CHAPTER V

DISCUSSION

Plumbago indica L. is a medicinal plant containing plumbagin, an important naphthoquinone with a broad range of biological activities. The biosynthetic pathway of plumbagin involves an early formation of a hexaketide chain, that is catalyzed by a polyketide synthase enzyme (Figure 1) (Duran and Zenk, 1971). So far, a polyketide synthase (PKS) enzyme and gene involved in plumbagin biosynthesis have not yet been discovered. Therefore, in this study, we aimed to find the enzyme and gene in this plumbagin-producing plant of *P. indica*.

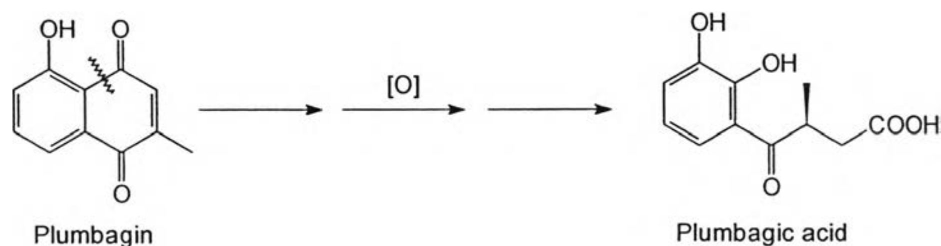
To obtain suitable materials for the study, various *P. indica* tissue cultures were first established and examined for their potential in producing plumbagin. These included callus culture, root culture and micropropagated plantlets. It appears that these three types of *in vitro* cultures appeared to need different media and hormonal composition. The callus culture can be initiated and maintained on MS medium with 1.0 mg/l 2,4-D and 0.1 mg/l BA. The root culture can be induced on B5 medium containing 1.0 mg/l NAA and 0.1 mg/l kinetin and maintained in the hormone-free MS medium. The regenerated plantlets, on the other hand, can be successfully induced and maintained on LS medium without addition of plant growth regulators. In literature, MS media has been the most commonly used for plant cell culture and have proved to be effective for the growth of variety of plants (Dixon, 1985), including callus, cell cultures (Komaraiah et al.,2003a; 2003b) and *in vitro* propagation of *P. indica* (Satheeshkumar and Seeni, 2003). B5 medium have been reported previously for root induction from *P. indica* leaf explants (Panichayupakaranant and Tewtrakul,

2002). For the effect of plant growth regulators on explant regeneration, it has been known that the medium containing high auxin levels will induce callus formation. Inclusion of cytokinins with auxins may be beneficial for promotion of callus formation in some species. Lowering the auxin concentration and increasing the cytokinin concentration is traditionally performed to induce shoot organogenesis. Rooting is usually obtained from cultured shoots or plantlets by inclusion of low levels of auxins or hormone-free MS medium (Dixon, 1985).

In terms of plumbagin content, it appears that the plantlets contain plumbagin higher than the callus and root cultures. The highest plumbagin content is, however, found in whole root of *P. indica* plants (Figure 23 and Table 3). Plumbagin has been reported to produce from *in vitro* cultures of various plants. The production of plumbagin was found to be 4.9 mg/g dry weight (DW) in callus culture (Komaraiah et al., 2003a) and 0.129 $\pm$ 0.0139% DW in root cultures of *P. indica* (Panichayupakaranant and Tewtrakul, 2002). The hairy root culture of *P. zeylanica* contained plumbagin in yield of 0.042% fresh weight (FW) (Verma et al., 2002) and suspension cultures of *Drosophyllum lusitanicum* were 1-1.5 % FW (Nahálka et al., 1996).

In addition to plumbagin, we also found plumbagic acid and its glucoside in our *in vitro* cultures. Both compounds have previously been reported to be present in the root of *P. zeylanica* (Lin et al., 2003). In this study, plumbagic acid and its glucoside were detected in both the root cultures and plantlet roots of *P. indica* (Figure 23).

