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Purification and characterization of deacetylipecoside synthase from *Alangium lamarckii* Thw.

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Abstract

Deacetylipecoside synthase (DIS), the enzyme catalyzing the condensation of dopamine and secologanin to form the (*R*)-epimer of deacetylipecoside, has been purified 570-fold from the leaves of *Alangium lamarckii* and partially characterized. The isolated enzyme is a single polypeptide with *Mr* 30, 000, and has a pH optimum at 7.5 and a temperature optimum at 45°C. The apparent K_m values for dopamine and secologanin are 0.7 and 0.9 mM, respectively. DIS exhibits high substrate specificity toward dopamine, whereas neither tyramine nor tryptamine are utilized. The enzyme activity is not inhibited by its substrate dopamine, but is inhibited by alangimakine and dehydroalangimakine with similar I_{50} values of 10 μM. DIS presumably provides (*R*)-deacetylipecoside for the formation of tetrahydroisoquinoline monoterpene glucosides that also possess an (*R*)-configuration at the same chiral center.

Keywords: *Alangium lamarkii*; Alangiaceae; Deacetylipecoside; Deacetylipecoside synthase; Isoquinoline monoterpenoid biosynthesis; Purification; Characterization

1. Introduction

We have recently discovered in cell free extracts of *Alangium lamarckii* Thw. (Alangiaceae) two new enzyme activities that are involved in the condensation of dopamine and secologanin to form two epimers of deacetylipecoside (*R*-form, **1**) and deacetylisoipecoside (*S*-form, **2**) (De-Eknamkul et al., 1997). Based on their activities, the two enzymes have been called deacetylipecoside synthase (DIS) and deacetylisoipecoside synthase (DIIS), respectively. This finding appears to be consistent with the results of administration experiments reported more than 20 years ago showing that secologanin was condensed with dopamine in a Pictet-Spengler manner to form the two epimers (Battersby et al., 1978; Nagakura, et al., 1978). Furthermore, it has been found that secondary products with tetrahydroisoquinoline-monoterpene skeletons are present in *A. lamarckii* both in the *R*-configuration of nitrogenous glucosides (Itoh, et al., 1994, 1995, 1996) and the *S*-configuration of emetine-type alkaloids (Fujii and Ohba, 1983; Buckingham and Southon, 1994). Therefore, it has been proposed that **1** and **2** are, respectively, the first intermediates of the biosynthetic pathways of these two alkaloid groups (Nagakura et al., 1978; De-Eknamkul et al., 1997) (Fig. 1). As part of our interest in the elucidation of the biosynthetic enzymes involved in these pathways, we aim to purify and characterize both condensing enzymes. It was found, however, that DIS was relatively stable and could be purified by column chromatography whereas DIIS was too labile for purification (De-Eknamkul et al., 1997). This paper reports the results on purification and characterization of the former enzyme.

2. Results

2.1. Enzyme purification

The crude protein extracts prepared from the leaves of *A. lamarckii* were first precipitated by $(NH_4)_2SO_4$ (40–60% saturation) to obtain a concentrated solution of DIS. This $(NH_4)_2SO_4$ fractionation reduced a major portion of the proteins (92%) from the crude extract,

