CHAPTER IV

RESULTS

1. Tissue cultures of *Plumgago indica* Linn.

Tissue cultures of *P. indica* were established in forms of callus, root culture and *in vitro* plantlets as described below.

1.1 Establishment of callus culture

Callus formation was successfully induced from young stem segments on MS medium supplemented with 1.0 mg/l 2,4-D and 0.1 mg/l BA. Within two weeks, each stem segment was found to enlarge and callus tissues were then initiated at the cutting area with friable yellow color. These callus cultures were maintained by using the same medium with regularly subculturing at 4-week interval. The resulting callus cultures appeared to be friable with greenish-yellow color of the tissue as shown in Figure 20. They were used subsequently for evaluating their ability in producing plumbagin (see section 2).

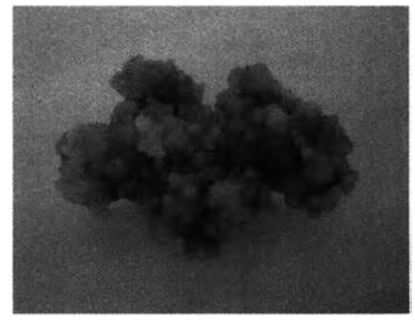

Figure 20 Callus culture of *P. indica* on MS medium supplemented with 1.0 mg/l 2,4-D and 0.1 mg/l BA.

1.2 Establishment of root culture

The root cultures of *P. indica* were first initiated from young leaf segments by culturing on B5 medium supplemented with 1.0 mg/l NAA and 0.1 mg/l kinetin (Figure 21A). After 8 weeks of the initiation, the induced roots were observed. They were then excised from the explants and transferred to MS liquid medium containing no plant growth regulator. The root cultures were maintained in MS liquid medium which appeared to support active proliferation of the healthy roots (Figure 21B, C and D). The stable root cultures were used for the determination of their plumbagin content.

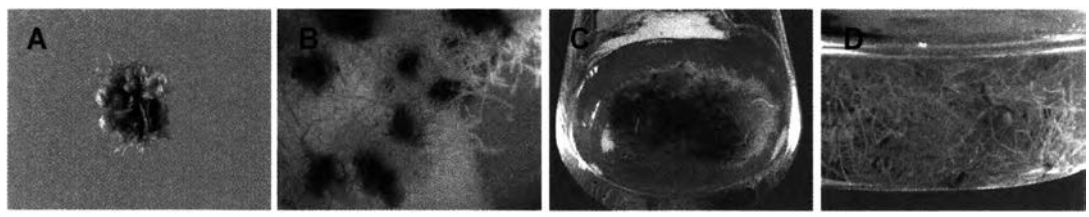

Figure 21 Root cultures of *P. indica*. A: Induction of roots from leaf explants on B5 medium supplemented with 1.0 mg/l NAA and 0.1 mg/l kinetin after 4 weeks of culturing, B: Development of the induced roots derived from leaf explants grown on MS liquid medium after 3 weeks of the initiation. C and D: The root cultures on MS liquid medium after 8 weeks of culturing.

1.3 *In vitro* propagation

Micropropagation of *P. indica* was performed with nodal segments of the plant. Firstly, the ability of these nodal segments on shoot induction were examined by using two different media: 1) MS medium supplemented with 0.1 mg/l IAA and 3.0 mg/l BA and 2) MS medium supplemented with 2.0 mg/l BA. The results showed that multiple shoots were induced from a lateral bud of a nodal segment on both hormonal conditions of the MS media. However, the number of shoots in the

presence of only BA was found to be less than that in the combined BA and IAA and thus the MS medium supplemented with 2.0 mg/l BA was used for the shoot induction. As shown in Figure 22A and B shoot elongation was observed as, nodal segments were transferred to the BA medium. After eight weeks of the culture, the regenerated shoots were separated and transferred to LS medium which contained no growth regulators for shoot elongation and root induction. Root development appeared to occur from the base of shoots after four weeks of culturing (Figure 22C). The percentage of root induction from shoots was found to be 100%. For long-term micropropagation, the nodal segments (1-1.5 cm in length) excised from the *in vitro* plantlets were cultured on the hormone-free LS medium and were subcultured for 12-week interval (Figure 22D and E). For subsequent experiments, both the aerial and root parts of the plantlets were used.

2. Detection and content determination of plumbagin and its derivatives

2.1 Detection of plumbagin in various tissue cultures of *P. indica*

In order to examine for the ability to produce plumbagin in various *in vitro* cultures, dried tissues of callus, cultured root and plantlets of *P. indica* were extracted under reflux with ethanol. After being evaporated to dryness, the residue was dissolved in methanol and plumbagin detected by reversed phase HPLC as described in Materials and Methods (section 3.2.2). In this experiment, the authentic plumbagin dissolved in methanol was also run parallelly in order to compare its retention time. Figure 23 shows the HPLC patterns of various tissue cultures of *P. indica*. Plumbagin could be detected in all the ethanolic extracts of the callus culture, root culture, and the aerial and root parts of the *in vitro* plantlets. These results suggested that the biosynthetic pathway of plumbagin could be expressed in various forms of *in vitro* cultures of *P. indica*.

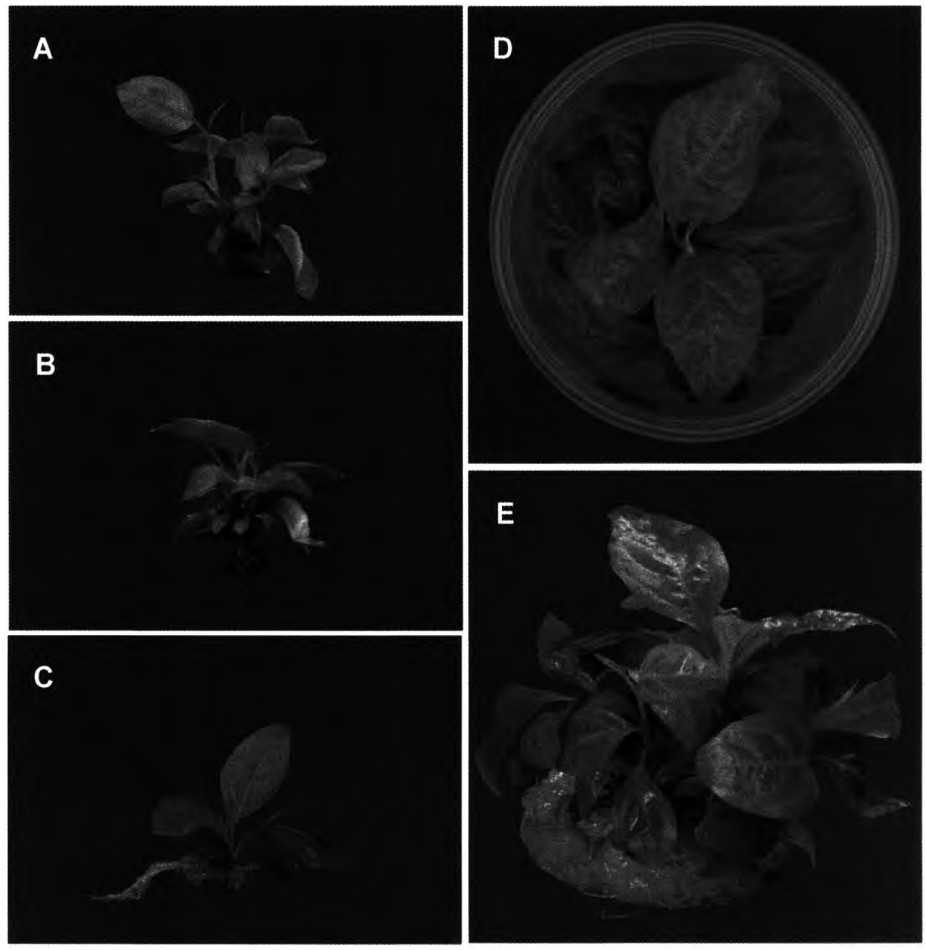

Figure 22 *In vitro* propagation of *P. indica*. A: Induction of multiple shoots from a nodal segment with a lateral bud on MS medium supplemented with 2 mg/l BA and B: on MS medium supplemented with 0.1 mg/l IAA and 3 mg/l BA after 8 weeks of culturing. C: Shoot elongation and root induction on LS medium without plant growth regulator, D and E: *In vitro* regenerated plantlets after 12 weeks of culturing on hormone-free LS medium.

