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ENZYMATIC CONDENSATION OF DOPAMINE AND SECOLOGANIN BY CELL-FREE EXTRACTS OF *ALANGIUM LAMARCKII*

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Key Word Index—*Alangium lamarckii*; Alangiaceae; deacetylipecoside; deacetylisoipecoside; demethylalangiside; demethylisoalangiside; dopamine-secologanin condensing enzyme; cell-free extract.

Abstract—Two novel enzyme activities involved in the condensation of dopamine and secologanin were discovered in the cell-free extracts prepared from the leaves of *Alangium lamarckii*. The extracts condensed dopamine and secologanin rapidly at pH 7.5 to form both (1*R*)-deacetylipecoside (**1**) and (1*S*)-deacetylisoipecoside (**2**) which were converted spontaneously to demethylalangiside (**3**) and demethylisoalangiside (**4**), respectively. Identification of the enzymatic reaction products was performed by using TLC, HPLC-photodiode array detector and LC-MS. A simple enzyme assay by a spectrophotometric method was developed for enzyme activity detection during enzyme purification. The discovery of these two enzyme activities in *A. lamarckii* suggested that the naturally occurring (*S*)- and (*R*)-forms of various tetrahydroisoquinoline-monoterpene alkaloids and nitrogenous glucosides found in this plant are determined by the first enzymatic step of dopamine and secologanin condensation which yields either the (1*R*) or the (1*S*)-condensation product.

INTRODUCTION

Alangium lamarckii Thw. (Alangiaceae) is a tropical medicinal plant that is widely distributed throughout India and southeast Asia. The root bark of this plant has long been used in folk medicine in the treatment of many diseases [1, 2]. Phytochemical studies on *A. lamarckii* have shown that the plant contains a number of alkaloids [1, 3] and nitrogenous glucosides [4–7], most of which are characterized by the presence of a tetrahydroisoquinoline-monoterpene skeleton in the molecules. These tetrahydroisoquinoline-monoterpene alkaloids [e.g. emetine (**7**), cephaeline (**8**), tubulosine (**9**), etc.] and glucosides [e.g. alangiside (**5**), isoalangiside (**6**), etc.] are biosynthetically interesting, particularly with respect to the acquisition of their absolute configuration at the chiral center of the tetrahydroisoquinoline ring. While the alkaloids isolated from natural sources have so far had an (*S*)-configuration, the glucosides, on the other hand, have been mostly found to possess an (*R*)-form at the chiral center. Very recently, the glucosides with an (*S*)-form, such as isoalangiside (**6**) and its derivatives have been reported [6].

Biosynthetically, it has been proposed, based on feeding experiments, that secologanin is condensed with dopamine in a Pictet–Spengler manner to form two epimers, namely (1*R*)-deacetylipecoside (**1**) and (1*S*)-deacetylisoipecoside (**2**) (Fig. 1) [8]. The (*R*)-epimer of deacetylipecoside, is presumably converted further to alangiside-type glucosides whereas the (*S*)-epimer of deacetylisoipecoside is transformed in *A. lamarckii* not only to the alkaloids, but also to the isoalangiside-type glucosides [6]. So far, the proposed biosynthetic scheme has not been investigated at the enzymatic level. This study, therefore, aims to evaluate the pathway by searching for the activities of the first enzymes catalysing dopamine–secologanin condensation.

RESULTS

Detection of enzymatic activity in cell-free extracts

When a crude enzyme extracts prepared from the leaves of *Alangium lamarckii* were incubated with dopamine and secologanin at pH 7.5, a reaction prod-