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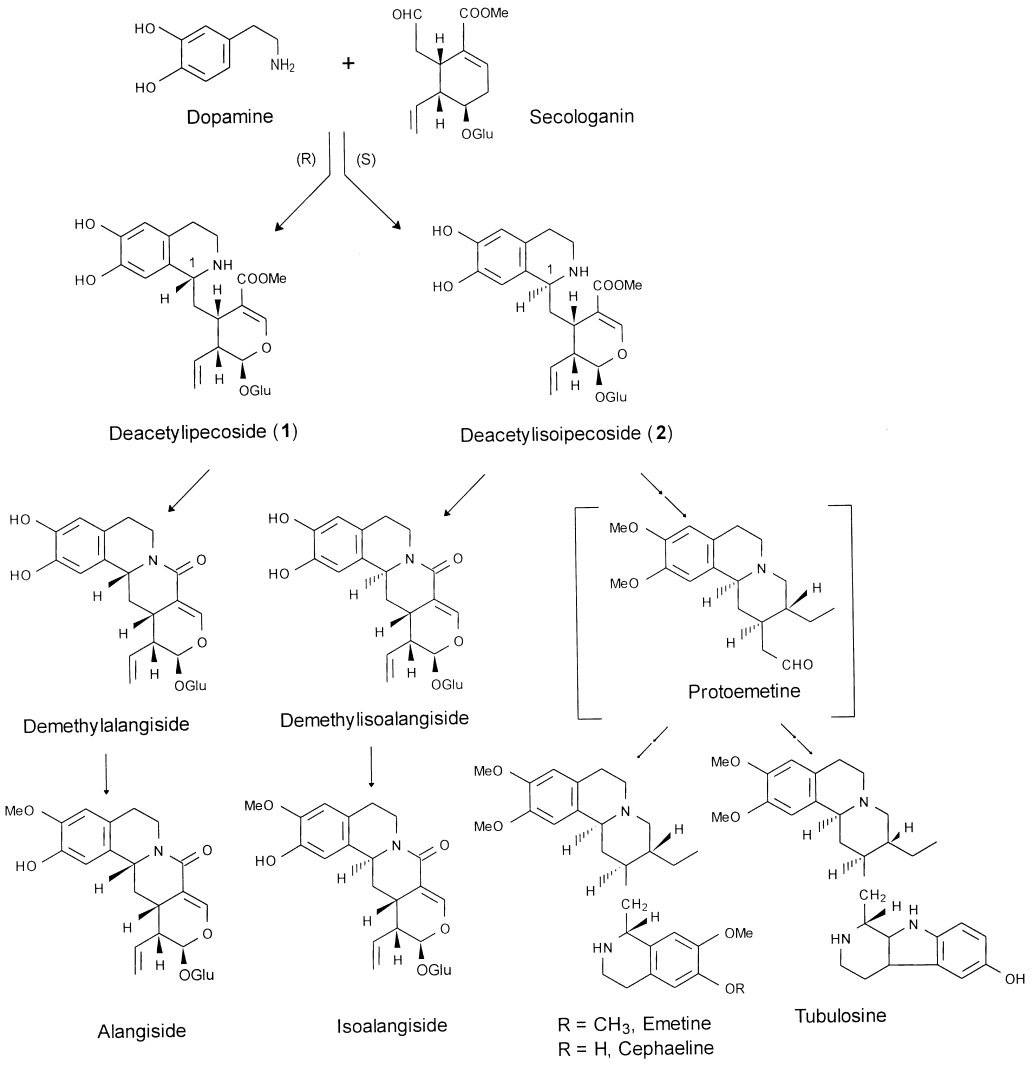

Fig. 1. Proposed biosynthetic sequence for the biosynthesis of the alkaloids and nitrogenous glucosides in *Alangium lamarckii*.

although more than 75% of DIS activity was also lost in this step. This activity loss was presumably due to the presence of high amounts of secondary compounds such as alkaloids and phenolics that destabilize the enzyme. Upon passage of the 40–60% $(NH_4)_2SO_4$ protein fraction solution through a phenyl-sepharose column, a large amount of protein passed through, and DIS activity was retained. The enzyme was eluted from this column with >10-fold purification. When the enzyme preparation was subjected to DEAE-Sephacel chromatography, the bulk of the enzyme activity was retained. DIS was eluted with a major protein peak and this step gave an additional 4-fold purification. The resulting enzyme preparation was finally passed though a Superose-6 gel filtration column (Fig. 2) and the active enzyme fraction was pooled, then concentrated by ultrafiltration to give the purified preparation.