The presence of plumbagic acid and its glucoside in plumbagin-containing plants has been reported in *Ceratostigma willmottianum* (Yue et al., 1997) and *P. zeylanica* (Lin et al., 2003). So far there has been no report on the biosynthetic studies of plumbagic acid and its glucosides in plants. Based on their structures, however, it might be possible that plumbagic acid is a degradative product of plumbagin which occurs through an oxidative reaction:



Attempts were made to find the enzyme activities of polyketide synthase in various types of tissue cultures. However, no enzymatic products were detected in any cases that we tried. It is possible that PKS enzyme in the crude protein extracts is inactive when it is removed from its native environment as an intracellular protein as a result of the breakdown of subcellular compartmentalization. Furthermore, the low concentration of enzymes in the crude protein extracts might also limit the detection of the enzyme activity.

The unsuccessful search of PKS enzyme activity in the crude enzyme extracts led us change to use the method of molecular cloning for studying the enzyme. As a result, a full-length cDNA clone *PinPKS* with 1622 bp was isolated successfully (Figure 29). The enzyme PinPKS appears to contain the important conserved Cys-His-Asn catalytic triad (Figure 30). However, three other residues which play an important role in shaping the architecture of the active site cavity appears to be changed as compared with the enzyme chalcone synthase (CHS). Instead of being Thr197, Gly256 and Ser338 (numbers were specified according to *M. sativa* CHS2), PinPKS contains the amino acids Ala, Leu and Thr in these positions, respectively. The three residues are sterically altered in a number of functionally diversity type III PKSs. These includ *Gerbera hybrida* 2-PS (Thr197Leu/Gly256Leu/Ser338Ile) (Eckermann et al., 1998), *Aloe arborescens* pentaketide chromone synthase (PCS) (Thr197Met/Gly256Leu/Ser338Val) (Abe et al., 2005b), *Aloe arborescens* octaketide

synthase (OKS) (Thr197Gly/Gly256Leu/Ser338Val) (Abe et al., 2005a) and *Rheum palmatum* ALS (Thr197Ala/Gly256Leu/Ser338Thr) (Abe et al., 2004).

In the heptaketide-producing *R. palmatum* ALS, the three residues, Ala197, Leu256, and Thr338 (numbers according to *M. sativa* CHS2) are also found in PinPKS (Figure 30). These active site residues, lining the initiation/elongation cavity, have been shown to control the steric modulation of enzyme facilitates ALS to utilize the small acetyl-CoA starter while providing adequate volume for the additional polyketide chain extensions (Abe et al., 2004; 2006).

Further examination on the relationship between PinPKS and related polyketide synthases by phylogenetic analysis of type III PKSs (Figure 31) suggests that PinPKS is closely related to *R. palmatum* ALS. The phylogenetic tree also shows PinPKS is located near the groups of non-chalcone-forming enzyme including *G. hybrida* 2-PS and *A. arborescens* PCS and OKS.

Expression of the full-length gene in *E. coli* BL21 (DE3) followed by enzyme characterization shows that the recombinant PinPKS has its molecular mass of 43 kDa (Figures 32). This is consistent with the structural plant type III PKS enzymes that have been reported to be a homodimer of 40-45 kDa proteins (Schröder, 1999; Austin and Noel, 2003). The enzyme catalyzes the condensation reaction of acetyl-CoA (one molecule) with malonyl-CoA (up to five molecules) to produce a series of polyketide pyrones, including triketide, tetraketide, pentaketide (one each) and three hexaketide pyrones. Among these, the triketide pyrone appears to be the major product. The triketide and tetraketide pyrones have been described previously as the products of *C. alata* CHS (Samappito et al., 2002) and *Rheum tataricum* STS (Samappito et al., 2003). For the hexaketide pyrone, the one (Rt = 9.60 min) with the parent ion *m/z* 233 $[M-H]^-$, appears to be identical with a hexaketide reported from an

A. arborescens OKS mutant (Abe et al., 2005a). Its structure of compound was determined to be 6-(2,4-dihydroxy-6-methylphenyl)-4-hydroxy-2-pyrone.

The putative hexaketide pyrone (Rt = 3.23 min) with the parent ion peak $[M-H]^-$ at *m/z* 251 gave the fragment ions 159 $[M-(2xOH)-(CH_3CH_2OHCH_2)]^-$, 117 $[M-159-CH_2CO]^-$, and 73 $[M-117-CO_2]^-$. The results suggest that the compound is a 6-(4'-oxo-2', 6'-dihydroxy-6-methyl-4, 5-cyclohexene)-4-hydroxy-2-pyrone (Table 4).

