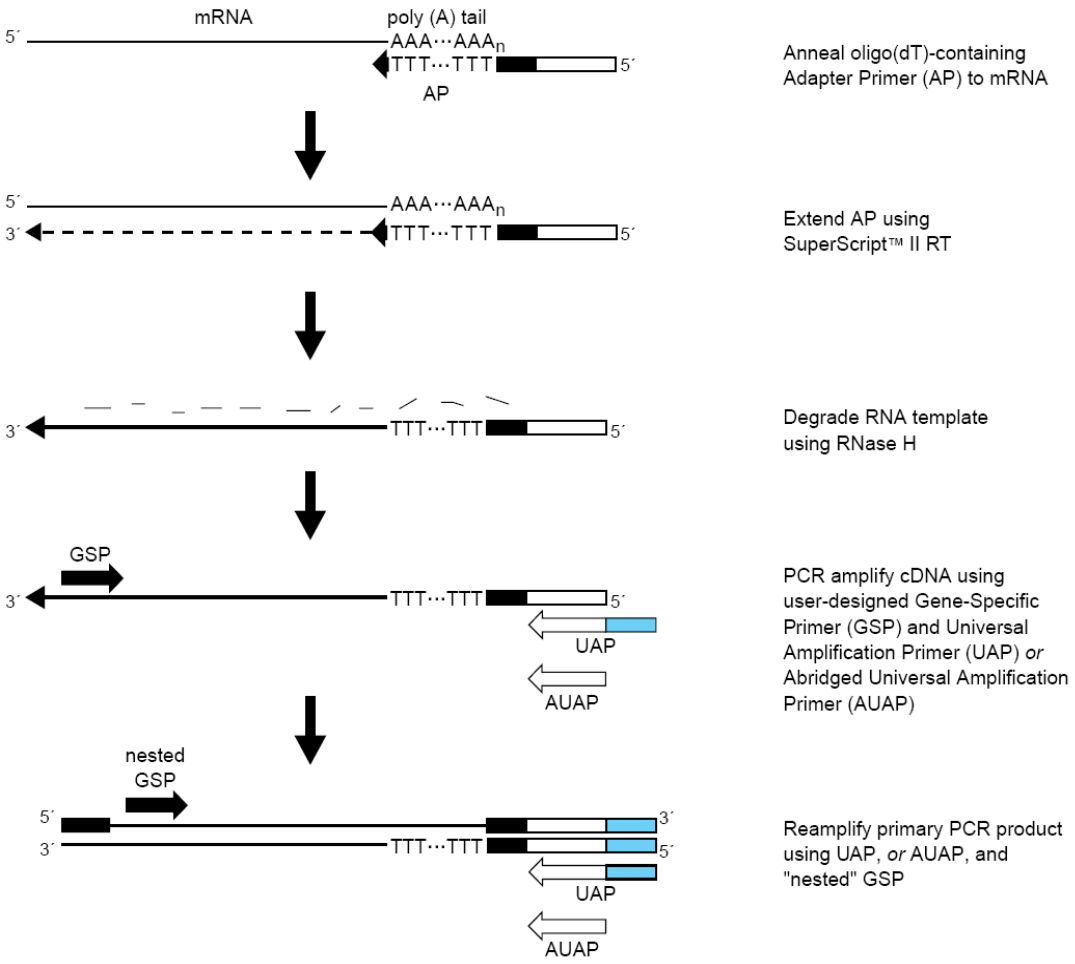


Fig. 9 Overview of the 3'RACE System procedure. The figure is available for downloading from Invitrogen company's World Wide Web site (http://www.invitrogen.com/site/us/en/home.html).

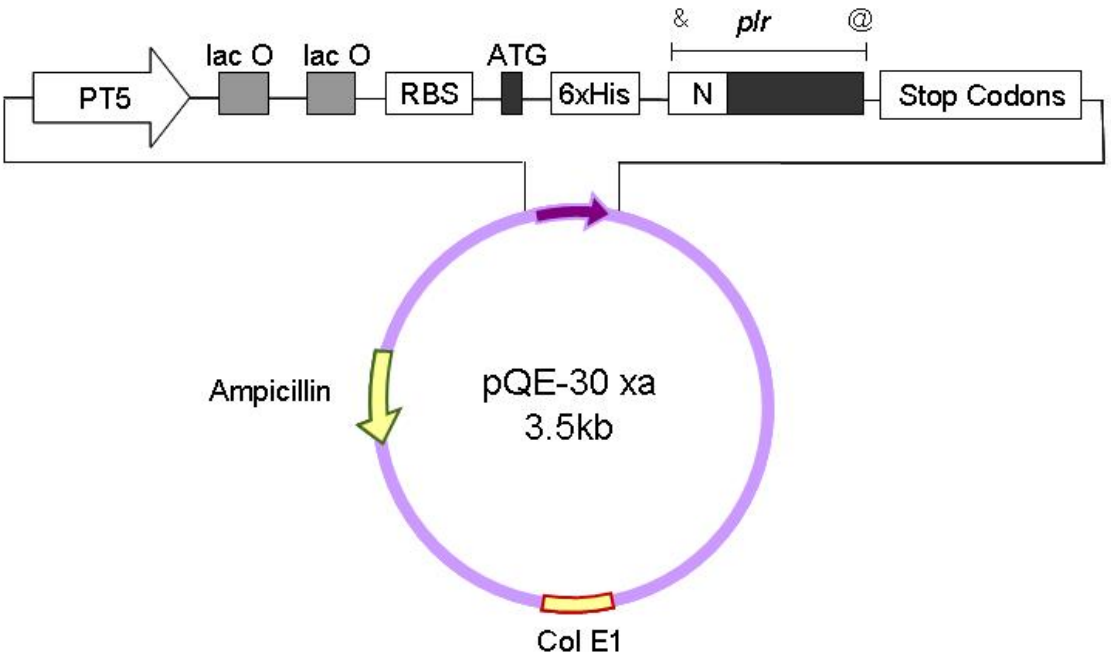


Fig. 10 Gene map of pQEPLR construct. PT5: phage T5 promoter, lac O: lac operator, RBS: rivosome binding site, ATG: translation start site, H: 6 x His-tag, N: N-terminal membrane anchor, *plr*: pinoresinol lariciresinol reductase encoding sequence, &: *Sac*I restriction site, @: *Pst*I restriction site.