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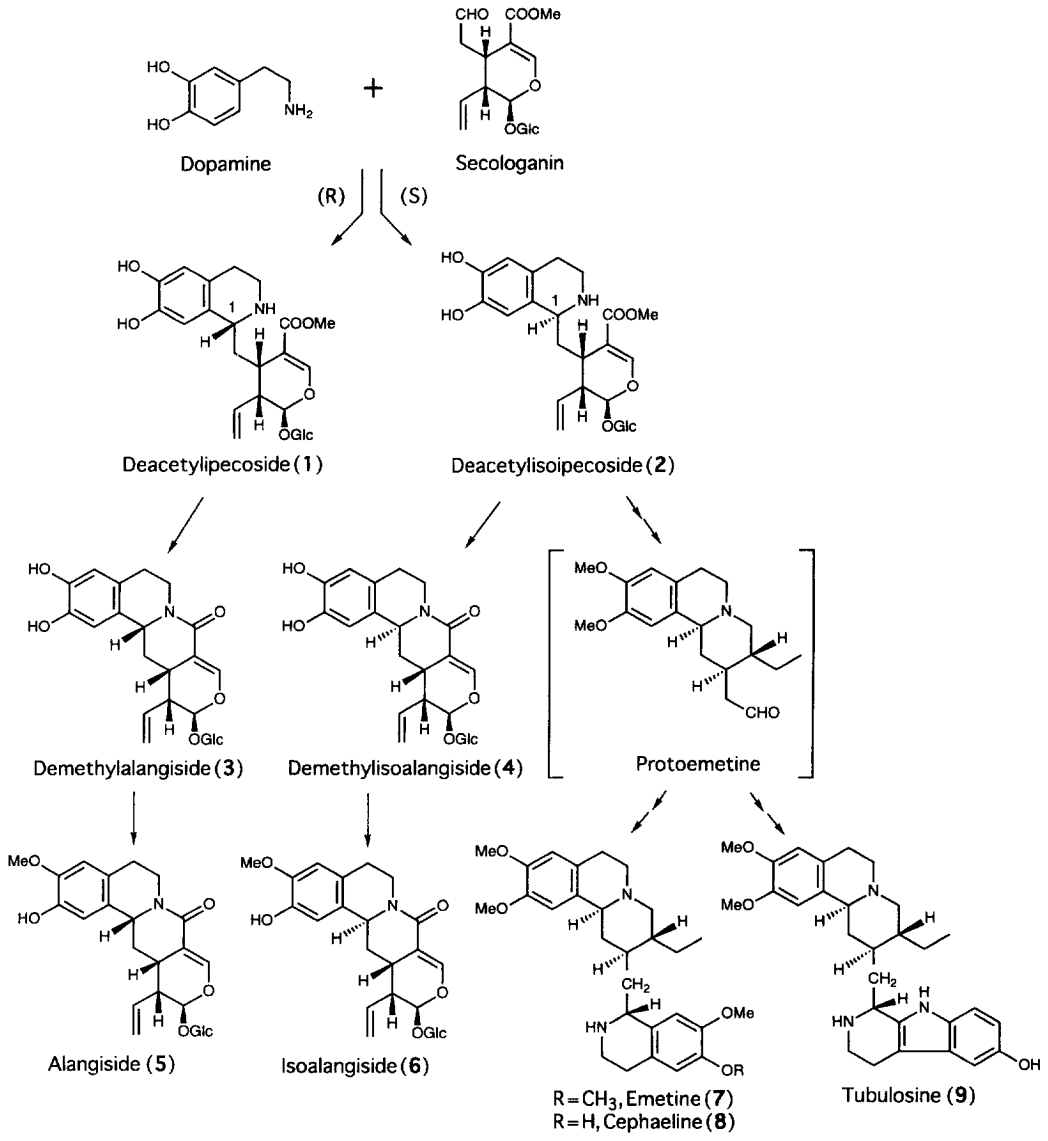

Fig. 1. Proposed biosynthetic sequence for the biosynthesis of the alkaloids and nitrogenous glucosides in *Alangium lamarckii*.

uct appeared in the incubation mixture as detected by the technique of TLC-densitometry. As shown in Fig. 2, the TLC-chromatogram of the reaction mixture obtained after 30-min incubation revealed the presence of a new peak at R_f 0.75 (Fig. 2B). No such peak was detected in the boiled control [Fig. 2(A)] or in the crude extract containing no substrates [Fig. 2(C)]. When the new peak position on the TLC plate was scanned the resulting UV-absorption spectrum showed combined UV-absorption characteristics of both dopamine and secologanin (Fig. 3). Furthermore, when the TLC plate was sprayed with Dragendorff's alkaloidal reagent, the plate showed an orange spot at the same position as the new peak. These results clearly suggested the existence of enzyme activity in the crude protein extract of *A. lamarckii* leaves that rapidly catalysed the condensation of dopamine and secologanin into an alkaloidal product.