The findings of the three structures of the hexaketide pyrones are very interesting for the following 3 reasons. First, their condensed products of polyketide chains (from one acetyl-CoA plus 5 malonyl-CoA) are the same length as the plumbagin's polyketide chain. No longer carbon chains are found as the PinPKS products. This suggests that the recombinant enzyme might be the same one as the plumbagin PKS. Second, based on their structures, it seems that the three hexaketide pyrones are related to each other in terms of their sequence of conversion. As shown in Figure 39, it is likely that the side-chain the hexaketide pyrone is converted into a ring form of aromatic pyrone. These structures fit very well with their mass spectra data.

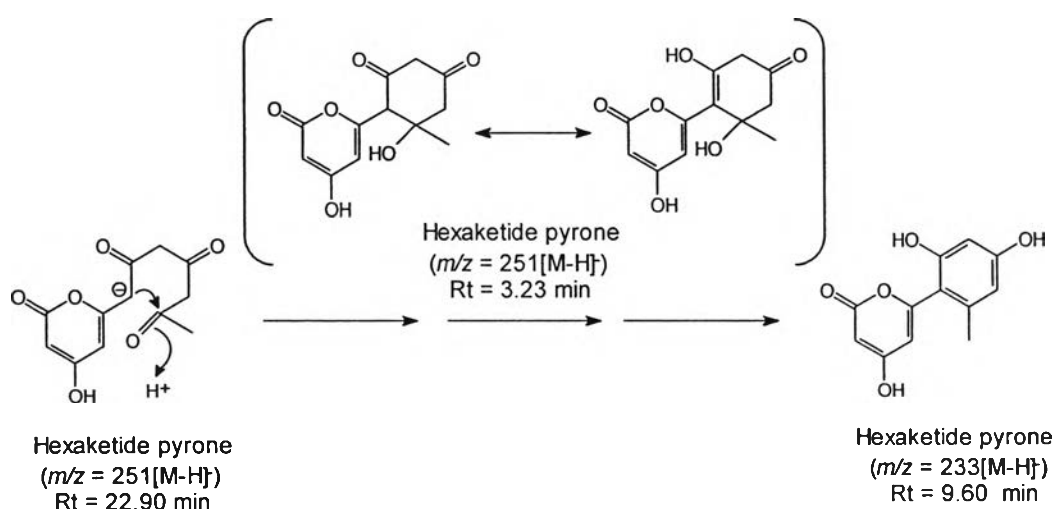


Figure 39 Proposed sequence of conversion of the three hexaketide pyrones produced from the recombinant PinPKS enzyme under the assay condition.

Third, the ability of the recombinant PinPKS in aromatizing the hexaketide pyrone precursor suggests that the enzyme itself is a multifunctional enzyme. This property has also been shown in the case of *R. palmatum* ALS (Abe et al., 2004). *A. arborescens* PCS (Abe et al., 2005b) and OKS (Abe et al., 2005a).

Based on the findings of various polyketide pyrone products, we proposed the whole reaction sequence catalyzed by the purified recombinant PinPKS. As shown in Figure 40, the enzyme catalyzes five steps of malonyl-CoA condensation. Each step of the intermediate formed (starting from the triketide) leads to the formation of its corresponding pyrone. The biggest one, hexaketide pyrone, with seven-carbon side chain can be further cyclized to form a six-membered ring pyrone which is finally reduced to its corresponding aromatic ring of the final product as observed.

The reason why the recombinant PinPKS can catalyze the formation of various pyrone intermediates is still not clear. It might be possible that the protein folding of the recombinant PinPKS is not as tight as the native enzyme in its physiological conditions. Leakage of the polyketide intermediates from the enzyme's active site in form of polyketide pyrone can take place and lead to the diversity of the products.

In addition, the aspect of the misfolding of the recombinant PinPKS or the missing of some unknown factors may also be the reasons of why the products are the pyrones instead of plumbagin (Figure 41). It has been proposed that the absence of interaction with unidentified tailoring enzymes the recombinant PinPKS just afforded hexaketide pyrones as shunt products as in the case of *A. arborescens* OKS (Abe et al., 2005a). The aromatic octaketide SEK4/SEK4b has been proposed to be the shunt products of OKS that catalyzed seven decarboxylative condensation of malonyl-CoA (Abe et al., 2005a).