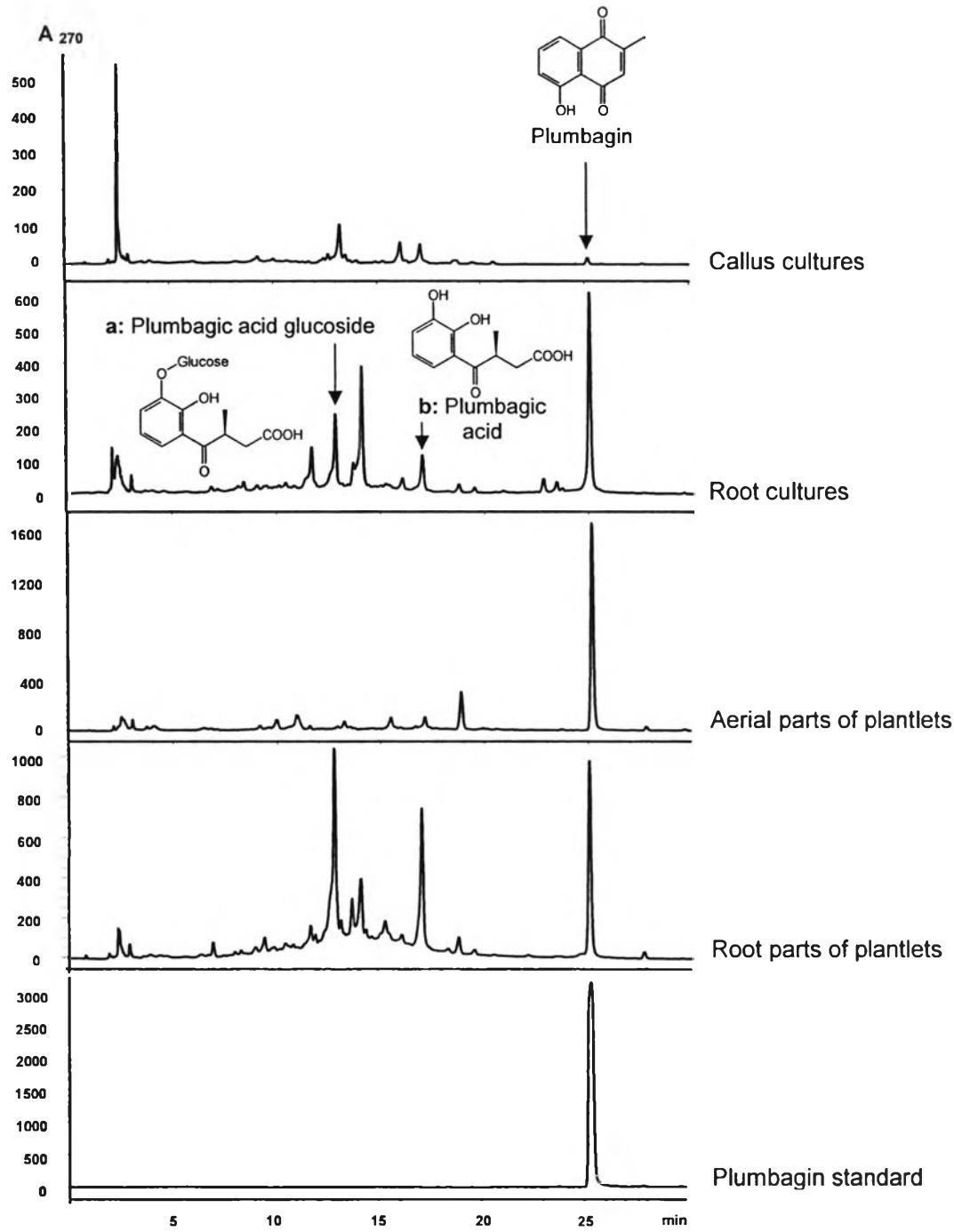

Figure 23 HPLC chromatograms of the ethanolic extracts of various *in vitro* cultures of *P. indica*. a: plumbagic acid glucoside and b: plumbagic acid.

2.2 Detection and identification of plumbagic acid and its glucoside in *P. indica* root culture and plantlet roots

In addition to plumbagin, attempts were also made to identify some other secondary metabolites present in the ethanolic extracts of the *in vitro* cultures, Among these the root cultures and roots of plantlets which showed to have a number of compounds were of particular interest. From the HPLC chromatograms shown in Figure 23, two peaks of **a** (Rt = 12.878 min) and **b** (Rt = 17.150 min), detected in the from crude extract from dried roots of *P. indica* plantlets, were prepared by preparative HPLC. By using LC-MS analysis as described in Materials and Methods (section 3.4), compound **a** showed a parent ion peak $[M+H]^+$ at *m/z* 387, and a fragment ion at *m/z* 225 corresponding to $[M+H-162]^+$, suggesting the loss of a hexosyl moiety from the molecular ion. Compound **b** showed its the molecular ion peak $[M+H]^+$ at *m/z* 225 and can therefore be identified as aglycone of compound **a**. The ESI-CID-MS spectra of **a** and **b** displayed a key ion at *m/z* 137 which seems to be typical for plumbagic acid. By comparison with previously reported data (Lin et al., 2003), it was found that the information was consistent with that of plumbagic acid glucoside (**a**) and plumbagic acid (**b**). Therefore, it can be concluded that plumbagic acid and its glucoside were produced in both the root cultures and plantlet roots of *P. indica*. For the other peaks in the HPLC chromatogram, their structure elucidation was not carried out.

2.3 Determination of plumbagin content

The quantitative determination of plumbagin was carried out by HPLC. The results of plumbagin content in various tissue cultures compared with the root part of *P. indica* plant grown in greenhouse are shown in Table 3. It can be seen that plumbagin contents in the aerial parts (0.79 $\pm$ 0.13 mg/g dry weight) and the roots (0.87 $\pm$ 0.09 mg/g dry weight) of *P. indica* plantlets were significantly higher than

those in the callus (0.013 ± 0.002 mg/g dry weight) and root cultures (0.28 ± 0.02 mg/g dry weight). In whole roots of the plants grown in the greenhouse, however, their plumbagin content appeared to be the highest (1.45 ± 0.25 mg/g dry weight). These results showed that various tissue cultures of *P. indica*, could essentially accumulate plumbagin in their tissues, and therefore, could be used as the starting materials for studying the enzyme and gene of polyketide synthase involved in the biosynthesis of plumbagin.

Table 3 Plumbagin content of roots of *P. indica* grown in the greenhouse and tissue cultures.

Plant material	Plumbagin[a] (mg/g dry weight)
Callus culture	0.013 ± 0.002
Root culture	0.28 ± 0.02
In vitro propagated plantlets	
- Aerial parts	0.79 ± 0.13
- Roots	0.87 ± 0.09
Whole roots of *P. indica* plants	1.45 ± 0.25

[a]The values are means of triplicate analyses ± standard deviation.

However, to confirm the identity of plumbagin detected in each sample, the peak of plumbagin in each HPLC chromatogram was routinely identified from its UV spectrum. As shown in Figure 24, plumbagin which had the same retention time in HPLC chromatogram as standard plumbagin, showed identical absorption spectrum with λ_{max} 210, 266, 420 nm. The peak was also confirmed by GC-MS based on base peak integration. The mass spectrum of plumbagin was shown in Figure 25.

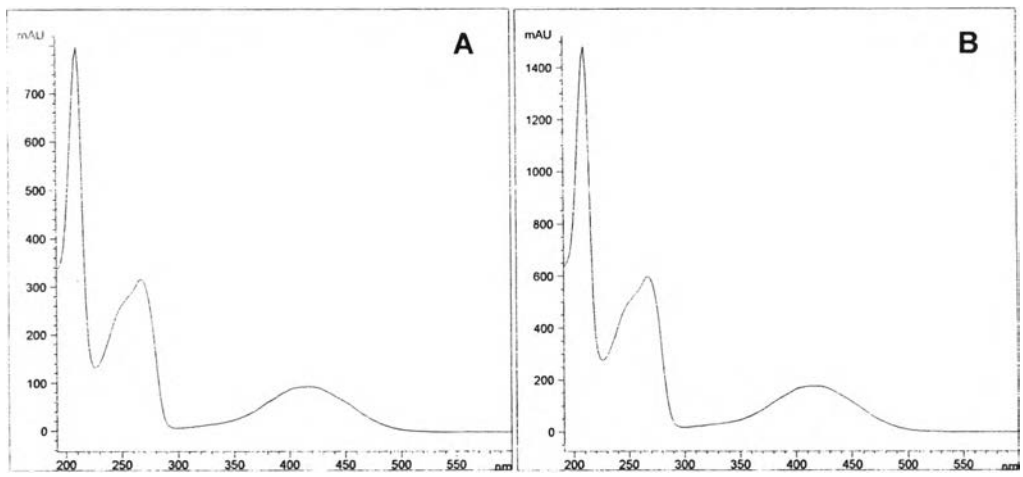

Figure 24 UV absorption spectra of authentic plumbagin (a) and the compound of similar retention time to plumbagin obtained from the ethanolic extract of *P. indica* roots (b)

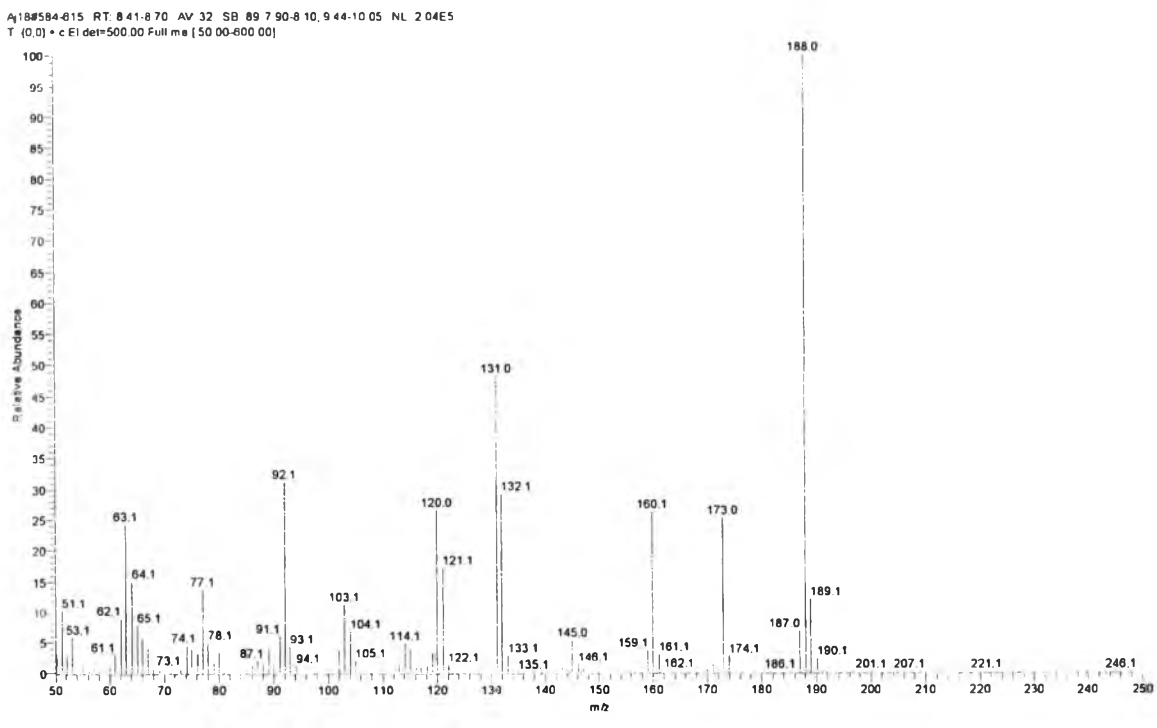

Figure 25 GC-MS spectrum of plumbagin

3. Detection of plant polyketide synthase activity in crude protein extracts

For an enzyme assay of plant polyketide synthase in crude protein extracts of *P. indica*, acetyl-CoA was used as a starter molecule and the radio-labeled [2-^{14}C] malonyl-CoA was used as the extension molecules for condensation to produce plumbagin. The reaction presumably needs NADPH for the complete function of the enzyme (Figure 26).