Time-course of the enzymatic dopamine–secologanin condensation

A study of the time-course of the reaction product formation was carried out under the same incubation condition as above. The appearance of the enzymatic reaction product during the course of incubation was again monitored by TLC-densitometry using a wavelength of 290 nm. This wavelength could also detect the spot of dopamine but not secologanin (Fig. 3). The resulting chromatograms showed that, during a 2-hr incubation, there was a rapid increase of the alkaloidal reaction product which occurred sim-

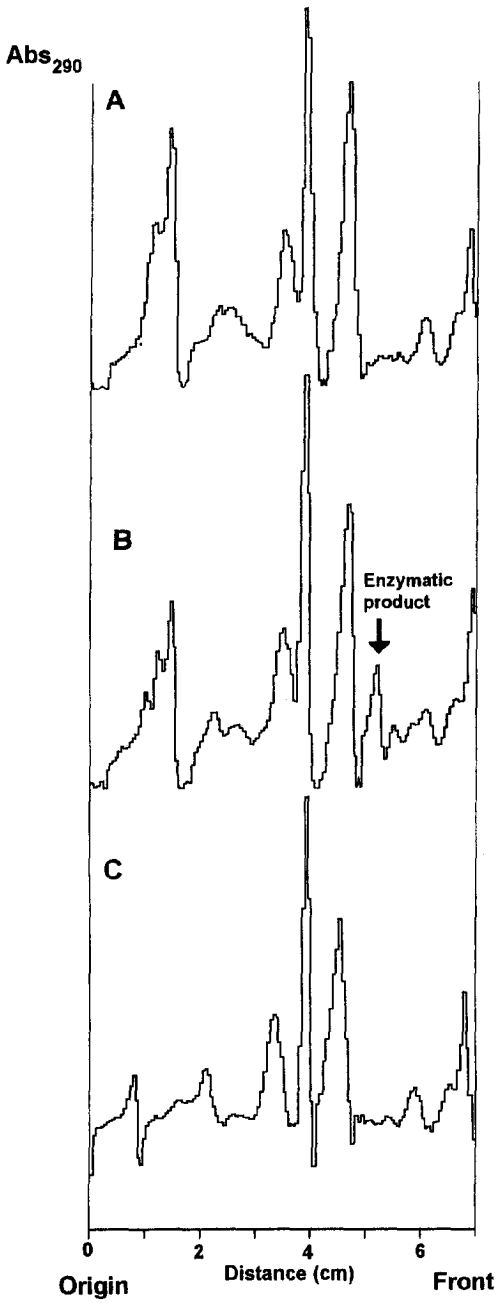

Fig. 2. TLC-densitometric chromatograms of: (A) reaction mixture containing boiled enzyme; (B) complete reaction mixture; and (C) crude enzyme extract.

ultaneously with a decrease of the substrate dopamine (Fig. 4). It should be noted that during the first 60 min there appeared an increase of two peaks close to each other around an R_f value of 0.75. The lower R_f-value peak seemed to increase more rapidly than the one with the higher R_f-value. After 60 min, however, only one big peak was observed (Fig. 4). These results suggested that there might be more than one reaction product generated in the reaction mixture during the incubation.

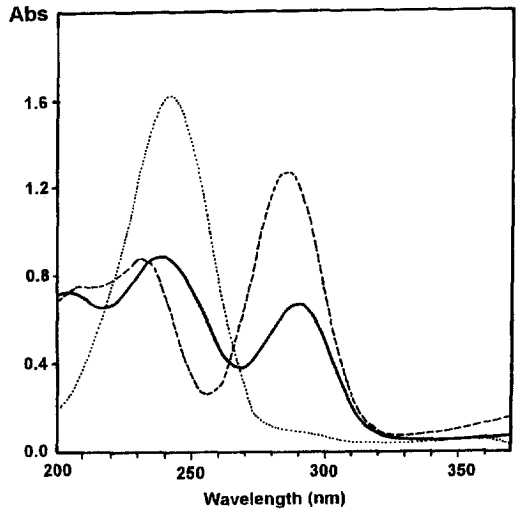

Fig. 3. UV-absorption spectra of dopamine (------), secologanin (.....) and enzymatic product (——). Each spectrum was obtained by wavelength-scan of each spot on TLC plate using TLC-densitometer.