As summarized in Table 1, the purification procedure gave about a 570-fold purification with 6% recovery of

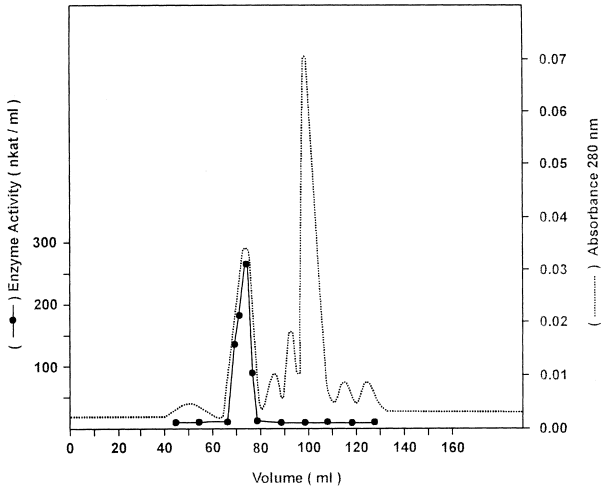

Fig. 2. Superose-6 gel filtration of DIS preparation obtained from the step of DEAE-Sephacel chromatography.

Table 1
Summary of the purification of deacetylipecoside synthase from *Alangium lamarckii* leaves

Purification step	Volume (ml)	Protein (mg)	Total act. (nkat)	Spec. act. (nkat mg^{-1})	Yield (%)	Purification (-fold)
Crude extract	550	462	575	1.3	100	1
40–60% $(NH_4)_2SO_4$	105	39	125	3.1	22	3
Phenyl-sepharose	60	1.20	42	34	8	27
DEAE-Sephacel	33	0.45	61	135	11	108
Superose 6	8	0.05	36	717	6	573

the enzyme activity. The specific DIS activity of the purified preparations varied from 650 to 750 nkat mg^{-1} protein. The purity of the final enzyme preparation was checked by SDS-PAGE as compared to the protein patterns of other preparations obtained from the sequential steps of the column chromatography. As shown in Fig. 3, the purified enzyme migrated as a single band showing no major protein contamination. Furthermore, the gel also showed increasing concentrations of the protein band along the steps of the chromatography consistent with the purified enzyme being the band with the DIS activity.

2.2. Properties of deacetylipecoside synthase

The *Mr* of DIS was determined by employing a Superose-6 precalibrated gel filtration column. It was found that the DIS activity was eluted at a volume corresponding to a protein of *Mr* 30,000. Under denaturing conditions, the purified enzyme also showed a protein band at ~*Mr* 30,000 on a SDS polyacrylamide gel (Fig. 3). These results indicate that DIS is a monomeric protein.

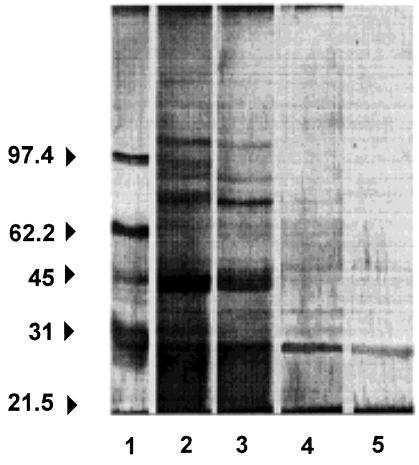

Fig. 3. SDS-polyacrylamide gel electrophoresis of various enzyme preparations obtained during enzyme purification of deacetylipecoside synthase from *A. lamarckii*. Lane 1, marker proteins; lane 2, 40–60% ammonium sulphate; lane 3, phenyl-sepharose CL-4B; lane 4, DEAE-Sephacel; lane 5, Superose 6. Arrows indicate the distribution of marker proteins and molecular mass ($\times 10^{-3}$).

The pH optimum of the enzyme was found to be 7.5. Under the conditions of the standard assay, the formation of deacetylipecoside was linear with time for at least 40 min. The optimal temperature for catalytic activity was determined to be 45°C. For enzyme stability, it was found that DIS stored at either 4 or −20°C lost half of its activity in 5 days, and all of its activity in 7 days. The enzyme appeared to exhibit high substrate specificity towards dopamine. No reaction could be detected with tyramine or tryptamine as substrate. Furthermore, addition of either of these two compounds in the assay's reaction mixture appeared to have no interference on the ability of the enzyme to utilize dopamine (Table 2). The K_m values for both dopamine and secologanin were also determined for the enzyme. By keeping the secologanin concentration at 5 mM and varying the dopamine concentration from 0.25 to 7.5 mM, the K_m for dopamine was determined as 0.7 mM. When the dopamine concentration was fixed at 5 mM and the secologanin concentration varied from 0.2 to 3 mM, the K_m value for secologanin was determined to be 0.9 mM.

