CHAPTER V

DISCUSSION

1. Purification and Characterization of Dopamine-Secologanin Condensing Enzyme from *Alangium salviifolium* Wang Leaves

The biosynthetic pathway of tetrahydroisoquinoline monoterpene alkaloids involved the first step of dopamine and secologanin condensation to form both the (*R*)-epimer of deacetylipecoside and (*S*) deacetylisoipecoside have been proposed base on feeding experiment since 1978 by Nagakura *et al.*. This pathway have had no experimental support at the enzyme level until very recently. De-Eknamkul *et al.* (1997) have been found the two enzyme activities responsible for such a condensation to form the two epimers. Both enzyme activities were detected in the cell free extracts prepared form the fresh leaves of *Alangium salviifolium* Wang, the tropical plant widely distributed in Thailand. Attempts to induce production of the alkaloids have been made in *Alangium* cell culture system in order to be used as the enzyme source. Unfortunately, no alkaloid product was detected in this cell cultures under various conditions of hormonal and nutritional factors manipulation. The leaves of *A. salviifolium* were therefore used as materials for this enzymological study.

Although the two dopamine-secologanin condensing enzymes have both been found in the crude enzyme extraction, the previous study has shown that the stereoselective enzyme that catalyses the condensation of dopamine and secologanin to yield (*R*)-deacetylipecoside is more stable than the other enzyme, and could be purified by column chromatography. Therefore, this study is concentration on the purification and characterization of the stereoselective enzyme that catalyses the formation of (*R*)-deacetylipecoside. According to this activity, we name this enzyme as (*R*)-deacetylipecoside synthase.

By using the radioassay to follow the enzyme activity and 4 steps of purification procedure, it was possible to purify the enzyme to apparent electrophoretic homogeniety with 6.2% recovery and the enzyme specific activity was approximately 717.0 pkat/mg protein. The low percentage recovery of enzyme activity was due to the loss of enzyme activity during purification processes. The purity of enzyme in each step was evaluated by SDS polyacrylamide gel electrophoresis. The results were summarized in Table 8. The fractionation by 40-60% $(NH_4)_2SO_4$ could get rid of undesired proteins but some of the enzyme activity was also lost in this step. The Phenyl Sepharose CL-4B and DEAE-Sephacel could efficiently separated deacetylipecoside synthase enzyme from other proteins. The most effective step of the purification scheme appeared to be Superose 6 HR16/50 gel filtration which could separate the impurity proteins and resulted to a homogenous enzyme. Characterization of the purified enzyme revealed that the enzyme had an apparent molecular weight of 30.05 kD on SDS polyacrylamide gel under denaturing condition. The native enzyme was also determinated to have similar size by employing a Superose 6 pre-calibrated gel filtration column. It was found that the enzyme activity was detected at a elution volume corresponding to a protein with molecular weight 30.38 kD. The results clearly indicated that the dopamine-secologanin condensing enzyme is a monomeric enzyme with a molecular weight of 30 kD. The optimum pH of the enzyme was found to be 7.5. The optimal temperature for its catalytic activity was 37°C. Under the condition of standard assay, the formation of deacetylipecoside was linear with time for at least 30 min. None of the metal ions test showed any effects on the enzyme activity. The enzyme appeared to exhibit high substrate specificity toward dopamine. No enzymatic reaction could be detected with tyramine or tryptamine as substrate. Furthermore, addition of either compounds in this assay reaction mixture appeared to have no interference on the enzyme activity utilizing dopamine, although using tryptamine at high concentration as 5 mM could reduce the enzyme activity by 50%. The study on the effect of some emetine derivatives including alangimarckine, dehydroalangimarckine, cephaeline, emetine and tubulosine on the enzyme activity showed that these alkaloids had various degrees of enzyme inhibitory activities.

Among these compounds, alangimarckine and dehydroalangimarckine effectively inhibited the enzyme activity. Their 50% inhibition concentrations were both approximately 10 μM. Although the reason of this observation is still not known, it is likely that the inhibition does not occur in *Alangium* cell. This may be deu to the different subcellular localization of the enzyme and alangimarckine or dehydro-alangimarckine. For the kinetic studies, K_m values for both dopamine and secologanin were also determined as pseudo-single substrate enzyme. By keeping the secologanin concentration at 5 mM and varying dopamine from 0.25 to 7.5 mM, the K_m and V_{max} values for dopamine were 0.69 mM and 7.09 pkat/mg protein, respectively. When dopamine concentration was fixed at 5 mM and secologanin concentrations were varied from 0.1 to 3 mM, the K_m and V_{max} values for secologanin were 0.92 mM and 8.33 pkat/mg protein, respectively. The enzymatic reaction product was determined by HPLC and radioactive analysis and found unambiguously as (*R*)-deacetylipecoside. With these properties, the enzyme was called "deacetylipecoside synthase" as the new enzyme.