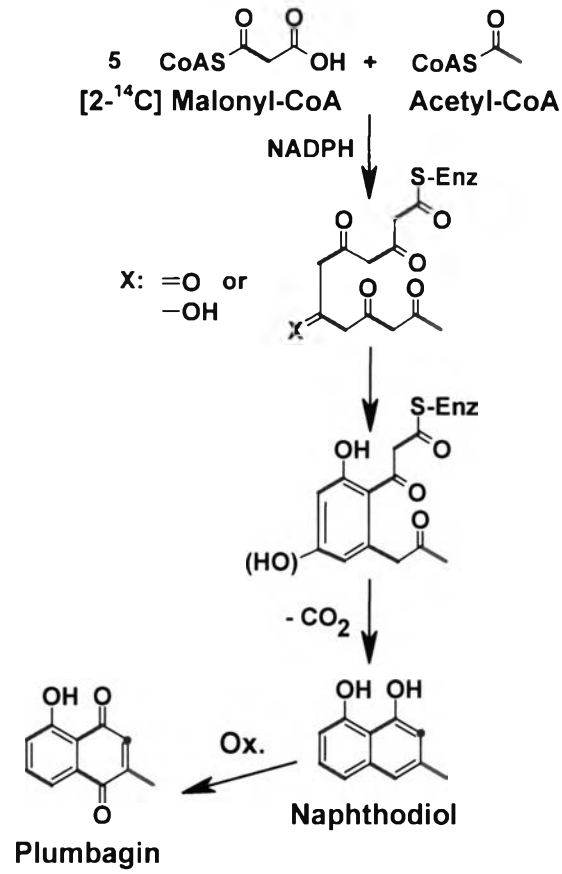

Figure 26 Proposed biosynthetic pathway of plumbagin.

Firstly, the aerial parts of *P. indica* plantlets were used as starting material for the preparation of crude protein extract. After desalting, the activity of the polyketide synthase in the crude extract was determined. The crude protein extract was incubated in the presence of acetyl-CoA and [2-^{14}C] malonyl-CoA using potassium phosphate buffer (pH 6.0) at 30 °C for 2 h. In some assays, the recombinant protein preparation of *Cassia alata* CHS (CalCHS1; Samappito et al., 2002) was used as reference enzyme for our assay system. After the reaction, the resulting radioactive products were then separated by TLC (silica gel 60 F254) with a solvent system of ethyl acetate: methanol: H_2O (100: 16.5: 13.5). The authentic compounds of plumbagin, diketide and 1,8-dihydroxy-3-methylnaphthalene were spotted on TLC to compare their Rf values for identification of the enzymatic product. The results from phosphorimaging of various reactions are shown in Figure 27. It can be seen that the resulting TLC radio-chromatogram of the reaction mixture with crude protein extract from *P. indica* plantlets had similar pattern to that of the boiled control. No appearance of the enzymatic products was detected as compared with the standard compounds and the main enzymatic product from CalCHS assay (triketide pyrone and tetraketide pyrone). These results suggested that the crude protein extract of *P. indica* plantlets could not catalyze the formation of plumbagin and other related enzymatic products.

In addition to the *in vitro* plantlets, crude protein extracts from the callus, root culture and root parts of plantlets were subsequently prepared and detected for the polyketide synthase activity, in comparison with their boiled controls. Again, after the process of incubation, solvent extraction, radioactive product separation and phosphorimaging detection, no enzyme activity was detected in all the enzyme sources (Figure 28). These results clearly showed that all the crude protein extracts prepared from plant tissue cultures of *P. indica* could not be detected for the plant polyketide synthase activity in our standard assay conditions.

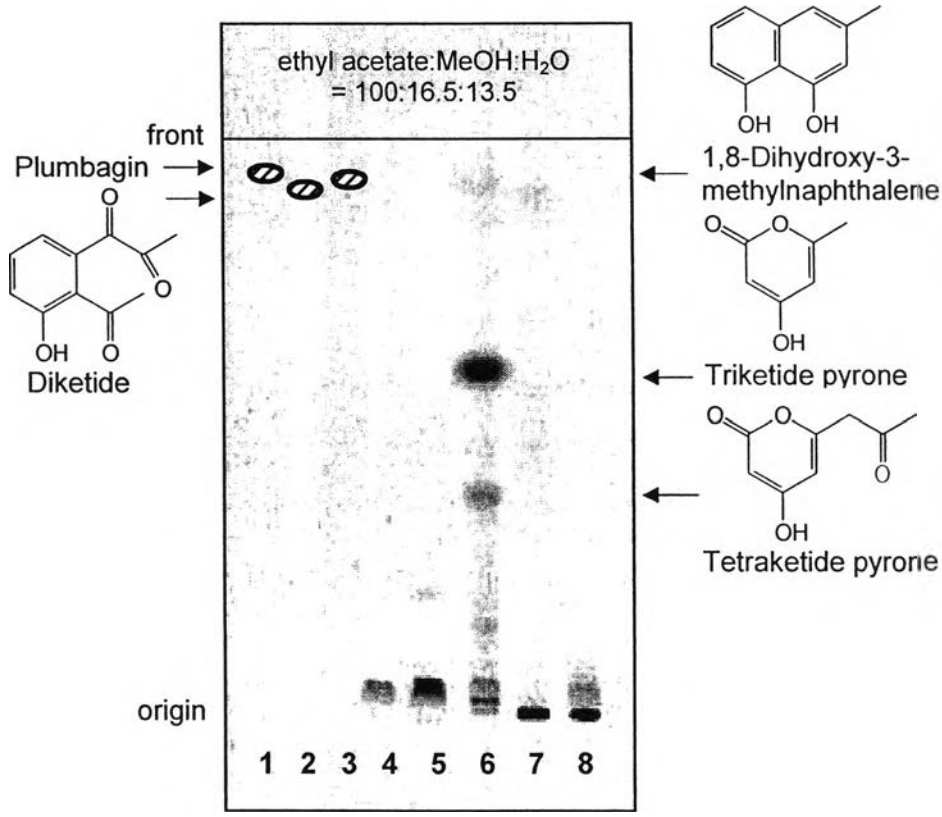

Figure 27 TLC-based analysis of radioactive products compared with authentic compounds of plumbagin (1), diketide (2) and 1,8-dihydroxy-3-methylnaphthalene (3) as reference standards. The reactions were performed with 30 µM acetyl-CoA, 70 µM [2-^{14}C] malonyl-CoA, 1 mM NADPH and 50 µl of desalted enzyme preparation obtained from the aerial parts of *P. indica* plantlets (4) and its boiled control (5). The recombinant protein CalCHS (6) was assayed as a standard protein. [1-^{14}C] acetyl-CoA (7) and [2-^{14}C] malonyl-CoA (8) were starter-CoA esters used in the reaction mixtures.

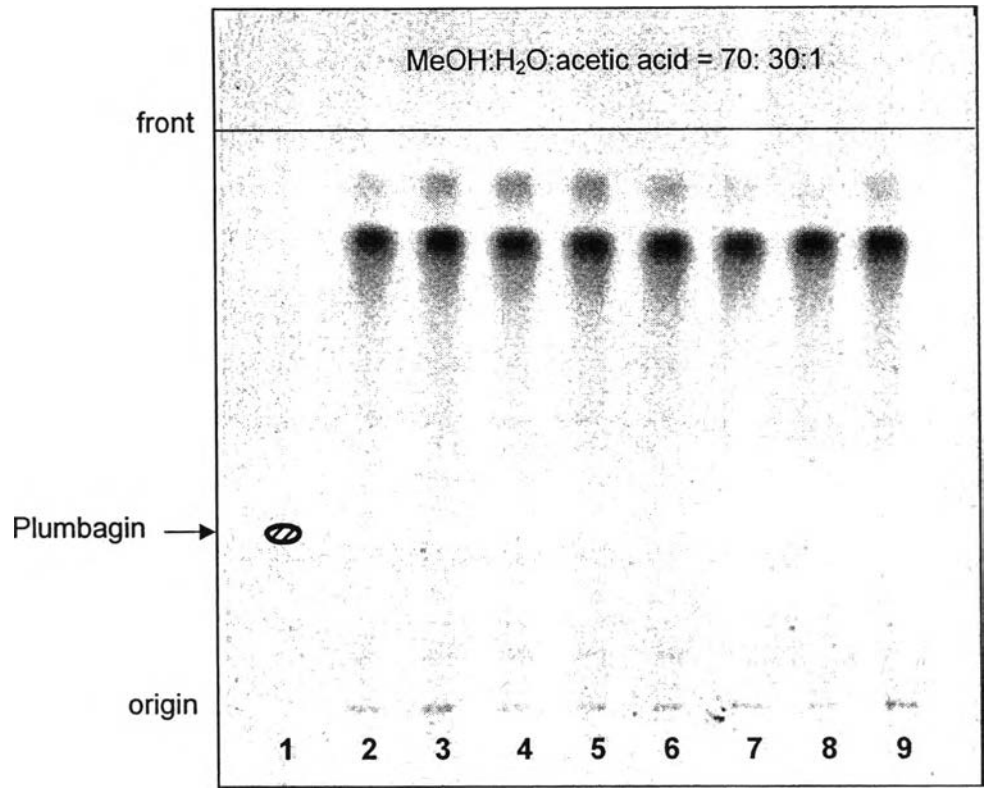

Figure 28 Reversed-phase TLC analysis of radioactive products compared with authentic plumbagin (1). The reactions were performed with 30 µM acetyl-CoA, 70 µM [2-^{14}C] malonyl-CoA, 1 mM NADPH and 50 µl of desalted enzyme preparation obtained from callus (2 and 3), root cultures (4 and 5), aerial parts (6 and 7) and root parts (8 and 9) *P. indica* plantlets. The crude enzymes (2, 4, 6 and 8) were boiled at 96 ºC for 30 min before being used.