Reaction product identification

In order to identify the enzymatic reaction product, a large-scale (200-fold) incubation of the crude enzyme extracts with dopamine and secologanin was carried out. The resulting incubation mixture was worked up by EtOAc extraction and preparative TLC purification to obtain a fraction containing the reaction product. Identification of the reaction product was performed by both HPLC connected to a photodiode array detector and LC-mass spectrometry. On HPLC, the product preparation showed the presence of two major peaks that co-migrated with authentic demethylalangiside (**3**) (R_t = 8.3 min) and demethylisoalangiside (**4**) (R_t = 15.6 min) (Fig. 5). The UV-absorption spectrum of each peak produced by the photodiode array detector was also identical with its authentic spectrum (Fig. 5, inset). With LC-mass spectrometry, both peaks showed their Cl mass spectra with $[M+H]^+$ at m/z 492, corresponding to the molecular mass 491 of, again, both demethylalangiside (**3**) and demethylisoalangiside (**4**). These results clearly showed that there was an enzyme-catalysed condensation of dopamine and secologanin occurring in the reaction mixture. The immediate condensation products were likely to be (1*R*)-deacetylipecoside (**1**) and (1*S*)-deacetylisoipecoside (**2**) which were converted rapidly to **3** and **4**, respectively.

Development of enzyme assay

The assay system for determining the catalytic activity of the dopamine–secologanin condensing enzyme was developed based on the difference in solubility of the substrate and the enzymatic product. Dopamine is highly soluble in water, whereas the condensation product is more soluble in EtOAc. As a result, the reaction product could be extracted into

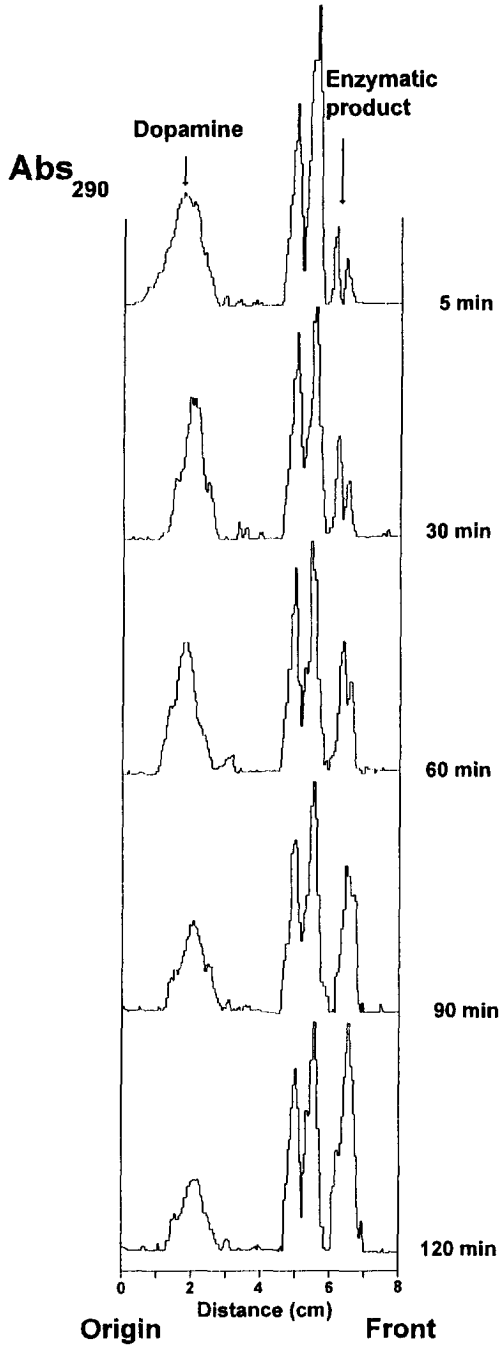

Fig. 4. TLC-densitometric chromatograms showing time-course of the enzymatic dopamine-secologanin condensation by *Alanglum lamarckii* crude enzyme extract.

EtOAc, whereas the unreacted substrate dopamine remained in the aqueous phase. Since the formation rate of the enzymatic product was relatively rapid (Fig. 4), the detection by spectrophotometric methods appeared to be sufficiently sensitive to determine the amount of the extracted product in the EtOAc phase. A wavelength of 290 nm was selected for reading the absorbance because it caused no interference from secologanin which was also extracted into the ethyl acetate phase. As shown in Fig. 3, absolutely no absorbance of secologanin occurred at this wavelength.