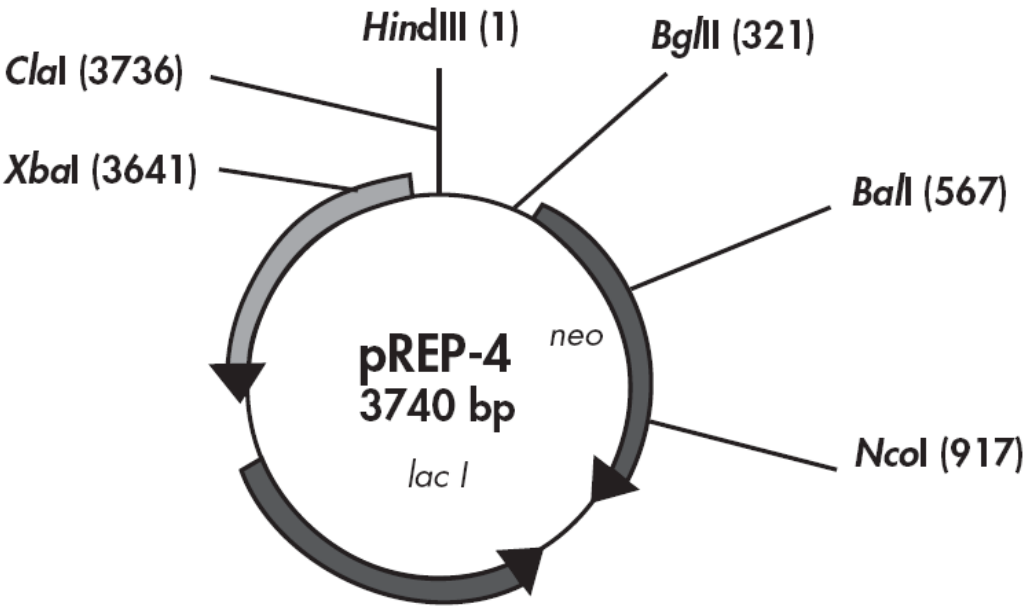


Fig. 11 The restriction map of pREP4 plasmid. The figure is available for downloading from Qiagen company's World Wide Web site (www1.qiagen.com).

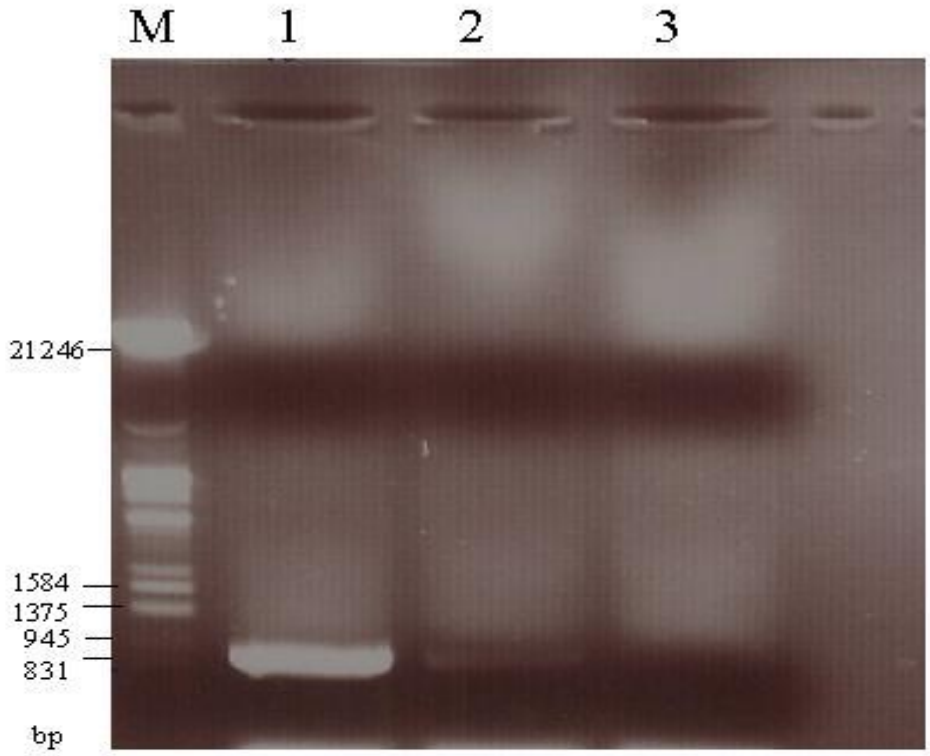


Fig. 12 RT-PCR of *sdh* with different annealing temperatures. M : marker. Lane 1: Annealing temperature was 45°C. Lane 2: Annealing temperature was 50°C. Lane 3: Annealing temperature was 55°C.

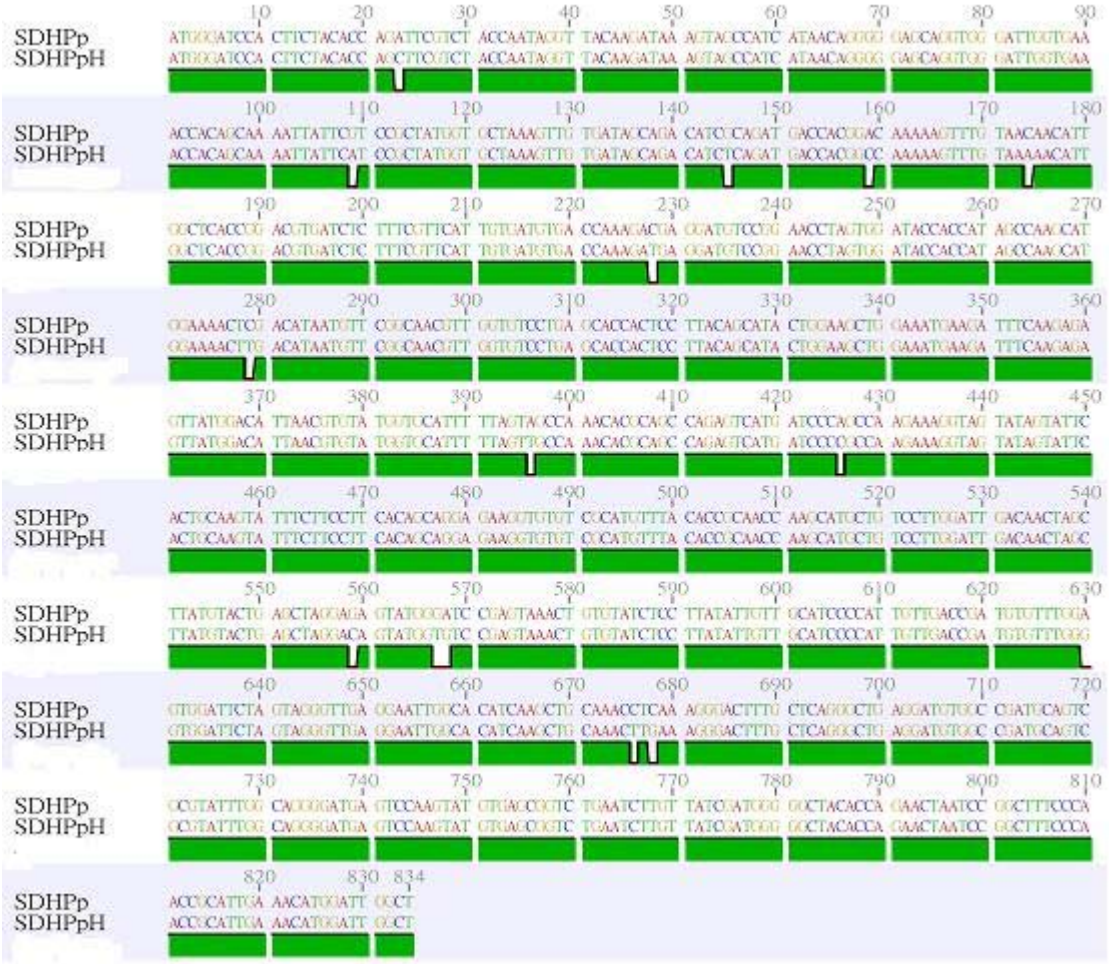


Fig. 13 The *sdh* cDNA sequence alignment between *Podophyllum pleianthum* Hance and *Podophyllum peltatum.* SDHPp: *sdh* cDNA sequence from *P. peltatum.* SDHPpH: *sdh* cDNA sequence from *P. pleianthum*. The identity of two cDNA sequence was 98.1%.