4. Molecular cloning of plant polyketide synthase gene from *P. indica*

The cDNA cloning of a polyketide synthase from *Plumbago indica* (*Pin*PKS) was performed previously by Dr. Supachai Samappito of Mahasarakham University, Mahasarakham, Thailand (unpublished results). A cDNA was isolated from a cDNA library prepared from RNA from root tissue of *P. indica*. Recombinant PinPKS was expressed in *E. coli* BL21 (DE3) and assayed for polyketide synthase activity as described previously (Samappito, 2002).

4.1 The full length cDNA encoding plant polyketide synthase from *P. indica*

Figure 29 shows the full length gene of *Pin*PKS (1622 bp) encoding complete reading frame of 396 amino acid residues. The predicted molecular weight for the encoded protein is 43.0 kDa.

Comparison of the translated nucleotide sequences of cDNA with those present in the GenBank/EMBL databases indicated that PinPKS shows 57% identity with chalcone synthase (CHS2) from *Medicago sativa* (Ferrer et al., 1999) and alosone synthase (ALS) from *Rheum palmatum* (Abe et al., 2004). Similar to other members of the CHS superfamily, PinPKS contains the conserved Cys-His-Asn catalytic triad. However, three other residues which play an important role in shaping the architecture of the active site cavity are changed in comparison with *M. sativa* chalcone symthase (CHS2). Instead of Ter197, Gly256 and Ser338, PinPKS contains the amino acids Ala, Leu and Thr in these positions, respectively (Figure 30).

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1       ggcacgagggagctctctagcattcattatatattcctagagctaagctagtgcagttac        60
61      tgcatatagagatctagagatcgaagagcagtactgctactaactagctagctagcaaat        120
121     acatttgaaATGGCACCAGCAGTTCAATCTCAATCTCACGGTGGAGCATACCGCAGCAAT        180
                M  A  P  A  V  Q  S  Q  S  H  G  G  A  Y  R  S  N   -     17
181     GGTGAGAGGTCAAAAGGGCCAGCGACCGTGCTAGCCATTGCTACTGCTGTGCCACCAAAT        240
         G  E  R  S  K  G  P  A  T  V  L  A  I  A  T  A  V  P  P  N   -     37
241     GTATACTATCAGGATGAATATGCCGACTTTTTCTTCCGCGTCACCAACAGCGAGCACAAG        300
         V  Y  Y  Q  D  E  Y  A  D  F  F  F  R  V  T  N  S  E  H  K   -     57
301     ACTGCGATCAAGGAGAAGTTTAACCGAGTTTGCGGTACCTCGATGATTAAGAAGAGGCAC        360
         T  A  I  K  E  K  F  N  R  V  C  G  T  S  M  I  K  K  R  H   -     77
361     ATGTACTTCACCGAGAAGATGCTTAACCAAAACAAAAACATGTGCACCTGGGATGATAAA        420
         M  Y  F  T  E  K  M  L  N  Q  N  K  N  M  C  T  W  D  D  K   -     97
421     TCCCTCAACGCCCGTCAGGACATGGTGATCCCAGCAGTCCCCGAGCTCGGCAAAGAAGCC        480
         S  L  N  A  R  Q  D  M  V  I  P  A  V  P  E  L  G  K  E  A   -     117
481     GCCTTGAAGGCCATCGAGGAGTGGGGAAAACCACTCTCTAACATCACCCACCTCATCTTC        540
         A  L  K  A  I  E  E  W  G  K  P  L  S  N  I  T  H  L  I  F   -     137
541     TGCACCACAGCCGGTAACGACGCCCCTGGAGCAGACTTCAGGCTAACCCAGCTCCTTGGA        600
         C  T  T  A  G  N  D  A  P  G  A  D  F  R  L  T  Q  L  L  G   -     157
601     CTGAACCCATCAGTGAACCGGTACATGATCTACCAGCAGGGATGCTTCGCTGGAGCCACC        660
         L  N  P  S  V  N  R  Y  M  I  Y  Q  Q  G  C  F  A  G  A  T   -     177
661     GCACTCCGCATAGCCAAGGACCTTGCTGAGAACAACAAGGGTGCTCGTGTGCTCATTGTA        720
         A  L  R  I  A  K  D  L  A  E  N  N  K  G  A  R  V  L  I  V   -     197
721     TGCTGTGAGATCTTTGCTTTTGCATTCCGTGGACCTCATGAGGACCACATGGACTCTTTG        780
         C  C  E  I  F  A  F  A  F  R  G  P  H  E  D  H  M  D  S  L   -     217
781     ATTTGCCAGCTGCTGTTTGGGGATGGTGCAGCTGCTGTCATTGTCGGTGGTGATCCTGAC        840
         I  C  Q  L  L  F  G  D  G  A  A  A  V  I  V  G  G  D  P  D   -     237
841     GAGACCGAGAATGCACTCTTTGAGCTCGAGTGGGCCAACTCAACCATCATACCACAATCA        900
         E  T  E  N  A  L  F  E  L  E  W  A  N  S  T  I  I  P  Q  S   -     257
901     GAAGAGGCCATCACCCTTAGAATGCGCGAAGAAGGTCTCATGATCGGTTTGTCCAAGGAA        960
         E  E  A  I  T  L  R  M  R  E  E  G  L  M  I  G  L  S  K  E   -     277
961     ATCCCAAGGCTCCTAGGCGAACAGATCGAAAGCATTTTGGTCGAGGCTTTCACACCCCTT        1020
         I  P  R  L  L  G  E  Q  I  E  S  I  L  V  E  A  F  T  P  L   -     297
1021    GGAATTACTGACTGGAGCTCACTCTTCTGGATTGCCCACCCAGGTGGTAAGGCCATCCTT        1080
         G  I  T  D  W  S  S  L  F  W  I  A  H  P  G  G  K  A  I  L   -     317
1081    GAGGCACTGGAGAAGAAAATCGGCGTTGAAGGTAAGTTGTGGGCTTCGTGGCACGTTCTT        1140
         E  A  L  E  K  K  I  G  V  E  G  K  L  W  A  S  W  H  V  L   -     337
1141    AAAGAATATGGAAACTTGACCAGTGCTTGTGTGCTGTTCGCCATGGACGAAATGAGGAAG        1200
         K  E  Y  G  N  L  T  S  A  C  V  L  F  A  M  D  E  M  R  K   -     357
1201    AGGTCCATTAAGGAAGGGAAGGCCACTACTGGAGACGGACACGAATATGGTGTTCTCTTC        1260
         R  S  I  K  E  G  K  A  T  T  G  D  G  H  E  Y  G  V  L  F   -     377
1261    GGTGTCGGCCCGGGTCTTACCGTCGAGACAGTTGTGCTAAAAAGTGTGCCGCTTAACTAA        1320
         G  V  G  P  G  L  T  V  E  T  V  V  L  K  S  V  P  L  N  .   -     396
1321    gaagaagttagttcgttggtccgtccgtccgaatcgaaagtagctatatatatacccatt        1380
1381    tttattaagttatttaattatcgttgctgccgctgctggtggctactgctgctctactgc        1440
1441    tgctgcatgagaataaagtcatatatatattatgacaccgagctacgtacgtgcttcttt        1500
1501    agagctgcttctgtttcaacctctcgtgagtgaatccgtcgttgacaatgctctcttttg        1560
1561    tattgacgtgttctctttttgttttttttttttctttctttcttgcgctatcaaaaaaaa        1620
1621    aa                                                                  1622
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Figure 29 Nucleotide sequence and deduced amino acid sequence of the *P. indica* cDNA clone PinPKS.