Table 8 The properties of deacetylipecoside synthase from *A. salviifolium* Wang

Properties	Value
pI	nd
Optimum pH	7.5
Optimum temperature	37^0C
K_m value with respect dopamine	0.69 mM
K_m value wth respect to secologanin	0.92 mM
Relative M_r	
gel filtration	30,380 daltons
SDS-PAGE	30,053 daltons

nd = not determined

Obviously, deacetylipecoside synthase described here is analogous to the enzyme strictosidine synthase (SSS, EC 4.3.3.2) catalysing the condensation of the glucoiridoid secologanin and indole tryptamine to (*S*)-strictosidine, which is the key intermediate of indole alkaloids. So far, the characterized enzyme strictosidine synthase has been purified from the cell cultures of two members of plant family Apocynaceae, namely *Catharanthus roseus* (L.) G. Don. and *Rauwolfia serpentina* Benth. (Pfitzner and Zenk, 1989; Hampp and Zenk, 1988) and one in family Rubiaceae, *Cinchona ledgerina* Mores. (Steven, 1993). The M_r of strictosidine synthase from *C. roseus* and *R. serpentina* have been reported as 41,500 and 30,000, daltons, respectively (De waal *et al.*, 1995). As determined by polyacrylamide gel electrophoresis, the M_r of strictosidine synthase obtained from *C. ledgerina* was 35,000 daltons. By means of isoelectric points of this enzyme found in Apocynaceae were between 4.3 and 4.8 and the one in Rubiaceae was 6.5-7.5. The kinetic data of strictosidine synthase for substrates tryptamine and secologanin range in micromolar scale, except for *R. serpentina* which was in milimolar level. The enzyme was completely inhibited by the product of strictiosidine. In addition the enzyme found to be glycosylated proteins (Table 9).

Table 9 The properties of strictosidine synthase from various plant species

Values	*C. roseus*	*R. serpentina*	*C. ledgerina*
pI	4.3-4.8	4.5	6.5-7.5
pH	6.5-7.5	6.5	nd
Optimum Temperature	45°C	45°C	45°C
K_m value for tryptamine	9 μM	4 mM	8-15 μM
K_m value for secologanin	nd	4 mM	nd
M_r	41,500	30,000	35,000
Carbohydrate content	5.3 %	5.3 %	5.3 %

nd = not determined

Among the characteristics of strictosidine synthase in three plant species, *A. salviifolium* deacetylipecoside synthase appeared to be compareble with *R. serpentina* strictosidine synthase. Both enzymes have similar molecular size of approximately 30,000 daltons. No phenomenon of substrates inhibition was observed. And they also have high K_m values. It is interesting to compare the amino acid sequences of these two enzymes which there are any common portion which are responsible for their similar substrates condensation activity.

2. The Proposed Biosynthetic Pathway of Tetrahydroisoquinoline Monoterpene Alkaloids and Glucosides in *A. salviifolium* Plant

The effort to clarify the biosynthetic pathway of emetine alkaloids and the indole alkaloids took place in 1970s. Strictosidine synthase, the key enzyme of indole alkaloid pathway was first purified from the cell cultures of *Catharanthus roseus*, thereby opening the way for enzyme identification along the indole alkaloid biosynthetic routes. The elucidation of many steps in the biosynthesis of different types of indole alkaloid had been achieved and some key enzymes were characterized. The cDNA for strictosidine synthase obtained from *Rauwofia serpentina* has been cloned and expressed in *Escherichia coli* (Kutchan *et al.*, 1988; Kutchan, 1989). This is the first plant enzyme of alkaloid biosynthesis cloned and expressed in bacteria and this acheivement represents a major breakthough in plant biotectnology.