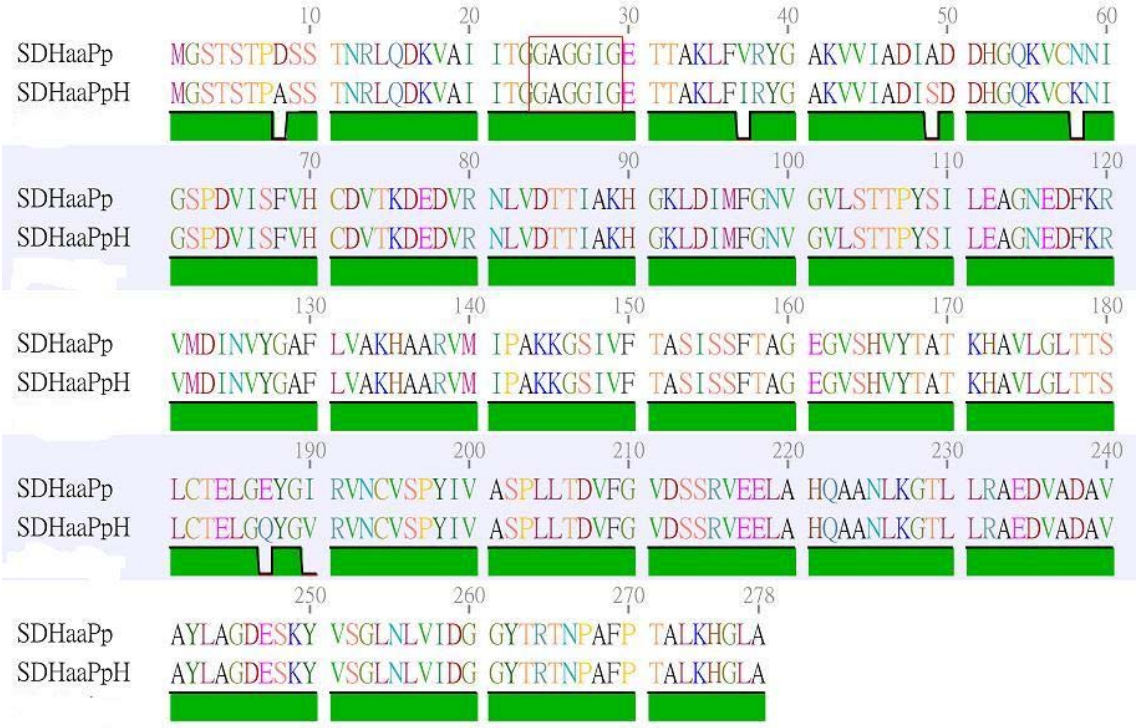


Fig. 14 The SDH amino acid sequence alignment between *Podophyllum pleianthum* Hance and *Podophyllum peltatum.* SDHaaPp: amino acid sequence of SDH from *P. peltatum.* SDHaaPpH: amino acid sequence of SDH from *P. pleianthum*. The identity of two amino acid sequence was: 98.2%. The conserved sequence ‘‘GxGGxG’’ of the NAD binding domain is circled.

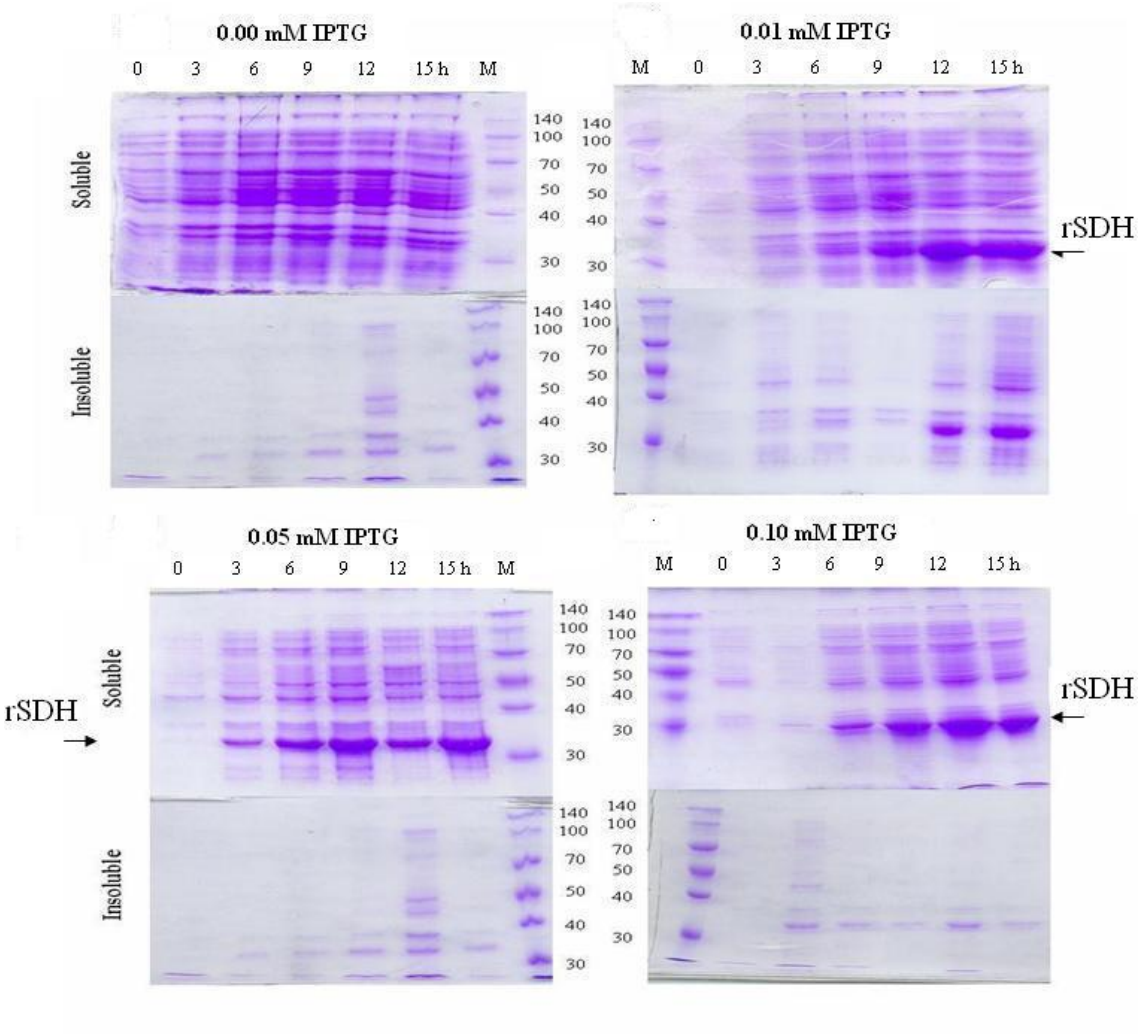


Fig. 15 SDS-PAGE analysis of pQESDH expressing in *E.coli* M15 at 25^{o}C induction. Time after induction and induction concentration were noted on top of each figure. The unit of molecular weight marker was kDa. The loading volume of molecular marker was 5 μL and for other samples were 10 μL. The rSDH was showed one SDS-PAGE as the arrow pointed.