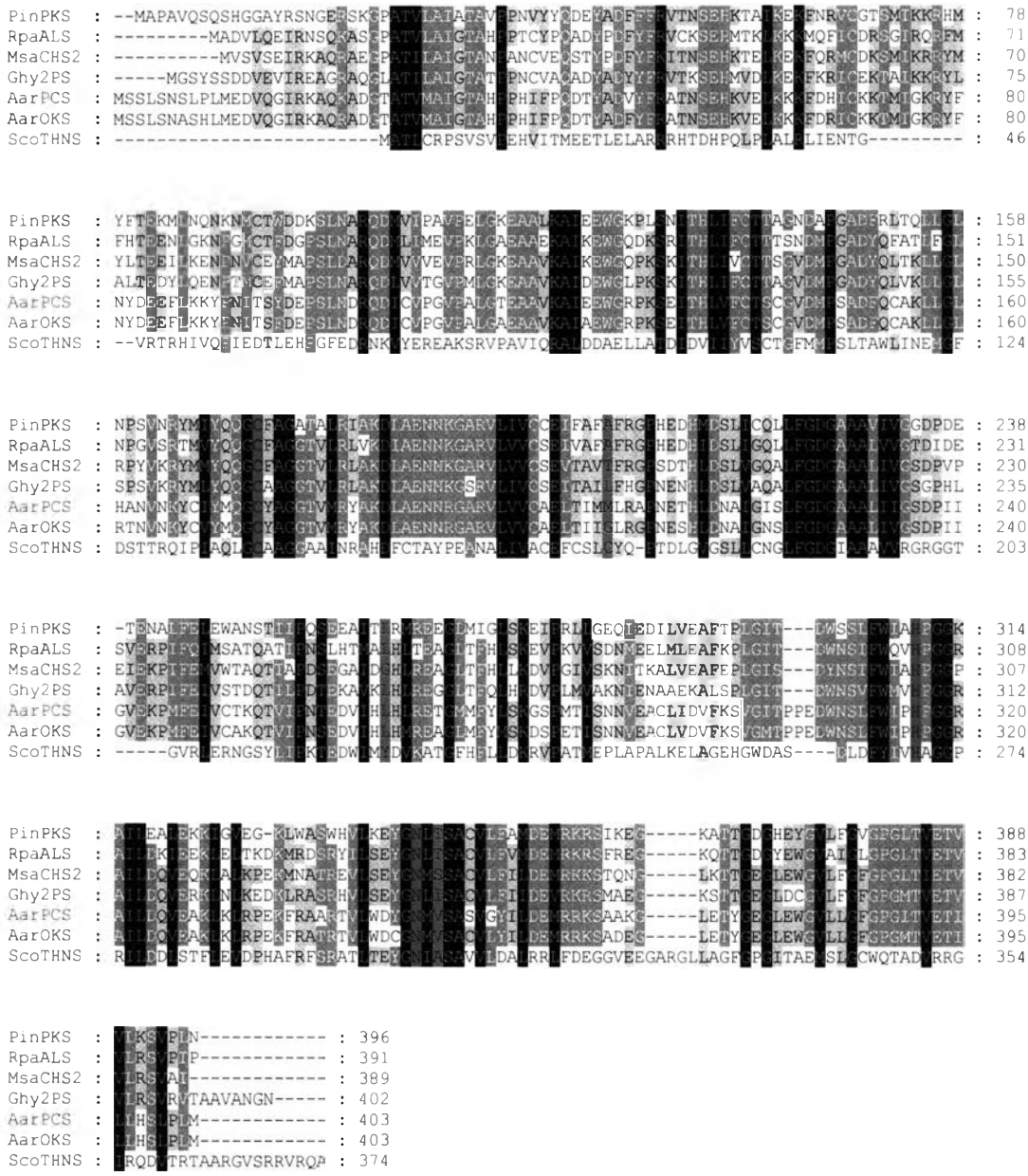


Figure 30 Alignment of PKS amino acid sequences. AarPCS, *Aloe arborescens* pentaketide chromone synthase (AAX35541); AarOKS, *Aloe arborescens* octaketide synthase (AAT48709); Ghy2PS, *Gerbera hybrida* 2-pyrone synthase (P48391); MsaCHS2, *Medicago sativa* CHS2 (P30074); PinPKS, *Plumbago indica* PKS; RpaALS, *Rheum palmatum* aloesone synthase (AAS87170); ScoTHNS, *Streptomyces coelicolor* THNS (NP_625495). Amino acids of the catalytic Cys-His-Asn triad are marked in red, positions crucial for the geometry of the active site are highlighted in blue.

4.2 Phylogenetic comparison of the deduced polypeptide sequence of *Pin*PKS

A phylogenetic tree was constructed using the Neighbour-Joining algorithm, and bootstrap values higher than 50 are shown The GenBank accession numbers of the plant and bacterial type III polyketide synthases used to construct the phylogenetic tree are *Aloe arborescens* OKS (AAT48709), *Aloe arborescens* PCS (AAX35541), *Amycolatopsis balhimycina* DpgA (CAC48378), *Arabidopsis thaliana* CHS (P13114), *Arachis hypogaea* CHS (AAO32821), *Aspergillus oryzae* csyA (BAD97390), *Aspergillus oryzae* csyB (BAD97391), *Cassia alata* CHS1 (AAM00230), *E. coli* FabH (1EBL_B), *Fusarium graminearum* FG08378.1 (XP_388554), *Gerbera hybrida* 2PS (P48391), *Hordeum vulgare* CHS1 (CAA41250), *Hordeum vulgare* CHS2 (CAA70435), *Humulus lupulus* VPS (BAA29039), *Hydrangea macrophylla* CHS (BAA32732), *Hydrangea macrophylla* CTAS (BAA32733), *Hydrangea macrophylla* STCS1 (AAN76182), *Hypericum androsaemum* CHS (AAG30295), *Hypericum androsaemum* BPS (AAL79808), *Magnaporthe grisea* MG04643.4 (XP_362198), *Medicago sativa* CHS2 (P30074), *Mycobacterium tuberculosis* PKS18 (AAK45681), *Pinus strobus* CHS (CAA06077), *Pinus strobus* STS1 (CAA87012), *Pinus sylvestris* CHS (CAA43166), *Pinus sylvestris* STS (AAB24341), *Pseudomonas fluorescens* PhlD (AAB48106), *Rheum palmatum* ALS (AAS87170), *Rheum palmatum* BAS (AAK82824), *Rheum tataricum* STS (AAP13782), *Ruta graveolens* ACS1 (S60241), *Streptomyces coelicolor* THNS (NP_625495), Zea *mays* CHS C2 (AAW56964) and Zea *mays* CHS WHP (CAA42763).

The relationships of PinPKS and other related polyketide synthases are shown in Figure 31. PinPKS seemed to be closely related to *R. palmatum* aloesone synthase (ALS) and thus seemed to form an isolated group. Other close neighbours are *G. hybrida* 2-pyrone synthase (2-PS) and *A. arborescens* pentaketide chromone synthase (PCS).

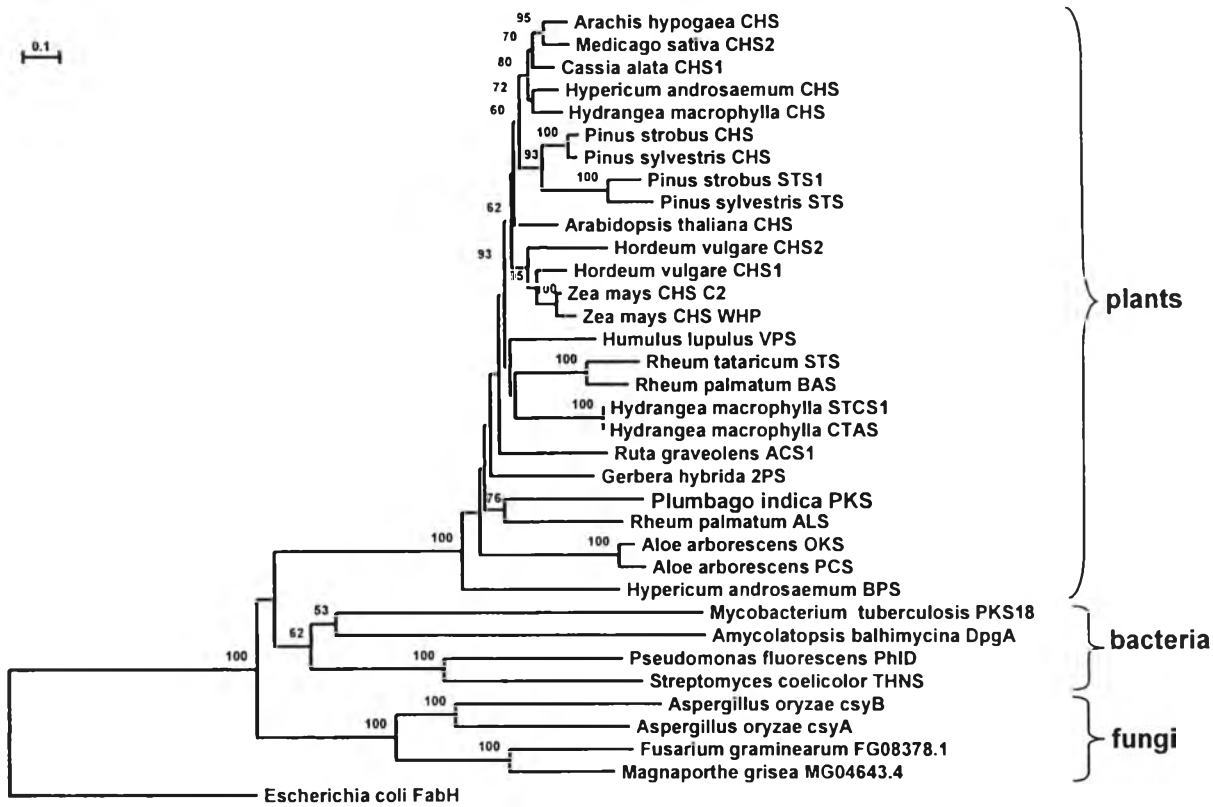


Figure 31 Phylogenetic comparison of the deduced polypeptide sequence of *Pin*PKS with type III PKSs from plants, fungi, and bacteria. *E. coli* β-ketoacyl synthase III (FabH) was used as outgroup. The phylogenetic tree was constructed using TREECON (Van de Peer and Wachter, 1994). Abbreviations: 2PS, 2-pyrone synthase; ACS, acridone synthase; ALS, aloesone synthase; BAS, benzalacetone synthase; BPS, benzophenone synthase; CHS, chalcone synthase; CTAS; *p*-coumaroyl triacetic acid lactone synthase; OKS, octaketide synthase; PCS, pentaketide chromone synthase; STCS, stilbenecarboxylate synthase; STS, stilbene synthase; VPS, valerophenone synthase; THNS, 1,3,6,8-tetrahydroxynaphthalene synthase.

4.3 Heterologous expression of plant polyketide synthase from PinPKS in *E. coli*

Expression of the full-length gene was performed in the *E. coli* pET expression system (Novagen). The open reading frame (ORF) of the putative polyketide synthase from *Plumbago* (*PinPKS*) was ligated into the pET-14b vector containing an amino terminal hexahistidine fusion tag. The expression vector was transformed in the expression host *E. coli* BL21 (DE3). The recombinant protein was then purified by immobilized metal affinity chromatography on cobalt resin as described in Materials and Methods. SDS-PAGE results are shown in Figure 32. It was found that the purified enzyme gave a single band with a molecular mass of 43 kDa.