To check the recovery of the product extraction, a known amount of authentic demethylalangiside (**3**) (0.1 and 1.0 μmol) was added into the standard reaction mixture (200 μl) and extracted by 1 ml EtOAc. It was found that 60% of **3** was extracted into the organic solvent. With the second EtOAc extraction, more than 90% recovery was observed. In practice, however, the assay was performed with only a single extraction and the values obtained were converted to 100% by calculation. A study on the relationship between the absorbance values and demethylalangiside concentration (in EtOAc) showed that a linear relationship occurred in the concentration range of 40–200 μM which gave the absorbance values of 0.06–0.15, respectively. Within this range, boiled controls usually gave values less than 0.04 absorbance units. Based on a standard absorbance curve, the catalytic activity of dopamine–secologanin condensing enzyme could be determined.

Preliminary study on enzyme purification and detection of enzymatic product

In order to evaluate its efficiency, the developed enzyme assay was used to follow the condensing-enzyme activity during column chromatography of the crude enzyme extract. The chromatography was performed on a Phenyl-Sepharose CL-4B column which was usually used as the first chromatographic step after crude extract precipitation by $(NH_4)_2SO_4$. The concentrated enzyme extract was loaded onto the column pre-equilibrated with 100 mM tricine buffer, pH 7.5 containing 0.2 M $(NH_4)_2SO_4$ and the bound proteins were eluted with the same buffer containing no salt. Under these conditions, it appeared that the enzyme could be detected in the column eluate. As shown in Fig. 6, enzyme activity was found only in three out of 15 fractions of the elution. This confirmed that the developed spectrophometric enzyme assay was sensitive enough for activity detection of the dopamine–secologanin condensing enzyme.

With the enzyme preparation from Phenyl-Sepharose chromatography, the reaction product formed in the reaction mixture was identified one more time. This was to check whether the active enzyme fraction contained both or either one of the condensing enzyme activities. Again, using the technique of HPLC that allows complete separation of both (*R*)- and (*S*)-epimers of the reaction products, it was found that only the (*R*)-epimer of demethylalangiside (**3**) appeared in the HPLC chromatogram (Fig. 7). No peak of demethylisoalangiside was detected. This indicated that the partially purified enzyme preparation contained only the activity of deacetylipecoside synthase.

DISCUSSION

The groups of emetine alkaloids and alangiside/isoalangiside-type glucosides are unique among