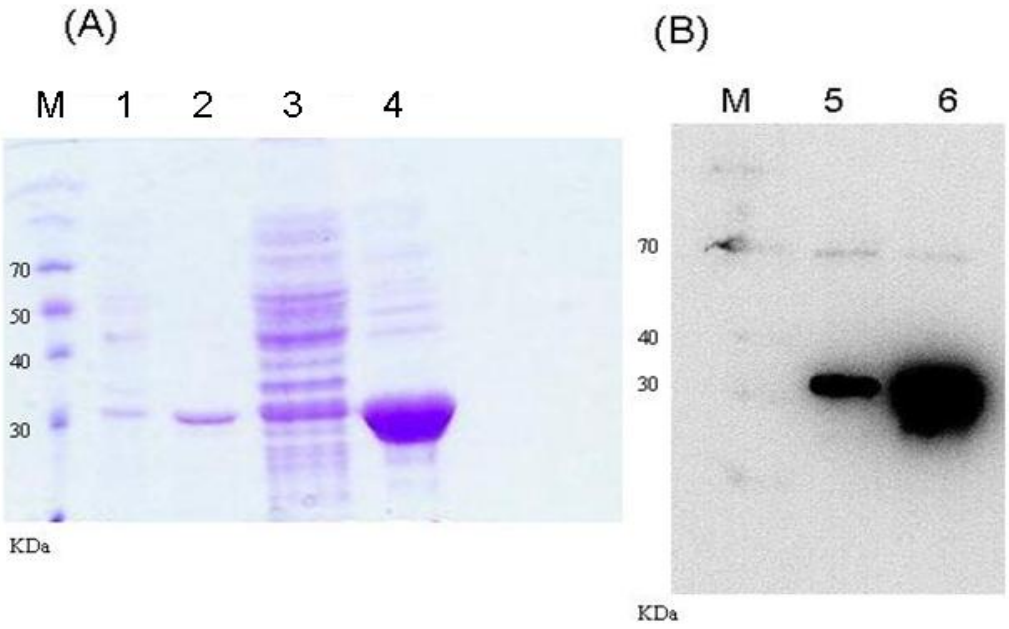


Fig. 16 (A) SDS-PAGE analysis of purified and non-purified rSDH protein. (B) Western blotting analysis of purified and non-purified rSDH protein. The induction condition was 0.01 mM IPTG induction for 9h. M: marker. Lane1: 1/20 dilution of cell lysate induction for 9h. Lane 2: 1/20 dilution of rSDH purified with His-tag. Lane3: cell lysate induction for 9h. Lane4: rSDH purified with His-tag. Lane 5: 1/20 dilution of cell lysate induction for 9h. Lane 6: 1/20 dilution of rSDH purified with His-tag. The loading volume of molecular marker was 5 μL and for other samples were 10 μL.

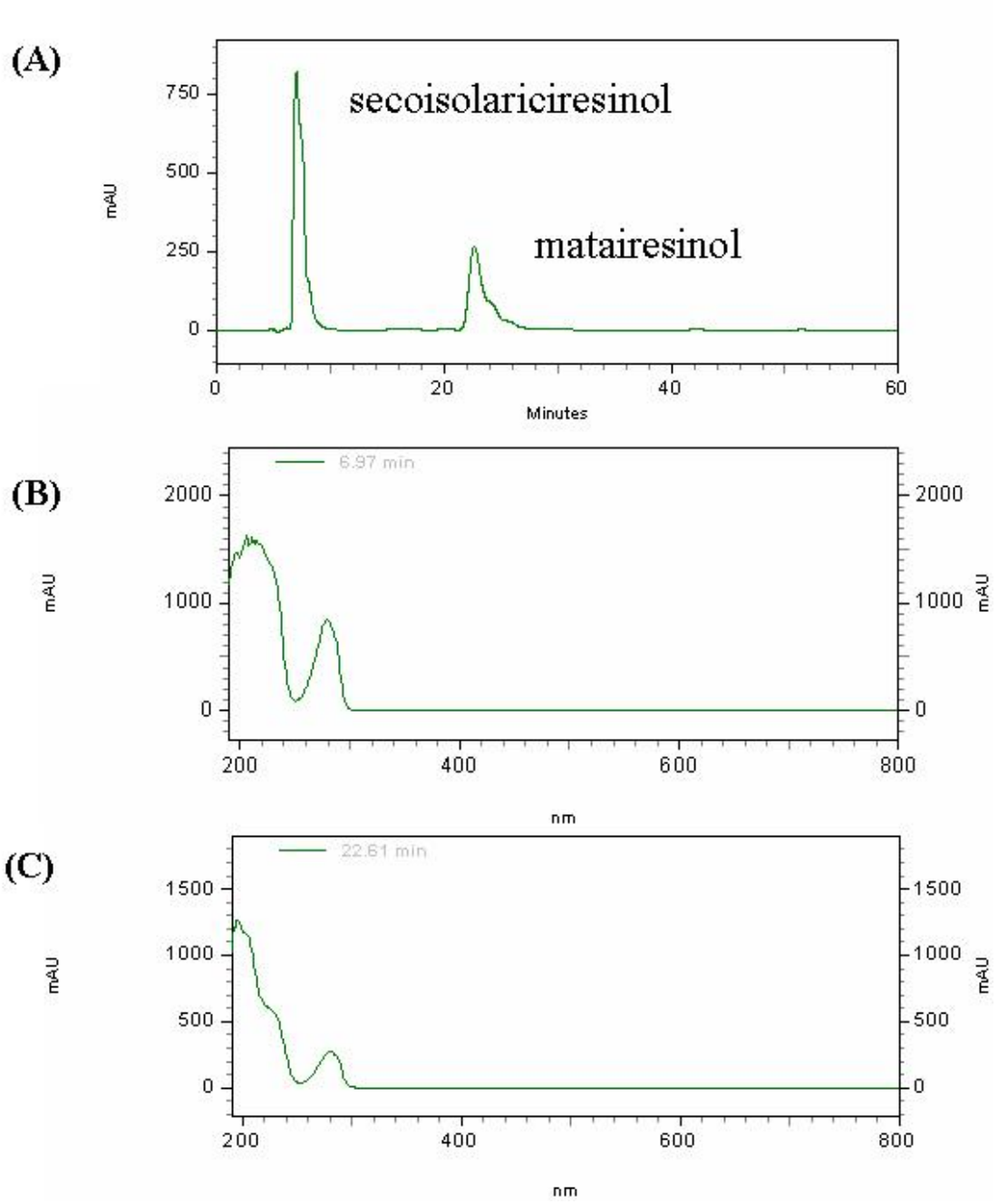


Fig. 17 HPLC analysis of authentic secoisolariciresinol and matairesinol. (A) HPLC chromatogram (at 280 nm absorption). (B) Ultraviolet absorption spectrum of authentic secoisolariciresino (6.97 min). (C) Ultraviolet absorption spectrum of authentic matairesinol (22.61 min).