Although the biosynthetic studies of tetrahydroisoquinoline monoterpene alkaloids and indole alkaloids seem to be analogous, little is known about the former biosynthetic pathway. This is due to the fact that the ultimate proof for the correct biosynthetic pathway can only be obtained from the studies with enzymes which catalyzed various steps of secondary metabolite biosynthesis and none of the enzymes in this pathway has been isolated and characterized. In this study, the first enzyme involved in this biosynthetic pathway has been purified and charaterized from cell-free extraction of *A. salviifolium* leaves for the first time. From our experiments , this enzyme is highly stereospecific in the condensation of dopamine and secologanin to

(*R*)-deacetylipecoside. Based on this discovery, the biosynthetic pathway of tetrahydroisoquinoline monoterpene alkaloids and glucosides in *Alangium* could be proposed as follows (Figure 29).

Secologanin, the monoterpenoid part, originates from geraniol. The first step leading to secologanin comprises the conversion of geraniol into 10-hydroxy geraniol. This reaction is catalysed by the P450-dependent enzyme, geraniol-10-hydroxylase (G10H). After this, a hypothetical pathway for the biosynthesis of secologanin has been postulated on the basis of feeding experiments, but most of the enzymes involved have not yet been identified (Meijer *et al.*, 1993). Dopamine, the other precursor is derived from tyrosine (Phillipson *et al.*, 1985).

The condensation of secologanin and dopamine in *A. salviifolium* is a competitive reaction between the two enzymes with opporsite stereochemical control namely, (*R*)-deacetylipecoside synthase and (*S*)-deacetylisoipecoside synthase (De-Eknamkul *et al.*, 1997). We have purified deacetylipecoside synthase in as described Chapter III. The deacetylipecoside synthase condenses the molecules of dopamine and secologanin to (*R*)-deacetylipecoside which is metabolically converted by lactam formation to alangiside. On the other hand, the deacetylisoipecoside synthase catalyses the some precursors to form (*S*)-deacetylisoipecoside which is further tranformed to several monoterpenoid isoquinoline alkaloids, including cephaeline and emetine, all posessing α−configuration.

Although the deacetylipecoside synthase was purified to homogeneity from cell-free extraction of *A. salviifolium* leaves. Many enzymes of the pathways have not yet been discovered, especially the similar enzyme in the first step of pathway leading to all of tetrahydroisoquinoline monoterpene alkaloids with α-configuration. In *A. salviifolium* cell-free extracts, the deacetylisoipecoside synthase is less stable. The investigation of this enzyme may further performed in *Cephelis ipecacuanha* root culture, the rich source of emetine alkaloid (Jha, *et al.*, 1991). In *A. salviifolium*, deacetylipecoside is converted to alangiside and deposits in roots, leaves and fruits.

But in *C. ipecacuanha*, it is tranformed to ipecoside and deposits only in roots. The comparative study of (*S*)-deacetylipecoside in *C. ipecacuanha* should be concerned and the enzymes involved in the next steps after deacetylipecoside are also interesing. As a consequence of this study, the amino acid sequencing of the deacetylipecoside synthase should be identified for further study in the molecular genetic level.