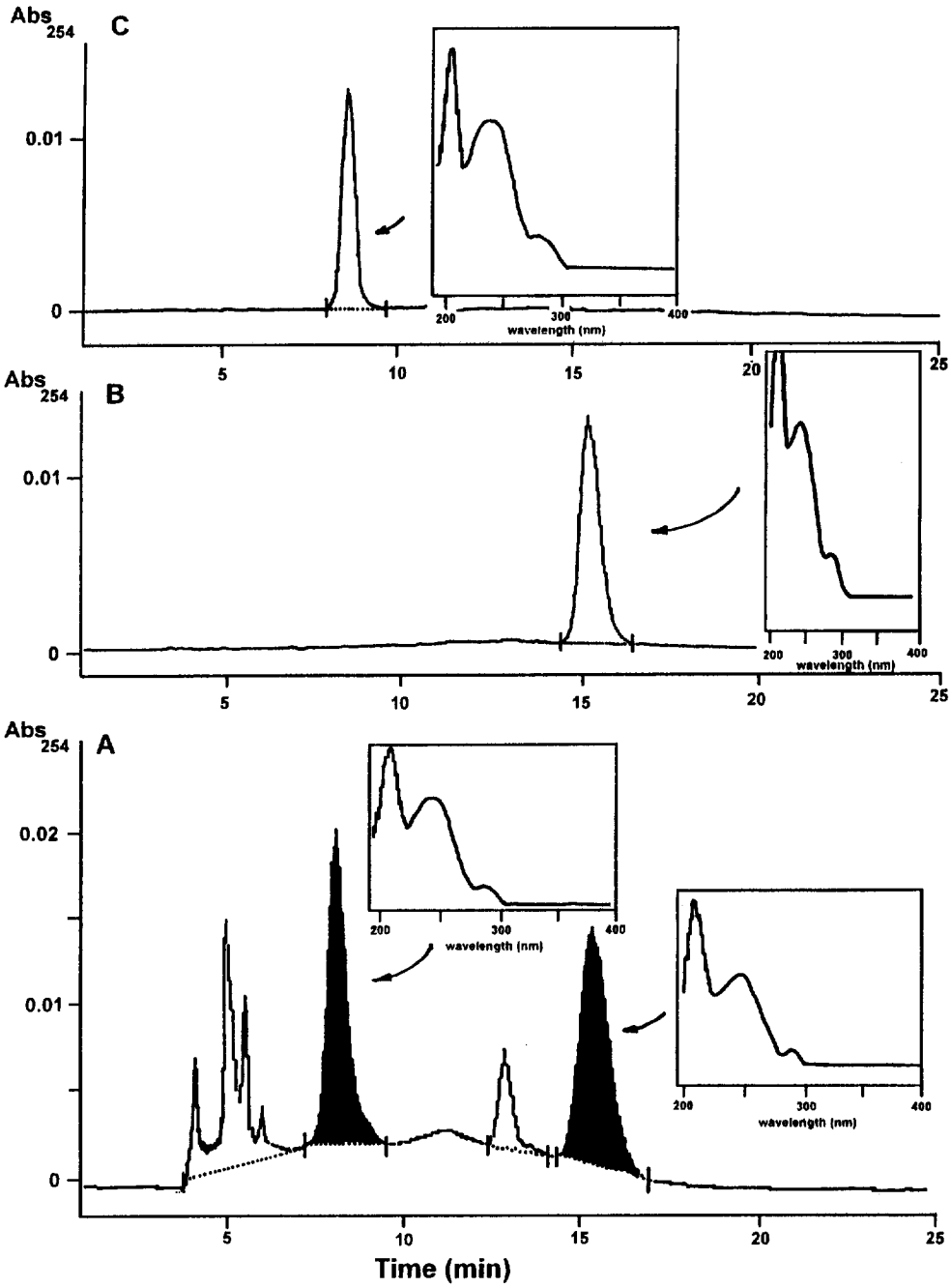

Fig. 5. Identification of the reaction products obtained from enzymatic dopamine-secologanin condensation by HPLC equipped with photodiode array detector. (A) HPLC chromatogram and UV-absorption spectra (inset) of the reaction products; (B) of authentic demethylisoalangiside; and (C) of authentic demethylalangiside.

the isoquinoline alkaloids in that they are biosynthesized from tyrosine (*via* dopamine) and a monoterpene unit (*via* secologanin). These natural products containing tetrahydroisoquinoline-monoterpene skeletons have so far been found to possess both (*S*)- and (*R*)-configurations at the tetrahydroisoquinoline chiral center [3]. The group with (*S*)-configuration includes the emetine alkaloids and nitrogenous glucosides related to isoalangiside, whereas the group with (*R*)-configuration includes the glucosides related to alangiside and ipecoside. In 1978, Nagakura *et al.* [8] made the important observation by feeding [1-^{3}H,3-^{14}C]-(1*S*)-deacetylisoipecoside and [1-^{3}H,3-^{14}C]-(1*R*)-deacetylipecoside into *Alangium lamarckii* and *Cephaelis ipecacuanha* plants that, in both plants, the labelled (*R*)-epimer was exclusively and specifically incorporated into the nitrogenous glucosides alangiside in *A. lamarckii* and ipecoside in *C. ipecacuanha*, each possessing the (*R*)-configuration. In contrast, the (*S*)-epimer was incorporated into the emetine alka-

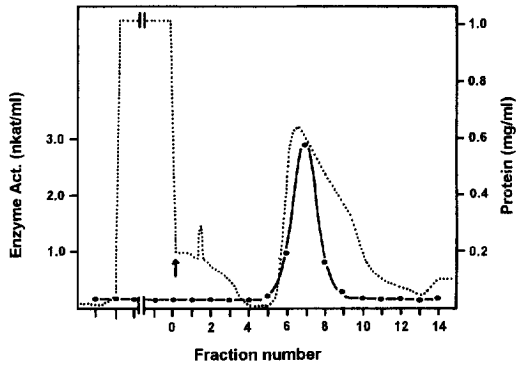

Fig. 6. Phenyl-Sepharose chromatography of *Alangium lamarckii* crude protein extract showing dopamine-secologanin condensing enzyme activity (——) and protein elution profile (.....).

loids with retention of configuration. Based on these findings, they proposed that the first step of the biosynthetic pathway of the isoquinoline-monoterpene alkaloids and glucosides involved the condensation of dopamine and secologanin with the formation of both the (*R*)-epimer deacetylipecoside and the (*S*)-epimer deacetylisoipecoside (Fig. 1). Indeed, the discovery described in this paper of both enzymatic condensation activities in the crucle cell-free extracts of *A. lamarckii* supports such a proposal.