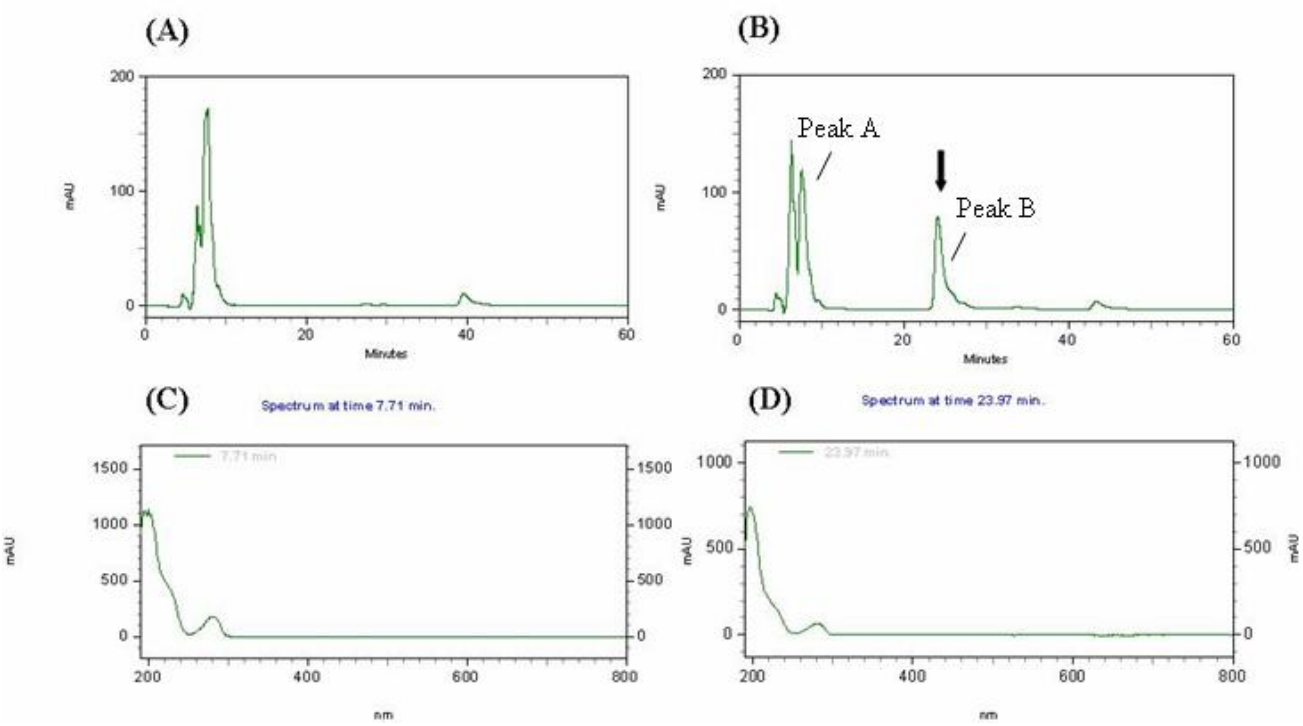


Fig. 18 HPLC analysis of *in vitro* biotransformation incubated with soluble protein fraction of M15 transformant. The condition was induction with 0.01 mM IPTG for 9 h. (A) Control test. (B) HPLC chromatogram (at 280 *nm* absorption). (C) Ultraviolet absorption spectrum of peak A at 7.71 min. (D) Ultraviolet absorption spectrum of peak B at 23.97 min.

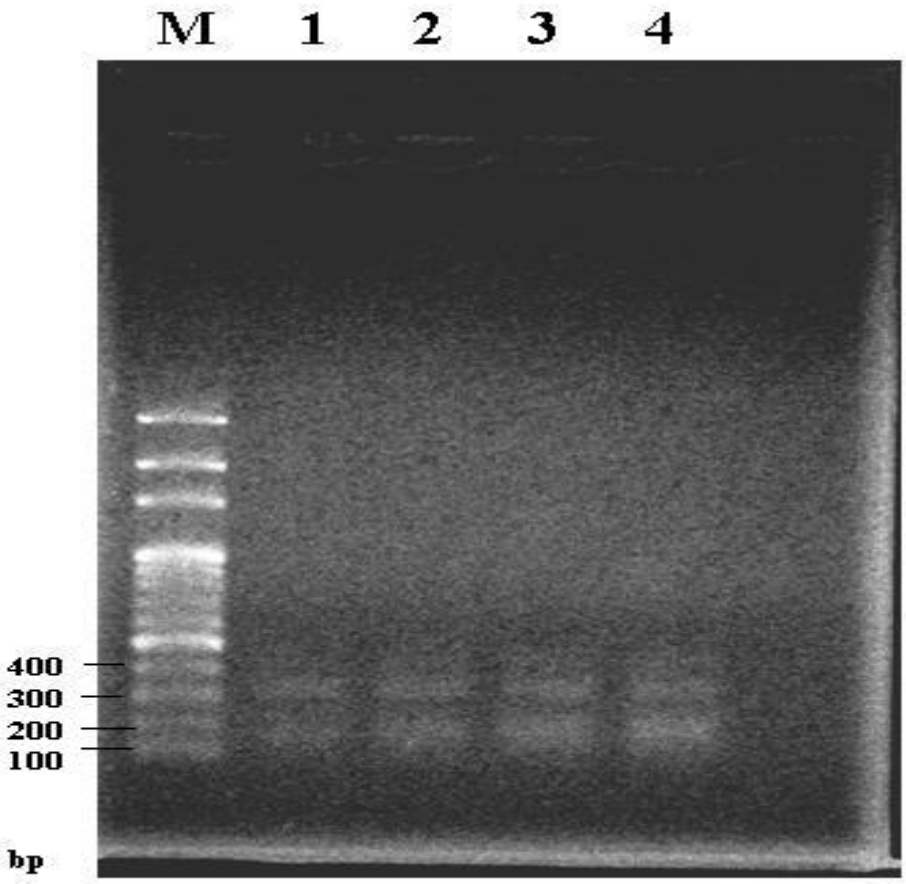


Fig. 19 RT-PCR of the middle sequence in *plr* with different annealing temperature. M: marker. Lane 1: 40^{o}C. Lane 2: 42.5^{o}C. Lane 3: 45^{o}C. Lane 4: 47.5^{o}C.