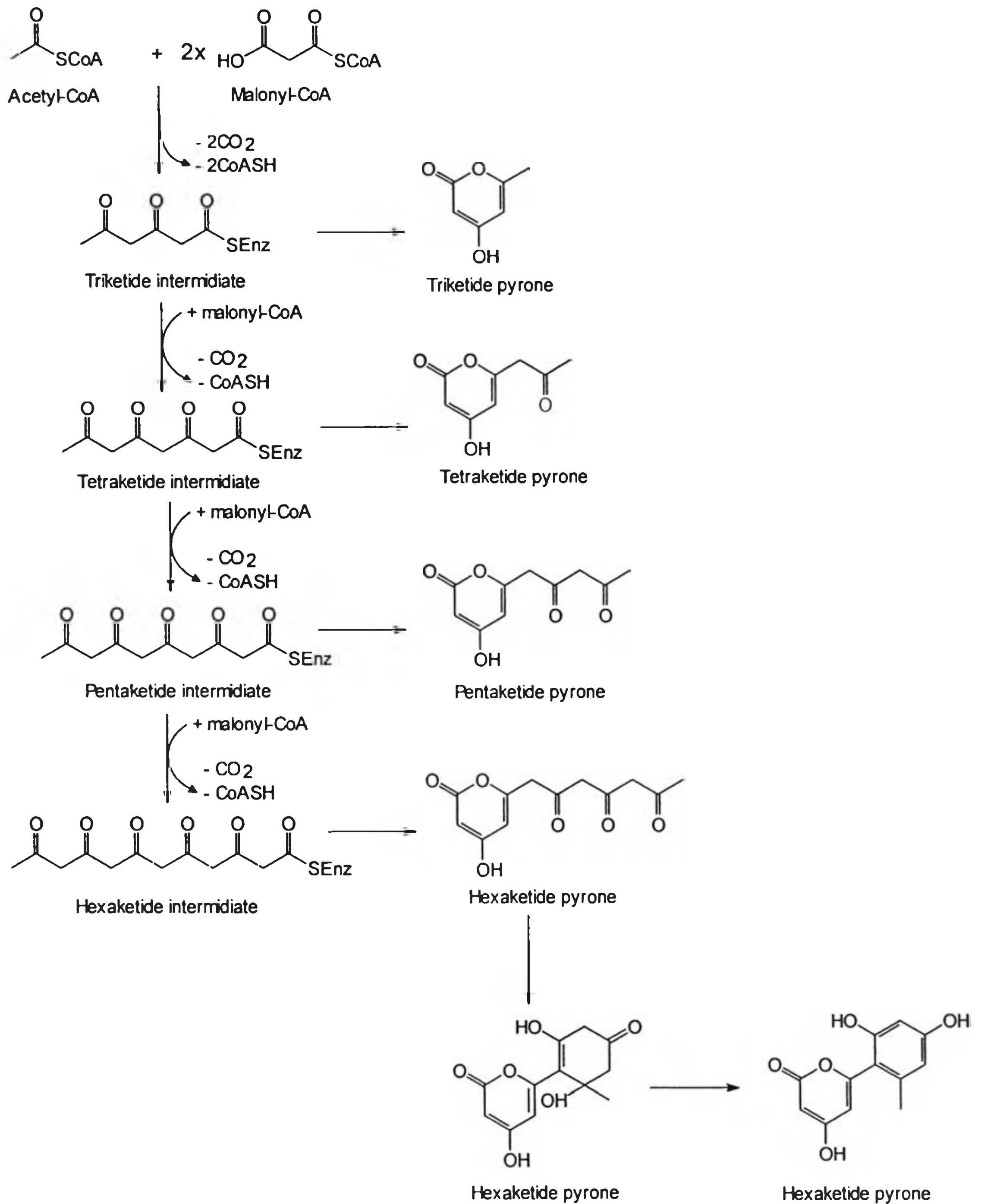


Figure 40 Proposed steps of the formation triketide, tetraketide, pentaketide and hexaketide pyrones from acetyl-CoA and malonyl-CoA in the standard reaction mixture catalyzed by the PinPKS enzyme.