In this study, the cell free-extract of *A. lamarckii* was prepared from the leaf part which has been reported to contain both the (*S*)-form alkaloids dehydroprotoemetine [9], protoemitinol [10], alangimarckine [11] and the (*R*)-form glucoside alangiside [4]. The presence of these tetrahydroisoquinoline-monoterpene natural products with both configurations makes the leaves an ideal starting material for detection of the condensing enzyme activities. Preparation of the crude enzyme extracts with active enzyme activities could be achieved by using 100 mM tricine–NaOH buffer, pH 7.5 containing 10 mM 2-mercaptoethanol. The crude extracts, after being desalted, still contained some alkaloids detectable by TLC (Fig. 2C) and Dragendorff's alkaloidal reagent. These alkaloids, however, did not interfere with the apparent increase of the condensation product in the incubation mixture as detected by TLC-densitometer at λ_{290} nm (Fig. 4). The formation of the reaction product was clearly the result of an enzymatic reaction since no such product was observed in the same incubation mixture containing boiled enzyme extracts [Fig. 2(A)]. Furthermore, replacement in the incubation mixture of dopamine by tyramine resulted in no formation of condensation products.

Identification of the enzymatic reaction product isolated by preparative TLC was performed by using both HPLC equipped with a photodiode array detector and LC-mass spectrometry. The results based on retention times, UV-absorption spectra and mass spectra revealed that the reaction product in fact consisted of two compounds which exhibited characteristics identical to authentic (*R*)-epimer demethylalangiside (**3**) and (*S*)-epimer demethylisoalangiside (**4**). Both epimers could be very well separated from each other by using a C18 column eluted with isocratic solvent of H_2O–MeOH (11 : 9) (Fig. 5). Based on peak area of both products, it appeared that the formation ratio of demethylalangiside and demethylisoalangiside was *ca* 11 : 9. The detection of both **3** and **4** in the reaction mixture indicated that there were, in *A. lamarckii* crude protein extracts, two different enzyme activities catalysing the condensation of dopamine and secologanin to form two opposite epimeric products. These two immediate condensation products were presumably (1*R*)-deacetylipecoside (**1**) and (1*S*)-deacetylisoipecoside (**2**) which could immediately undergo lactamization in the reaction mixture (pH 7.5) to form **3** and **4**, respectively. This spontaneous cyclization has been reported very recently by Itoh *et al.* [6].

In order to purify the dopamine–secologanin condensing enzymes, an enzyme assay was developed for determining their catalytic activities. This was accomplished by making use of different properties of the substrates and products, particularly their difference in solubility and λ_{max} of UV-absorption. The

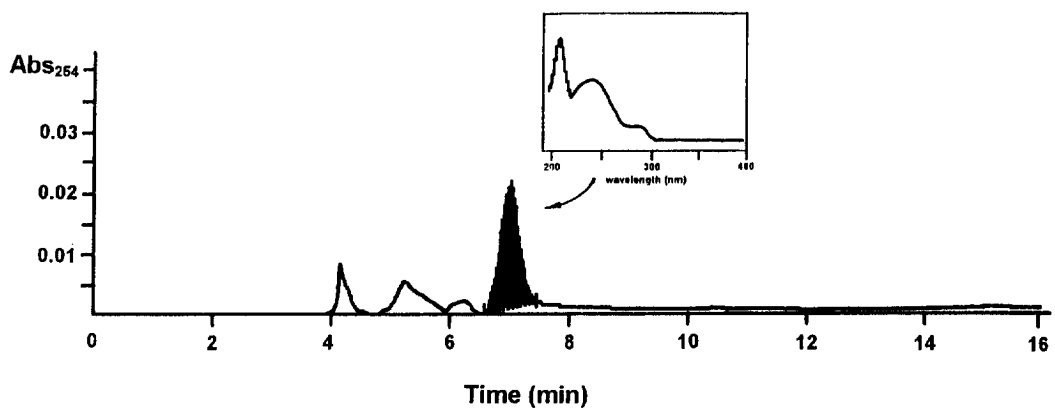

Fig. 7. HPLC chromatogram and UV-absorption spectra (inset) of the enzymatic reaction product obtained from the incubation mixture containing active enzyme fraction of Phenyl-Sepharose chromatography. The peak eluted at *R*, 7.2 is demethylalangiside.