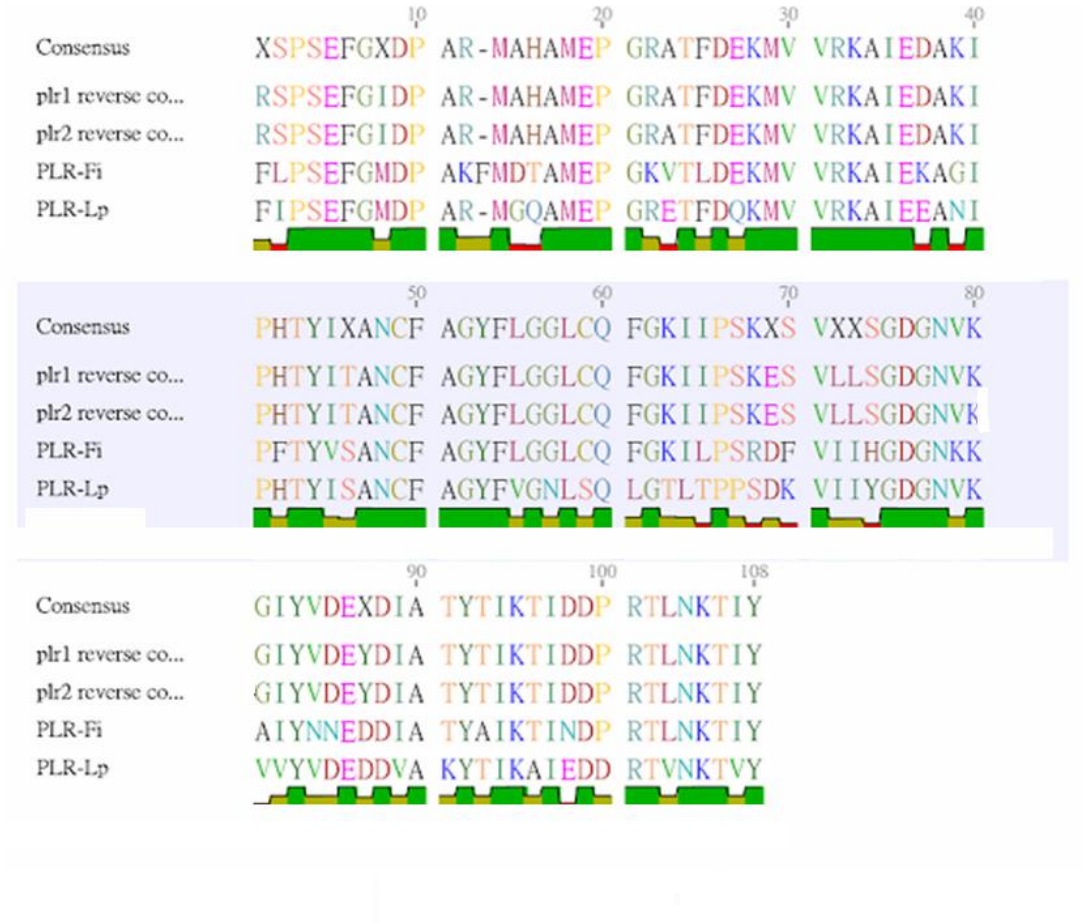


Fig. 20 The middle amino acid sequence in PLR alignment with *Forsythia intermedia* and *Linum perenne*. Consensus: the conserved sequence. plr 1 reverse co: one of the middle amino acid sequence of plr from *P. pleianthum* by RT-PCR. plr 2 reverse co : the other of the middle amino acid sequence of plr from *P. pleianthum* by RT-PCR. PLR-Fi: the middle amino acid sequence of plr from *F. intermedia*. PLR-Lp: the middle amino acid sequence of plr from *L. perenne*.

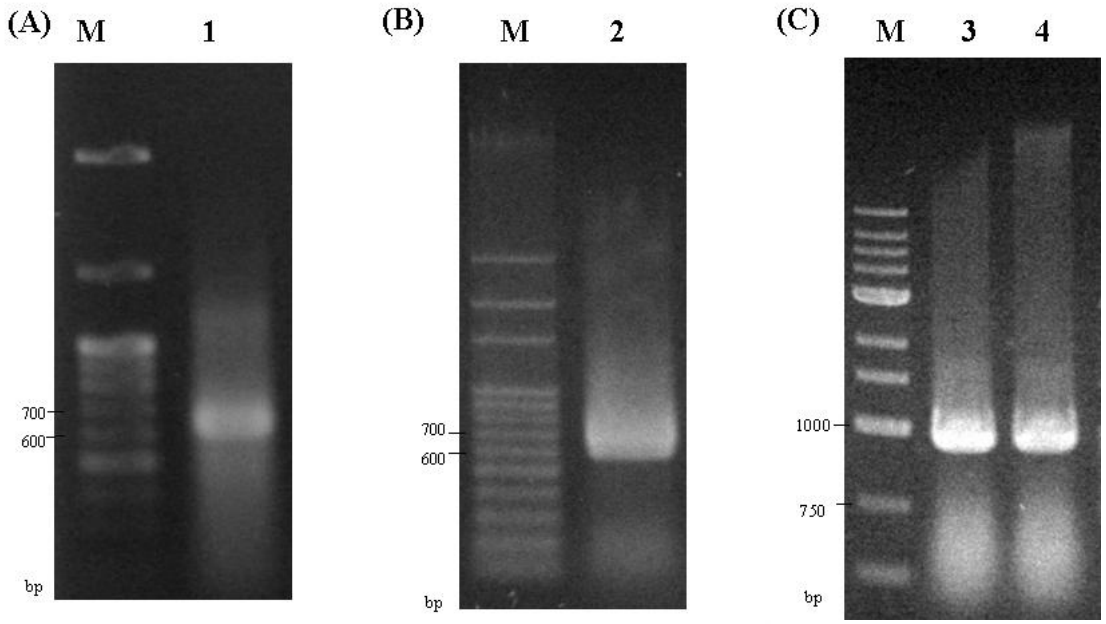


Fig. 21 (A) 5'RACE of *plr*. (B) 3'RACE of *plr*. (C) RT-PCR of *plr* with different annealing temperature. M: marker. Lane 3: Annealing temperature was 55^{o}C. Lane 4: Annealing temperature was 60^{o}C.

Fig. 22 The *plr* cDNA sequence alignment between *Podophyllum pleianthum* Hance and *Forsythia intermedia*. PLRPpH: *plr* cDNA sequence from *pleianthum* Hance. PLRFi: *plr* cDNA sequence from *Forsythia intermedia*. The identity of two cDNA was 68.9%.