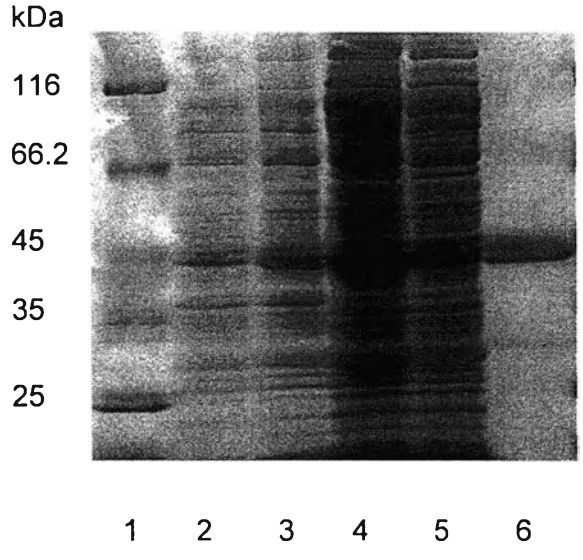


Figure 32 SDS-PAGE analysis of the purification of recombinant PinPKS. The protein from each individual purification step was applied on the 10% SDS gel and visualized with Coomassie Brilliant Blue R250. Lane 1: molecular mass markers (kDa), Lane 2: non-induced *E. coli* cells, Lane 3: induced cell, Lane 4: total cellular lysate, Land 5: Talon column flow-through, Lane 6: PinPKS purified by affinity chromatography with a Talon cobalt resin.

5. Enzyme assay and enzymatic product identification

5.1 Enzymatic products of PinPKS

The standard assay for the enzyme activity of PinPKS was again performed using acetyl-CoA and [2-^{14}C] malonyl-CoA as substrates, as described in Materials and Methods (section 16.1). The radioactive products were analyzed by TLC (silica gel 60 F254) with a solvent system of ethyl acetate: methanol: H_2O (100:16.5:13.5) and visualized by phosphorimaging as shown in Figure 33. It was found that the PinPKS produced two known pyrone compounds and several other unidentified products. However, plumbagin was not detected in this assay.

In order to confirm the enzymatic products, the reaction mixtures of PinPKS assay with acetyl-CoA and unlabeled malonyl-CoA was scaled-up as described in Materials and Methods (section 17.1). The enzymatic products were analyzed by reversed phase HPLC, compared with other assay conditions. As shown in the HPLC chromatogram (Figure 34), the reaction mixture of PinPKS assay showed the presence of the two major peaks at retention time = 10.746 min and 15.145 min which corresponded to triketide pyrone and hexaketide pyrone and several minor peaks of the unidentified products. No appearance of such peaks at the same retention times in the negative control and the reaction mixtures without acetyl-CoA and malonyl-CoA. The results clearly indicated that the products were formed by the enzymatic reaction. Again, plumbagin was not detected.

For identifying of the unknown enzymatic products, the technique of LC-ESI-MS was introduced. The resulting LC-ESI-MS spectra of various compounds (Figure 35) that there are showed parent ion peaks $[M-H]^-$ at *m/z* 125, 167, 233 and 251 (2 peaks). Between the two peaks of the $[M-H]^-$ at *m/z* 251, the one with the Rt = 22.90 min was identified as a long-side chain hexaketide pyrone where as the other one with the Rt = 3.23 min was identified was a cyclized hexapyrone.

The former showed its ions in the CID mass spectrum as: 251 (63), 209 (3), 207 (37), 183 (1), 165 (8), 141 (2), 125 (100), 123 (4), and 82 (2). The latter (cyclized hexapyrone) showed its mass spectrum as 251 (66), 191 (6), 177 (1), 159 (100), 133 (37), 117 (7), 115 (13), 99 (2), 91 (19), 85 (5), 73 (3), and 57 (17). In high resolution MS analysis, the one compound with the M^- peak at *m/z* 209.04505 was detected. The proposed structures of the products based on their mass fragmentation are summarized in Table 4. These included triketide pyrone, tetraketide pyrone, pentaketide pyrone and 3hexaketide pyrones. Therefore, it seemed that PinPKS catalyzed the condensation reaction of acetyl-CoA (one molecule) with malonyl-CoA sequentially up to five molecules to synthesize the various numbers of ketide pyrones and the putative hexaketides (*m/z* 251 and 233). It should be noted that in this experiment plumbagin was not detected.

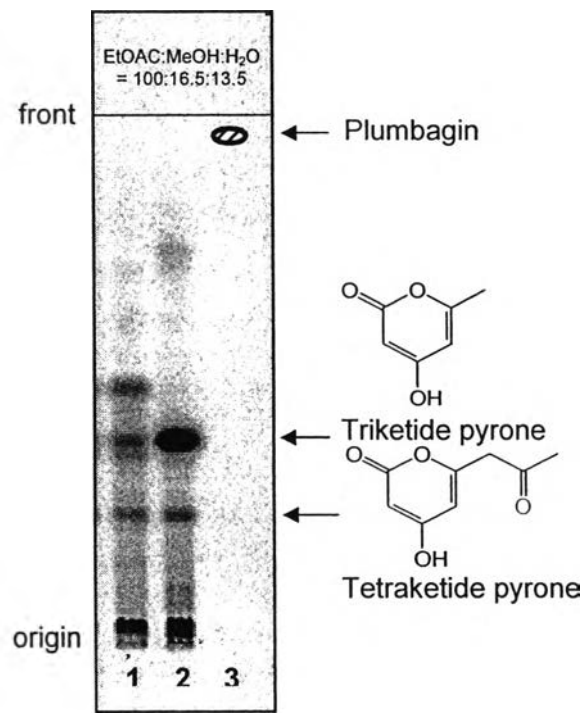


Figure 33 TLC analysis by phosphoimaging of PinPKS reaction. The reactions were performed with 30 µM acetyl-CoA, 70 µM [2-^{14}C] malonyl-CoA, 1 mM NADPH and 10 µg of recombinant PinPKS (1). The products were identified by comparing with CalCHS reaction products (2) and authentic plumbagin (3).

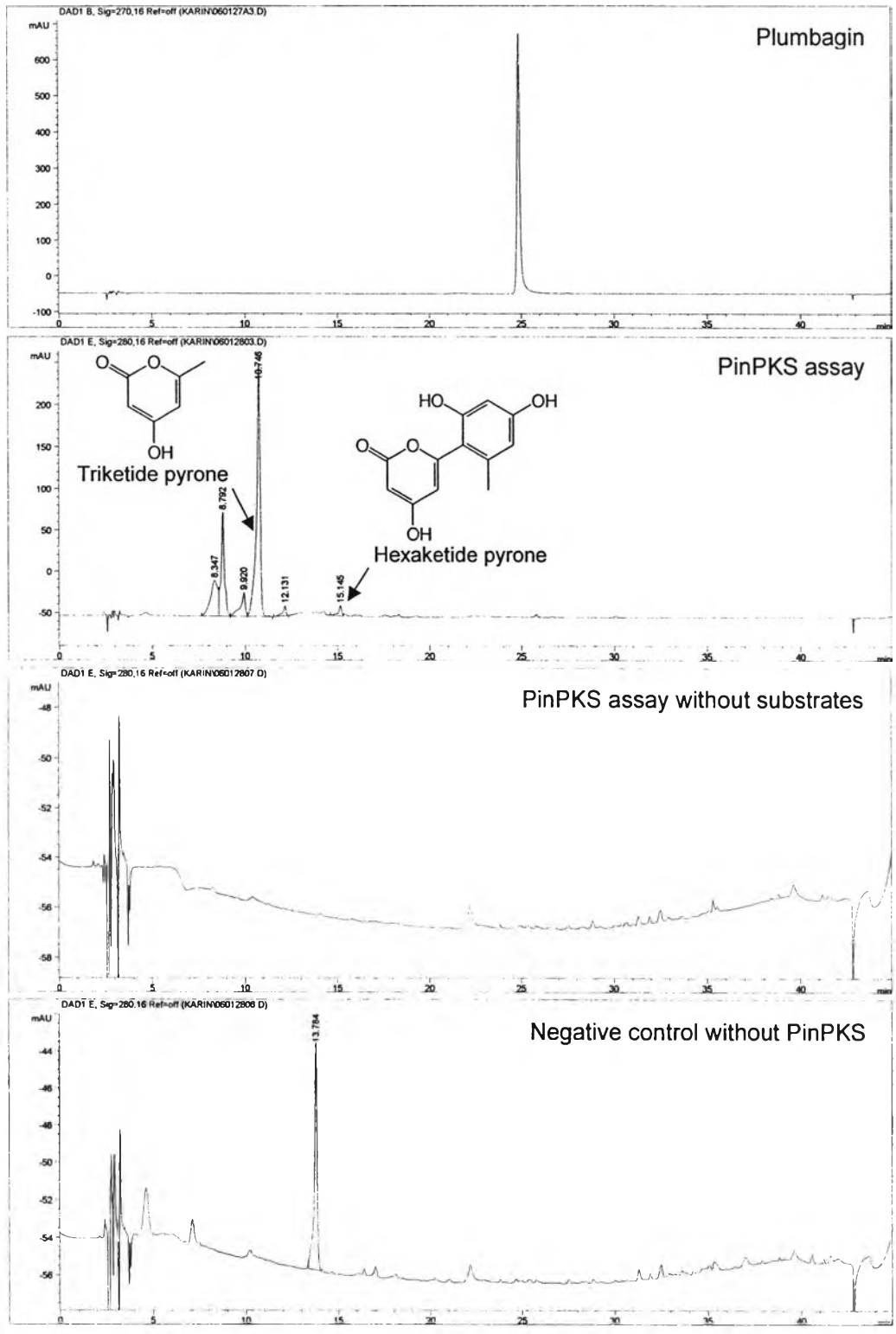


Figure 34 HPLC analysis of PinPKS reaction. The reactions were performed with 150 µM acetyl-CoA, 350 µM malonyl-CoA, 5 mM NADPH and 40 µg of PinPKS compared to the reaction mixtures without acetyl-CoA and malonyl-CoA as substrates and the negative control. The chromatograms were monitored at the wavelength of 280 nm.