A study on the effect of some compounds on DIS activity found that alangimakrine, dehydroalangimakrine, cephaeline, emetine and tubulosine showed different degrees of enzyme inhibition (Table 2). Among these, alangimakrine and dehydroalangimakrine appeared to effectively inhibit the enzyme activity. Concentrations resulting in 50% inhibition were both approximately 10 μM for both alkaloids.

Table 2
Inhibition of deacetylopecoside synthase by some related substrates and alkaloids

Compound	Inhibition (%)		
	0.01 mM	0.1 mM	1.0 mM
Tyramine	nd[a]	0	0
Tryptamine	nd	0	0
Cephaeline	0	0	50
Emetine	0	24	45
Tubulosine	0	11	52
Alangimakrine	40	73	100
Dehydroalangimakrine	51	84	100

[a] nd = Not determined.

3. Discussion

We have demonstrated the presence of the enzyme deacetylipecoside synthase in the leaves of *Alangium lamarckii*, a tropical plant widely distributed in Thailand. The enzyme catalyzes the condensation of dopamine and secologanin to form deacetylipecoside (**1**), a product with an *R*-configuration at its C-1 carbon (De-Eknamkul et al., 1997). It is likely that this first intermediate is the precursor of various *Alangium* tetralydroisoquinoline-monoterpene glucosides that also possess (*R*)-configurations at the same chiral center (Fig. 1). In this study, DIS was successfully purified from the crude protein extracts by using a combination of ammonium sulphate precipitation and three column chromatography steps. This procedure allows the enzyme to be purified 570-fold with 6% overall recovery. The enzyme appears to be a single polypeptide with a *Mr* of 30,000 as determined by both SDS-PAGE and gel filtration. The enzyme exhibits high substrate specifity toward dopamine with the apparent K_m value of 0.7 mM. The K_m value for the cosubstrate secologanin is 0.9 mM. The enzyme activity is not inhibited by its substrate dopamine but is inhibited by some tetrahydroisoquinoline-monoterpene alkaloids, especially alangimakrine and dehydroalangimakrine. The physiological significance of this alkaloid inhibition is not known.

Deacetylipecoside synthase is analogous to the enzyme strictosidine synthase catalyzing the condensation of tryptamine and secologanin, although the absolute configuration of the product, strictosidine, is the opposite. The latter enzyme has been isolated and characterized from *Catharanthus roseus* (Pfitzner and Zenk, 1989), *Rauvolfia serpentina* (Hampp and Zenk, 1988) and *Cinchona robusta* (Stevens et al., 1993). Table 3 shows comparative data of enzyme properties for *Alangium* DIS and strictosidine synthases from these sources. In general, DIS is similar to the strictosidine synthases with respect to molecular size, temperature optimum, pH optimum, exhibiting high substrate specificity and catalysing the same condensation reaction. These characteristics might suggest that a family of these synthase genes have evolved from a potential common primary metabolic ancestor. Both synthase groups differ in their characteristics of substrate specificity and stereo specificity. This might reflect the results of the evolution of substrate specific metabolic pathways leading to the type of alkaloid ultimately produced in each species. Ultimate characterization of clones will be useful to completely document this hypothesis. Since *A. lamarckii* DIS and *R. serpentina* strictosidine synthase are most comparable (Table 3), both enzymes have a similar molecular size of approx 30,000 and have high K_m values. It is of interest to compare the amino acid sequences of these two enzymes to see if there are common elements responsible for substrate condensation. This aspect of study is now under investigation in our laboratory.

4. Experimental

4.1. Plant material

Fresh leaves of *Alangium lamarckii* Thw. were collected from an 8-year-old plant growing in the field at the Faculty of Pharmaceutical Sciences, Chulalongkorn University, Bangkok, Thailand. A voucher specimen is deposited in the Herbarium Royal Forest Department in Bangkok, Thailand under No. 21867.