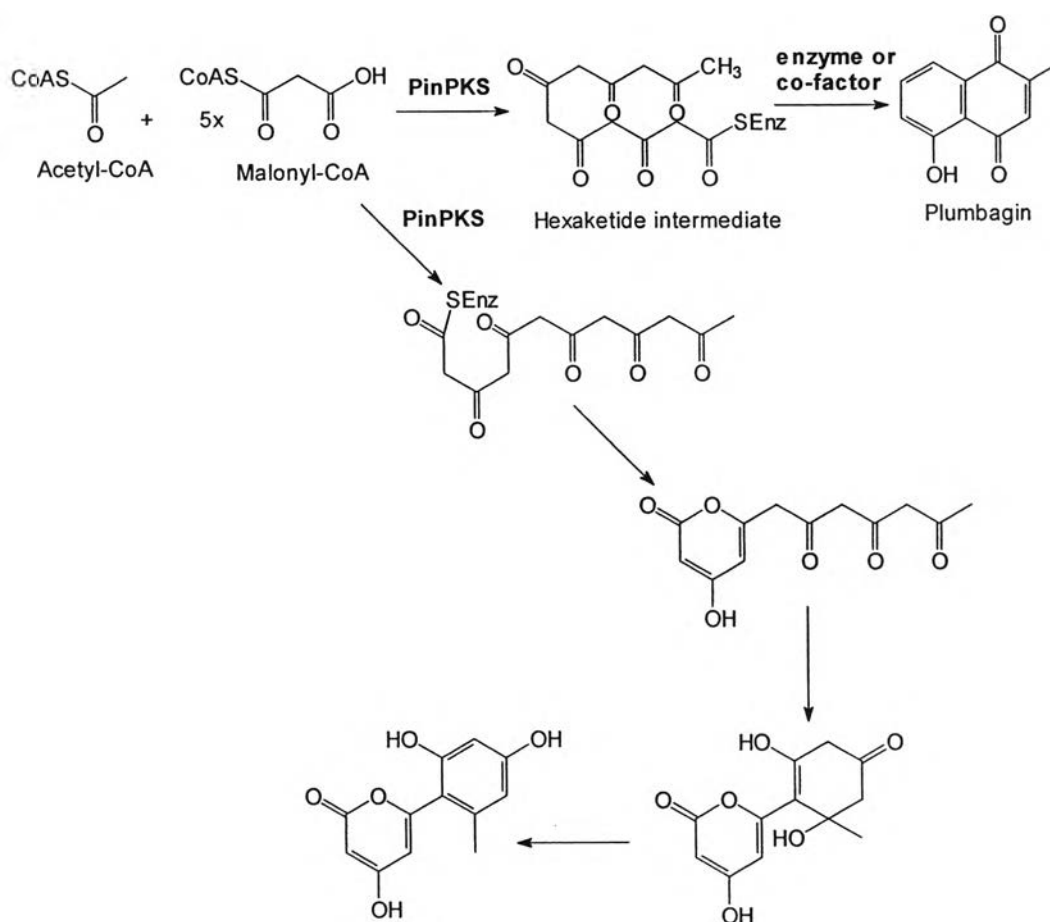


Figure 41 Proposed sequence taking place in the reaction mixture catalyzed by PinPKS.

CONCLUSION

From this research work of "polyketide synthase enzymes and genes in *Plumbago indica* ", The following conclusion can be drawn :

1. Three types of the *in vitro* cultures of *P. indica* including callus, root culture and *in vitro* plantlets have been successfully established for studying their potential of plumbagin formation.

2. These *in vitro* cultures can all produce plumbagin and root cultures and root parts of plantlets can also produce plumbagic acid and plumbagic acid glucoside. All tissue cultures can be used as a source for biosynthetic studies.

3. No enzyme activity of PKS has been detected in the enzyme assay of crude protein extracts prepared from *P. indica* tissue cultures.

4. One full-length cDNA clone from roots of *P. indica* encoding plant-specific type III PKS. PinPKS catalyze the formation of pyrone compounds (triketide, tetraketide, pentaketide and hexaketide pyrones) from acetyl-CoA and up to five molecules of malonyl-CoA and subsequent cyclization. Plumbagin is not formed in our assay as the hexaketide pyrones are shunt products of PinPKS.