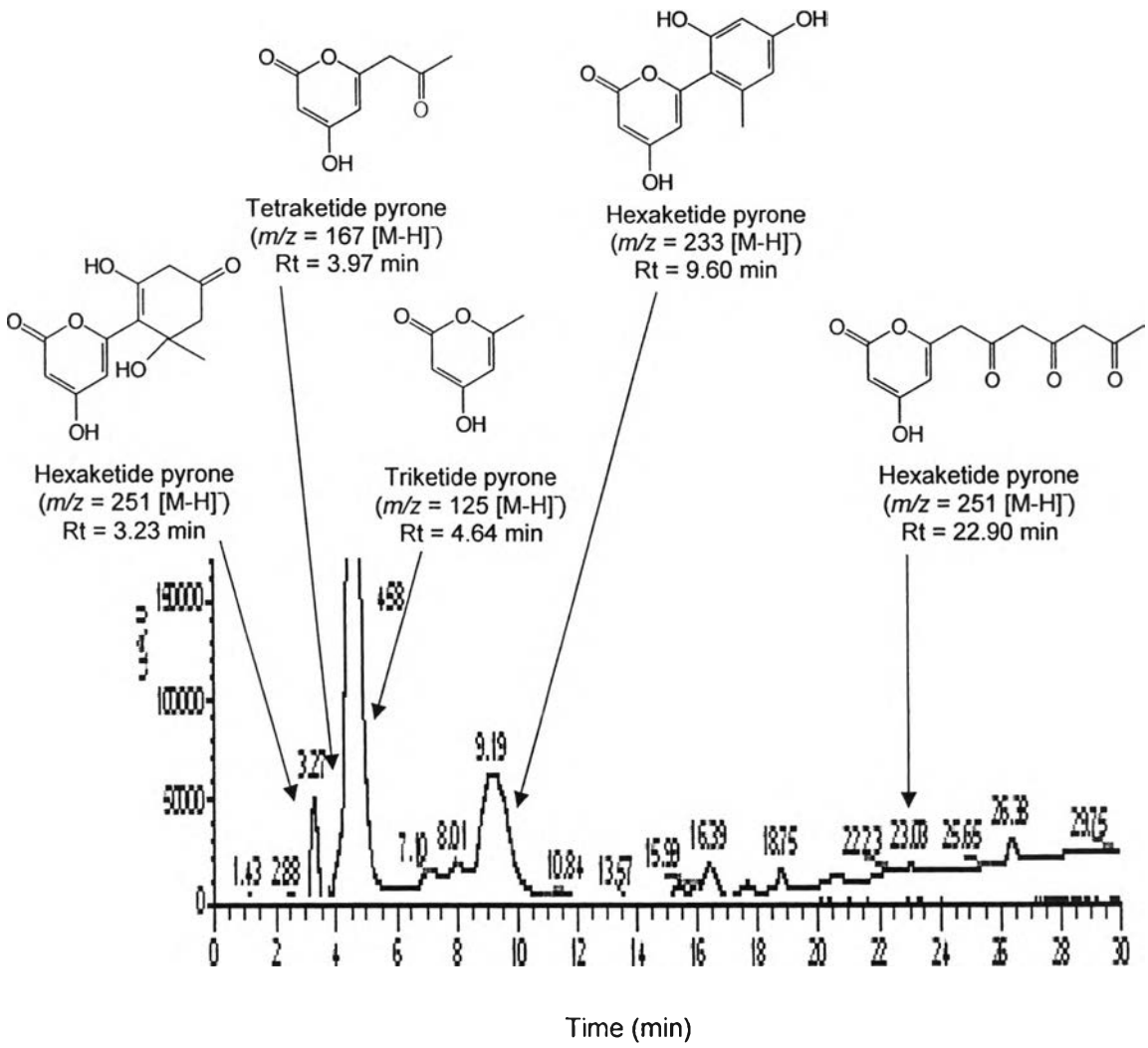
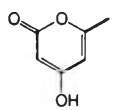
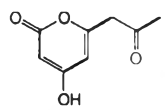
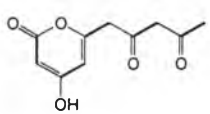
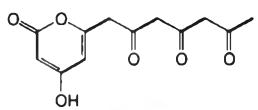
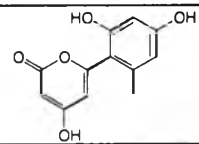
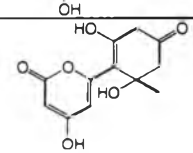


Figure 35 Total scan PDF obtained from LC-ESI-MS analysis of the enzymatic products produced from acetyl-CoA and malonyl-CoA as co-substrates by recombinant PinPKS. These peaks corresponded with the structure of enzymatic products as shown in Table 4.

Table 4 LC-MS analysis of the enzymatic products from acetyl-CoA and malonyl-CoA as co-substrates by recombinant PinPKS assay: Collision-induced dissociation (CID) mass spectra obtained from the $[M-H]^-$ ions by LC-ESI-MS and elemental composition obtained by HR-MS.

Product type	Rt (min)	$[M-H]^-$	Ions in the CID mass spectra (m/z), rel. int	CE (eV)	HR-MS Elemental composition	Proposed structure
Triketide pyrone	4.64	125	125 (42), 83 (5), 81 (100), 41 (4)	+20	not determined	
Tetraketide pyrone	3.97	167	167 (59), 125 (100), 123 (35), 99 (6), 83 (12), 81 (14), 75 (2), 65 (8), 57 (3)	+20	not determined	
Pentaketide pyrone	n.d.	209			m/z 209.04505 $[M-H]^-$ (calcd. For $C_{10}H_9O_5^-$ 209.04555	
Hexaketide pyrone	22.90	251	251 (63), 209 (3), 207 (37), 183 (1), 165 (8), 141 (2), 125 (100), 123 (4), 83 (2)	+20	m/z 251.05575 $[M-H]^-$ (calcd. For $C_{12}H_{11}O_6^-$ 251.05611	
Hexaketide pyrone	9.60	233	233 (50), 191 (12), 189 (100), 165 (51), 147 (35), 145 (2), 121 (1)	+20	m/z 233.04498 $[M-H]^-$ (calcd. For $C_{12}H_9O_5^-$ 233.04555	
Hexaketide pyrone	3.23	251	251 (66), 191 (6), 177 (1), 159 (100), 133 (37), 117 (7), 115 (13), 99 (2), 91 (19), 85 (5), 73 (3), 59 (17)	+20	not determined	

n.d. not detected

5.2 Enzymatic products of PinPKS assay coupled with crude protein extracts of *P. indica*

To examine additional unknown co-factors required in the reaction mixture for the formation of plumbagin, a coupled assay containing the recombinant *Pin*PKS and the crude protein extracts of *P. indica* were carried out. The crude protein extracts were prepared from callus, root culture and plantlets of *P. indica* and were added individually into the standard reaction mixtures. The reaction mixture contained 20 µM acetyl-CoA, 70 µM [2-^{14}C] malonyl-CoA, 1 mM NADPH and both 10 µl of recombinant *PinPKS* and 25 µl of crude protein extract prepared from each *P. indica* tissue culture. The radioactive products of each reaction were separated by TLC (silica gel 60 F254) using several solvent systems and viewed after subjected to phosphorimaging. As shown in Figure 36, it was found that a new spot of enzymatic product (Rf = 0.06) was formed in this coupled assay with all the crude protein extracts on TLC with the solvent system of toluene: formic acid (99:1). Among these, the coupled assay with the crude protein extract from the root culture produced higher radioactivity of the product than those of the other crude protein extracts. From these results, the root culture was used for the preparation and identification of the unknown enzymatic product.

With the crude protein extract from the root culture, it was found that the coupled assay containing acetyl-CoA, [2-^{14}C] malonyl-CoA and NADPH produced an unknown enzymatic product **1a** (lane 5, Figure 37). This product of **1a** was not detected when only the recombinant PinPKS assay was used (lane 4) and interestingly, in the absence of NADPH as well (lane 8, Figure 37). Furthermore, it can be seen that the appearance of **1a** occurred concomitantly with the disappearance the long side-chain hexaketide pyrone (**1b**) in the reaction mixture. Therefore, it seems to be that the unknown product was formed from **1b** by an oxidation-reduction reaction.

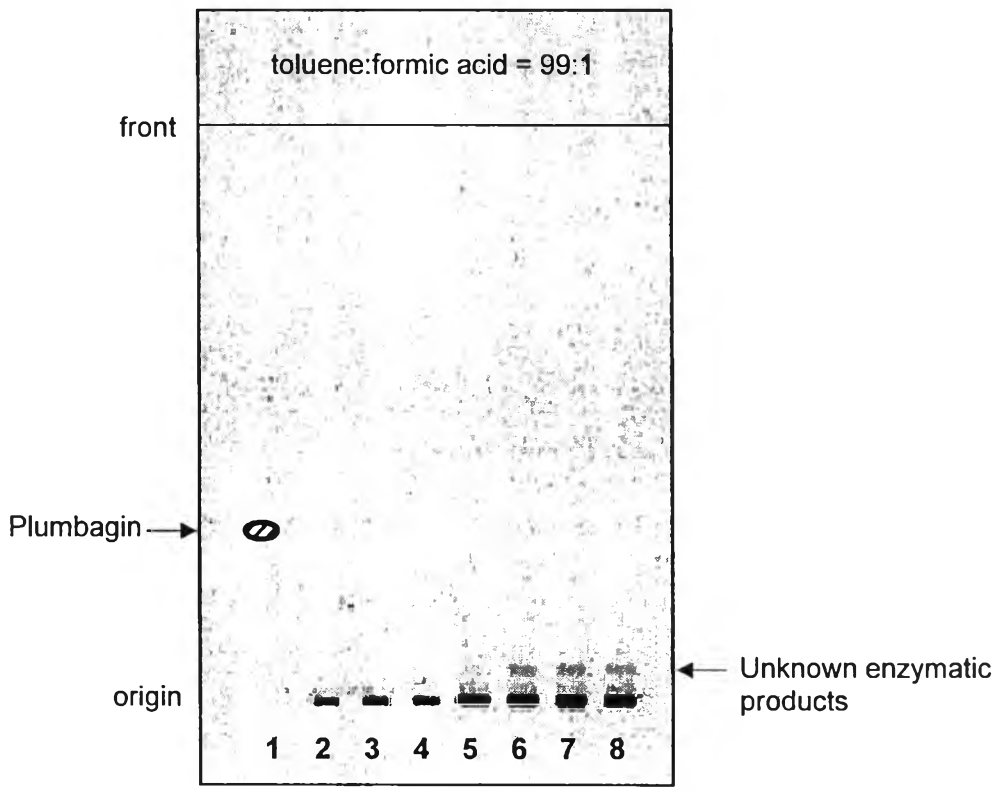


Figure 36 TLC analysis by phosphorimaging of the radioactive products of enzyme assays. The reactions were performed with 30 µM acetyl-CoA, 70 µM [2-^{14}C] malonyl-CoA, 1 mM NADPH and desalting enzyme prepared from crude protein extract of *P. indica* tissue cultures (2: callus, 3: root culture, 4: aerial parts of plantlets), recombinant PinPKS (5) and the coupled PinPKS and crude protein extract from *P. indica* tissue cultures (6: callus, 7: root culture, 8: aerial parts of plantlets).