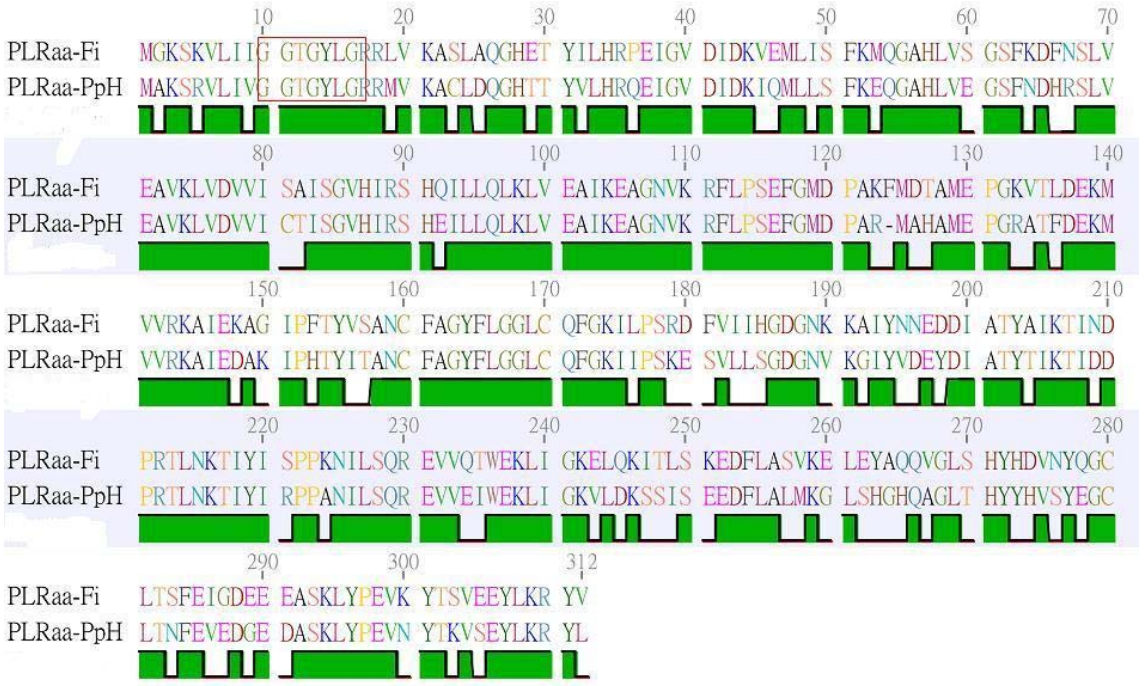


Fig. 23 The *plr* amino acid sequence alignment between *Podophyllum pleianthum* Hance and *Forsythia intermedia.* PLRaa-PpH: *plr* cDNA sequence from *pleianthum* Hance. PLRaa-Fi: *plr* cDNA sequence from *Forsythia intermedia*. The identity of two amino acid sequence was 68.9%, and the similarity was 85%. The conserved sequence ‘‘GGxGxxG’’ of the NADPH binding domain is circled.

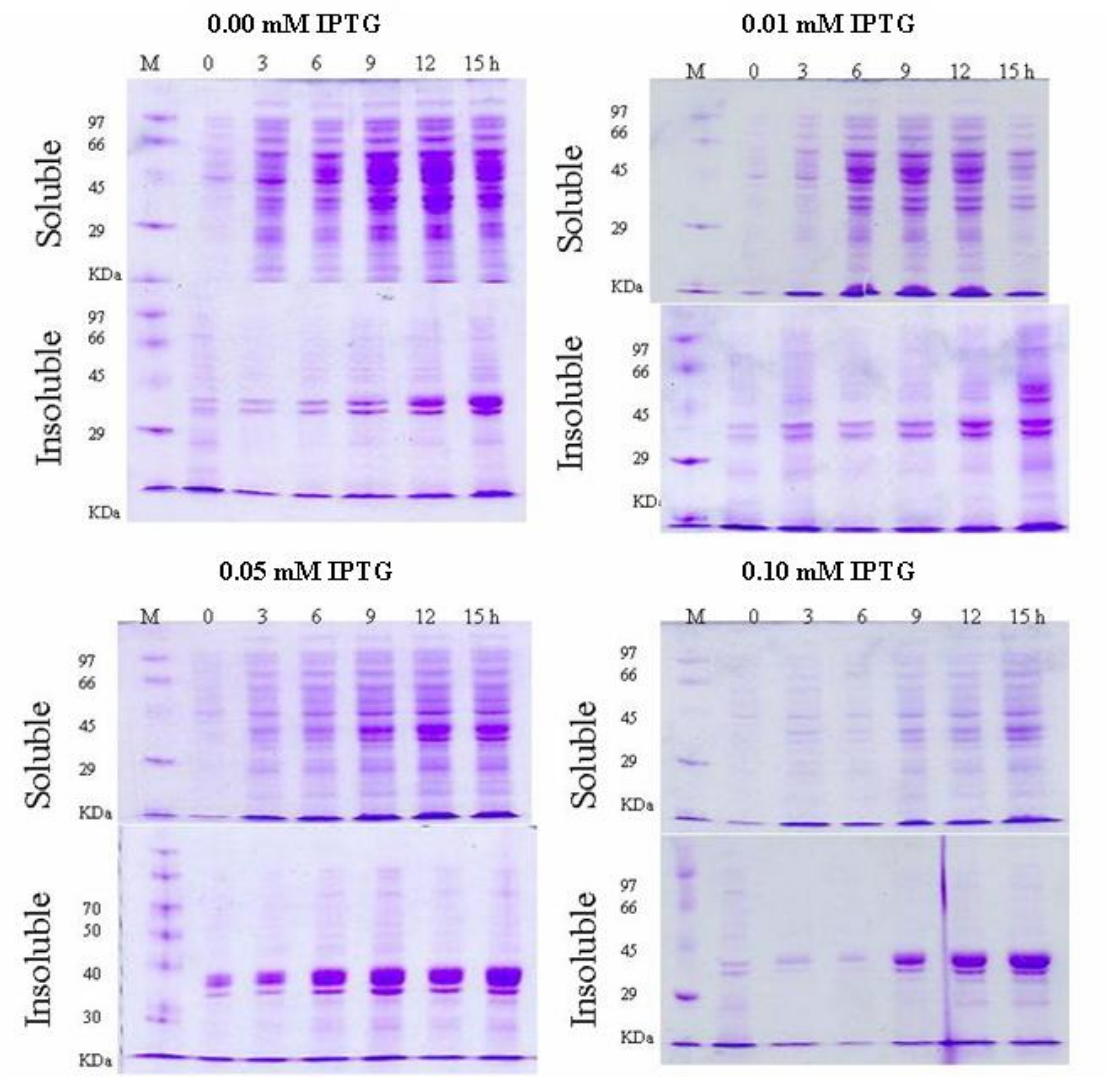
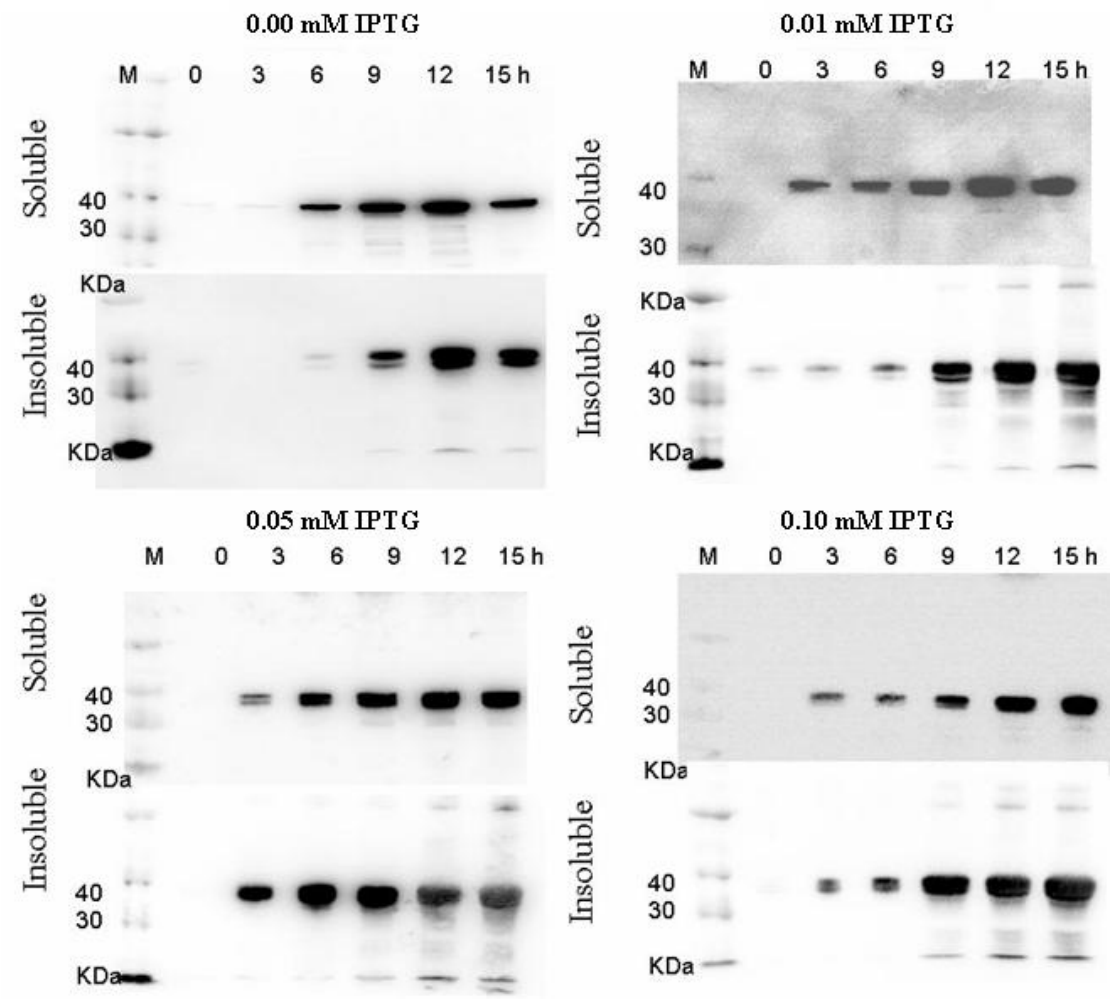