4.2. Chemicals

[2,5,6-^{3}H] dopamine was purchased from Amersham, England. Authentic demethylalangiside and demethylisoalangiside were prepared from dopamine and secologanin as described previously (Nagakura et al., 1978). Secologanin was isolated from dried leaves of *Lonicera tartarica* harvested during July/August 1998. The isolation procedure was followed as given in (Tietze et al., 1986) with 1.5 kg of leaf material being used.

Table 3
Comparison on the enzyme properties of deacetylipecoside synthase (DIS) from *A. lamarckii* and strictosidine synthases from some plant species

	DIS	Strictosidine synthase		
Enzyme property	*A. lamarckii*	*C. roseus* (form III)	*R. serpentina*	*C. robusta* 13
Substrate specificity	Dopamine	Tryptamine	Tryptamine	Tryptamine
Substrate inhibition	No	Yes	Yes	No
Product configuration	R-form	S-form	S-form	S-form
K_m for substrate	0.7 mM	1.9 mM	4 mM	8-15 μM
K_m for secologanin	0.9 mM	nd[a]	4 mM	nd
pH optimum	7.5	6.7	6.5	6.0–8.5
Temp. optimum	45°	45°	45°	45°
Mr	30,000	41,500	30,000	35,000
Carbohydrate content	nd	5.3%	5.3%	5.3%

[a] nd = Not determined.

4.3. Enzyme extraction

Fresh leaves (250 g) were ground in a mortar in the presence of liquid nitrogen. The resulting fine powder was thawed by stirring for 30 min at 4°C in 500 ml 100 mM tricine-NaOH buffer, pH 7.5 containing 10 mM 2-mercaptoethanol and 3 mM EDTA (extraction buffer). The suspension was pressed through four layers of cheesecloth and the filtrate was centrifuged at 10,000 *g* for 10 min at 4°C. The supernatant was then precipitated between 40 and 60% satn of $(NH_4)_2SO_4$ by using finely ground solid $(NH_4)_2SO_4$. The precipitate obtained was collected by centrifugation (10,000 *g*, 10 min) and dissolved in a minimal vol. of extraction buffer containing 0.4 M $(NH_4)_2SO_4$. The concd. enzyme soln. was then applied to a phenyl-sepharose CL-4B column (2.6 × 26 cm) pre-equilibrated with the same buffer. After washing with 700 ml of the buffer at a flow rate of 1.5 ml min^{-1}, the column was eluted with 300 ml extraction buffer and 10 ml frs were collected. Frs containing activities of (*R*)-deacetyl ipecoside synthase were pooled (90 ml) and applied to a DEAE-Sepharose column (2.6 × 15 cm) pre-equilibrated with extraction buffer. After washing with 200 ml of the same buffer at a flow rate of 1 ml min^{-1}, linear gradient of 0–70 mM KCI (in extraction buffer) was applied (in 280 min, flow rate 0.5 ml min^{-1}) while 5 ml fractions were collected. The active fractions were pooled (50 ml) and concd to 1.5 ml by using centriprep 10. The concd. protein solution was applied to a Superose-6 gel fitration column (1.6 × 50 cm). The column was pre-equilibrated and eluted with extraction buffer (flow rate : 0.4 ml min^{-1}, fraction size 2 ml). The fractions with the highest enzyme activity were pooled and used as the purified enzyme preparation.

4.4. Enzyme assay

The routine assay mixture contained 5 mM dopamine, 0.45 μCi [2,5,6-^{3}H]dopamine, 5 mM secologanin, 5 mM β-glucuronolactone, 0.1 M tricine-NaOH buffer, pH 7.5 and enzyme, in a total volume of 200 μl. The reaction was terminated by the addition of 10 μl 3.5 M NaOH and the radioactive product was extracted into 500 μl EtOAc. Tubes were vortexed for 30 s and the two phases were separated by centrifugation. The radioactive product of 200 μl of the organic layer was measured by scintillation counting.

4.5. Analytical procedure

Identification of reaction product was performed by HPLC as described previously (De-Eknamkul et al., 1997). Protein concentration was determined by the method of Bradford (1976) using BSA as a standard. Gel permeation chromatography for molecular mass analysis was carried out on a calibrated Superose-6 column (1.6 × 50 cm). SDS-polyacrylamide gel electrophoresis was carried out as described by Laemmli (1970) with 12.0% gels.

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