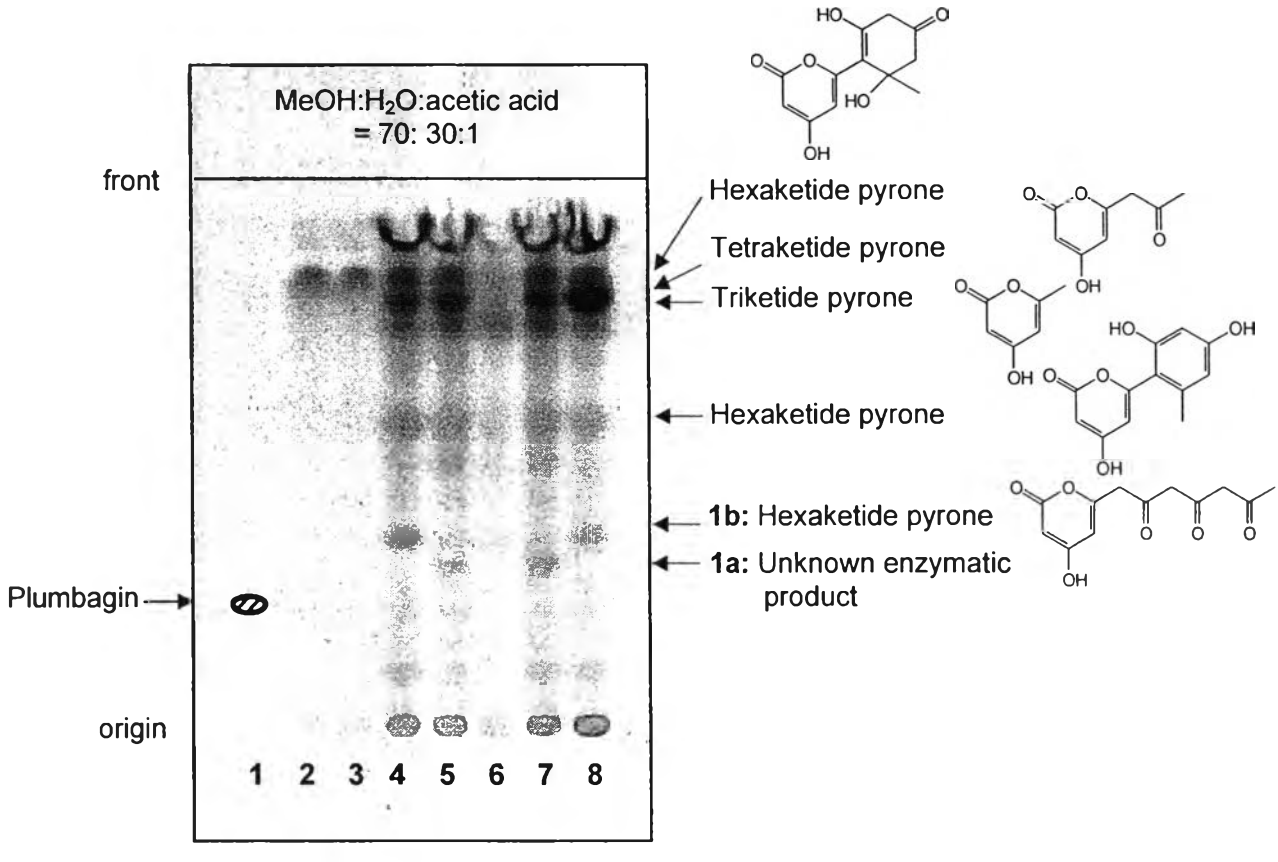


Figure 37 Reversed phase TLC analysis of the enzymatic products formed in different reaction mixtures compared with authentic plumbagin (1). The standard reactions were performed with 30 μM acetyl-CoA, 70 μM [2-^{14}C] malonyl-CoA , 1 mM NADPH with the crude protein extract of *P. indica* root culture (the boiled enzyme (2) and crude protein extract (3)), PinPKS assay (4) and the coupled assay of PinPKS with crude protein extract in different reaction mixtures (5-8). In the coupled assay, various substrates were used. [1-^{14}C] acetyl-CoA substituted [2-^{14}C] malonyl-CoA in the reaction 6. The coupled assays were performed without acetyl-CoA (7) and without NADPH (8).

To examine the formation of the unknown enzymatic product, the preparation of recombinant PinPKS assay with acetyl-CoA and radiolabeled malonyl-CoA was scaled-up. The radioactive products were analyzed by reversed phase HPLC connected with radio-detector. As shown in Figure 38, it was found that in the coupled assay, the enzymatic product **1b** was, again, was found to disappear concomitantly with the appearance of the unknown enzymatic product **1a**. No other peaks were changed under the assay conditions. It is likely that enzymatic product **1b** of PinPKS was used as substrates for the crude enzyme to produce the unknown enzymatic product **1a** in the coupled assay.

For identifying the enzymatic product of **1a**, the preparation of the coupled assay with PinPKS and crude protein extract, again, was scaled-up using unlabeled substrates followed by analysis of the product by LC-ESI-MS. However, due to insufficiency of the product for analysis, the structure of **1a** is still unknown.

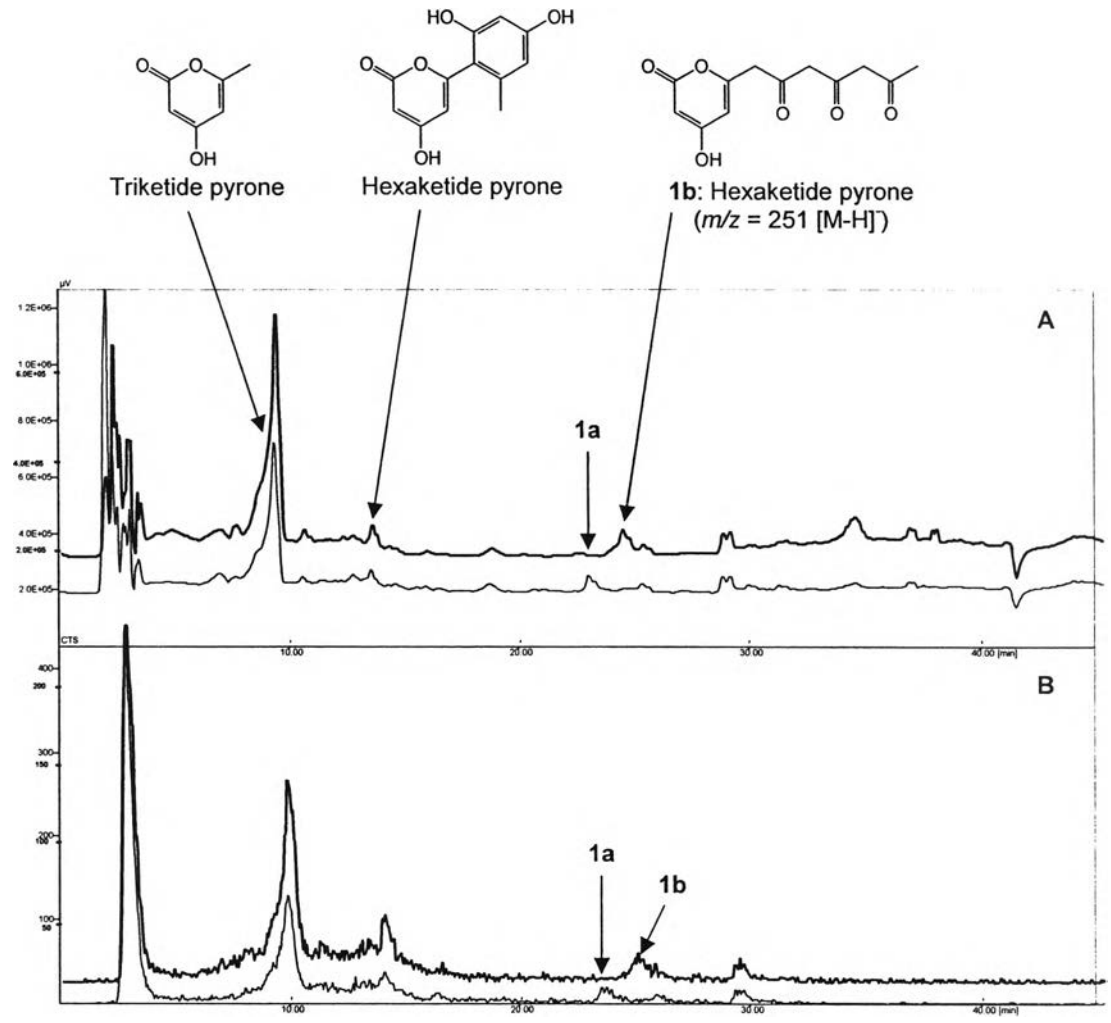


Figure 38 Radio-HPLC profiles of the enzymatic products of the reaction mixtures of PinPKS assay (—) and the coupled PinPKS with crude protein extract prepared from *P. indica* root cultures (—). The enzymatic products were detected at UV 280 nm (A) and the radioactivity of products were measured (B).