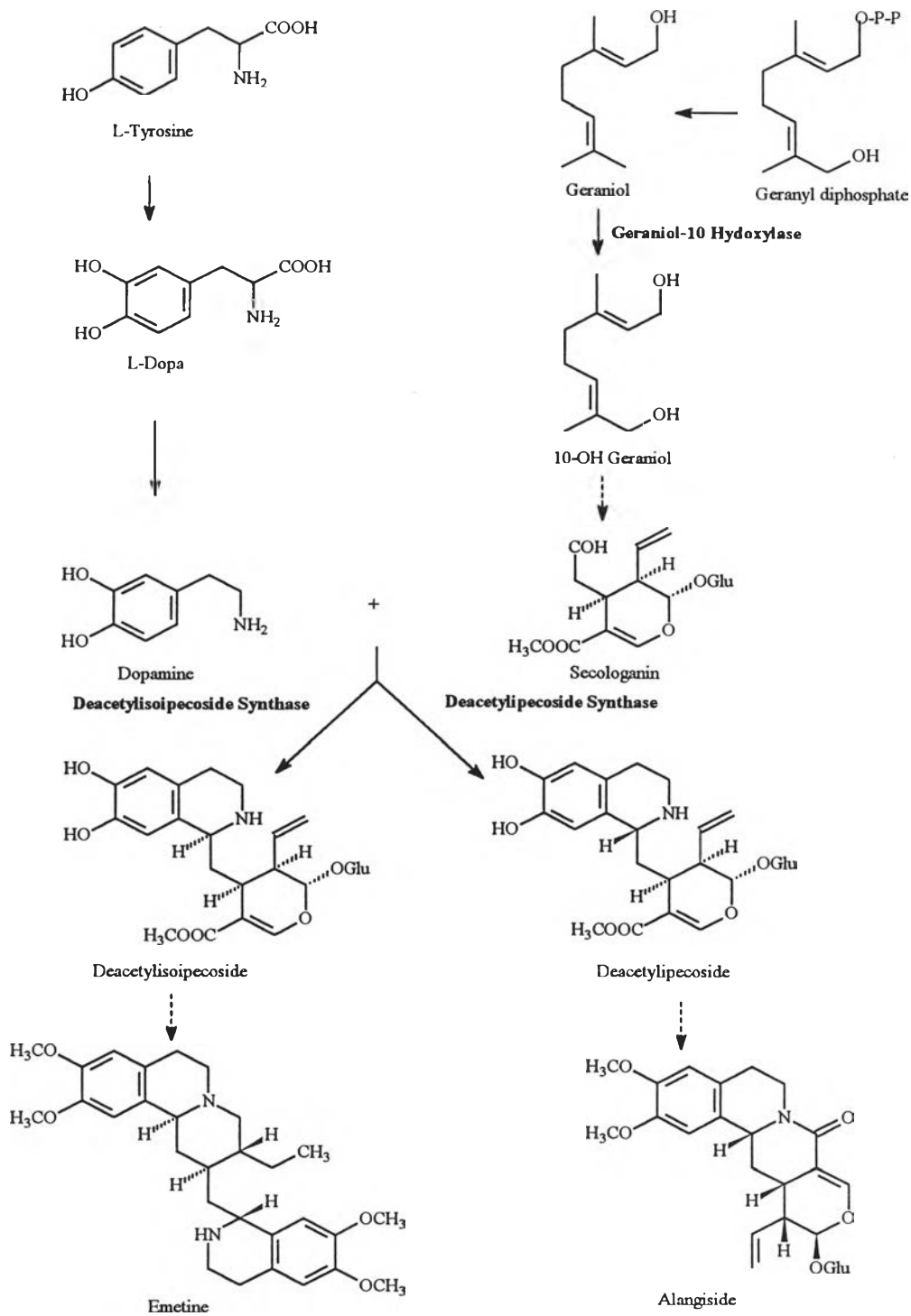

Figure 29 Proposed biosynthetic pathway of tetrahydroisoquinoline monoterpene alkaloids

CONCLUSION

Deacetylipecoside synthase enzyme, the novel enzyme catalysing the condensation of dopamine and secologanin to form the (*R*)-epimer of deacetylipecoside, has been purified from the leaves of *Alangium salviifolium* Wang. to apparent electrophoretic homogeniety by 40-60% ammonium sulfate fractionation precipitation and three subsequent column chromatography steps. The isolated enzyme is a single polypeptide with M_r 30,000 and has pH optimum at 7.5 and a temperature optimum at 37^0C. The apparent K_m values for dopamine and secologanin are 0.69 and 0.92 mM, respectively. The V_{max} values for dopamine and secologanin are 7.09 and 8.33 pkat/ mg protein, respectively. The enzyme exhibits high substrate specificity toward dopamine, neither tyramine nor tryptamine are utilized by the enzyme. The enzyme activity is not inhibited by substrates but inhibited by alangimarckine and dehydroalangimarckine with similar IC_{50} value of approximately 10 μM. Deacetylipecoside synthase presumably catalyses the provision of (*R*)-deacetylipecoside for the formation of tetrahydroisoquinoline monoterpene glucosides that possess also (*R*)-configuration at the same chiral center.

The successful purification and characterization of *A. salviifolium* deacetylipecoside synthase have lead to the way for enzyme identification along the tetrahydroisoquinoline monoterpene alkaloids pathway to completely biochemical understanding of this pathway, the technique of molecular genetic can be applied in a meaningful manner and potentially reveal the mechanism which control the biosynthesis of tetrahydroisoquinoline monoterpene alkaloids in *Alangium salviifolium* Wang.