Fig. 24 SDS-PAGE analysis of pQEPLR expressing in *E.coli* M15 and induction at 25^{o}C. Time after induction and induction concentration were noted on top of each figure. The unit of molecular weight marker was kDa. The loading volume of molecular marker was 5 μL and for other samples were 10 μL.



Fig, 25 Western blot analysis of pQEPLR expressing in *E.coli* M15 and induction at 25^{o}C. Time after induction and induction concentration were noted on top of each figure. The unit of molecular weight marker was KDa. The loading volume of molecular marker was 5 μL and for other samples were 10 μL.

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Appendices

A.1. Plasmid construction

PCR

Super-Therm DNA Polymerase (Bertec, Taipei, Taiwan)
dNTP mixture (Viogene, CA, USA)
10x PCR reaction buffer (Bertec, Taipei, Taiwan)

Restriction enzyme

*Kpn*I (Takara, Shiga, Japan)
*Pst*I (Takara, Shiga, Japan)
*Sac*I (Takara, Shiga, Japan)

TRIZOL® reagent (Invitrogen, USA)
QIAprep® Spin Miniprep Kit (Qiagen, Hilden, Germany)
Gel extraction kit (Geneaid, Taipei, Taiwan)

yT&A cloning vector kit (Yeastern Biotech, Taipei, Taiwan)
T4 DNA ligase (Takara, Shiga, Japan)

A.2. Microbe culture

Medium for *E. coli*

LB broth (1 L): 10 g Bacto®-tryptone, 5 g Bacto®-yeast extract, 10 g NaCl
LB agar (1 L): 10 g Bacto®-tryptone, 5 g Bacto®-yeast extract, 10 g NaCl, 15 g agar
SOC medium (1 L): 20 g Bacto®-tryptone, 5 g Bacto®-yeast extract, 10 mM NaCl, 2.5 mM KCl, 20 mM Mg^{2+} stock, 20 mM glucose
2 M Mg^{2+} stock (1 L): 203.3 g $MgCl_2 \cdot 6H_2O$, 246.5 g $MgSO_4 \cdot 7H_2O$

Antibiotics

Ampicillin (Sigma-Aldrich, MO, USA)
Kanamycin (Sigma-Aldrich, MO, USA)

A.3. Protein purification

Break buffer

25 mM Tris buffer contained 5 mM DTT, pH 7.5

Lysis buffer

50 mM $NaHPO_4$, 300 mM NaCl, 10 mM imidazole, adjust pH to 8.0 using NaOH

Wash buffer

50 mM $NaHPO_4$, 300 mM NaCl, 20 mM imidazole, adjust pH to 8.0 using NaOH

Elution buffer

50 mM $NaHPO_4$, 300 mM NaCl, 250 mM imidazole, adjust pH to 8.0 using NaOH

Protein quantity

Bradford (Bio-Rad, CA, USA)

A.4. Protein detection

SDS PAGE

A solution (1 L): 292 g acrylamide, 8 g N, N'-Methylene-bis-acrylamide (Bis)

B solution (1 L): 182 g Tris, 3.6 ml N, N, N', N'-Tetramethyl-ethylenediamine (TEMED), pH 8.8

C solution (1 L): 60 g Tris, 4 ml TEMED, pH 6.8

12.5% SDS-PAGE used in this study:

For running gel: 4.15 mL A solution, 2.5 mL B solution, 0.1 mL 10% SDS,3 mL H_2O, 0.05 mL 10% ammonium persulfate (APS) (total volume: 10 mL)

For stacking gel: 0.66 mL A solution, 1.24 mL C solution, 3 mL H_2O, 0.1 mL APS (total volume: 5 mL)

Protein electrophoresis buffer (5x) (1 L): 54.5 g Tris, 4.7 g EDTA · 2Na, 24.8 g boric acid, pH 8.4

SDS-sample buffer (5x): 0.225 M Tris-HCl (pH 6.8), 50% glycerol, 5% SDS, 0.05% bromophenol blue, 0.25 M DTT

Western blot

Western lightning-Chemical Reagent Plus (PerkinElmer Life Science, Inc., MA, USA)

Hybond-C Extra membrane (Amersham, London, UK)

Penta His HRP conjugated (Qiagen, Hilden, Germany)

Transfer buffer: 150 mM Glycine, 25mM Tris, 20% Methanol

10x TBS: 0.5M Tris Base, 9% NaCl, pH 7.6

Blocking buffer: 0.5%(w/v) blocking reagen, 1X blocking buffer, 0.1% Tween-20
Wash buffer (TBST): 1X TBS, 0.25 % Tween20

A.5. HPLC

CH_3CN (Merk, Darmstadt, Germany)
Acetic acid (Sigma-Aldrich, MO, USA)
Methanol (Merk, Darmstadt, Germany)