established UV-spectrophotometric assay was simple, rapid and sensitive. It allowed the activity of the condensing enzyme to be followed during purification as shown by Phenyl-Sepharose CL-4B column chromatography (Fig. 6). This chromatographic step showed only one peak of the enzyme activity which appeared associated with the enzyme deacetylipecoside synthase. This conclusion was based on the results of product identification by HPLC which showed only the major peak of demethylalangiside (**4**) (Fig. 7). A peak corresponding to demethylisoalangiside (**5**) was absent from the chromatogram. Therefore, it seems that, under the conditions used in this study, the enzyme deacetylipecoside synthase is more stable than deacetylisoipecoside synthase. Further investigation to stabilize the latter enzyme is needed to allow the two condensing enzymes to be simultaneously purified and characterized.

EXPERIMENTAL

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.

Chemicals. Authentic demethylalangiside and demethylisoalangiside were prepd from dopamine and secologanin as described in ref. [6]. Secologanin was isolated from dried leaves of *Lonicera tartarica* harvested during July–August. The isolation procedure was followed as given in ref. [12] with 1.5 kg leaf material being used.

Enzyme extraction. Fresh leaves (20 g) were ground in a mortar in the presence of liquid N_2. The resulting fine powder was thawed with stirring over 20 min at 4° in 20 ml 100 mM tricine–NaOH buffer, pH 7.5 containing 10 mM 2-mercaptoethanol (extraction buffer). The suspension was pressed through 4 layers of cheesecloth and the filtrate was centrifuged at 10000 *g* for 10 min at 4°. The supernatant was then desalted (Sephadex G-25) and used as the crude enzyme extract.

Detection of dopamine–secologanin condensing enzyme activity. The enzyme activity in the crude extract responsible for dopamine–secologanin condensation was detected in a reaction mixt. containing 1 mM dopamine, 1 mM secologanin, 60 mM tricine–NaOH buffer, pH 7.5 and crude enzyme extract, in a total vol. of 180 μl. After 30 min incubation, or during a time-course study of product formation, the reaction mixt. was analysed for the presence of the condensation product by TLC-densitometry. This technique involved fractionation of the reaction mixture (20 μl) on a silica gel 60 F_{254} plate using the solvent system $CHCl_3$–*n*PrOH–MeOH–H_2O, 3:1:4:2 ($CHCl_3$ phase). The TLC plate was then scanned to obtain chromatograms by TLC-densitometry using a wavelength of 290 nm. At the TLC spot position of the putative reaction product, it was scanned from 200 to 370 nm in order to obtain a UV-absorption spectrum.

Preparation of enzymatic reaction product. The enzymatic reaction product was prepared from a large-scale (200-fold) incubation mixture containing the same composition as described above. The reaction mixture (36 ml) obtained after 90 min was extracted 4× with EtOAc (18 ml for each extraction). The pooled EtOAc frs were evapd and fractionated on a prep. TLC (silica gel 60F_{254}S) using the solvent system $CHCl_3$–*n*PrOH–MeOH–H_2O, 3:1:4:2 ($CHCl_3$ phase). The product band with $R_f = 0.79$ was eluted with absolute EtOH and evapd *in vacuo* until dry before product identification.

Identification of enzymatic reaction products. Was performed by both HPLC connected to a photodiode array detector and LC-MS. For the former, the reaction product prepared from the prep. TLC was subjected to HPLC on a Nova-Pak C_{18} (5 μm) column (300 × 3.9 mm) using an isocratic solvent of H_2O–MeOH (11:9) at 0.5 ml min^{-1} flow rate and detected by a photodiode array detector. Under these conditions, authentic demethylalangiside (**3**) was eluted at $R_t = 8.3$ min and demethylisoalangiside (**4**) at $R_t = 15.6$ min. Both showed λ_{max} at 234 and 288 nm in their UV absorption spectra. For LC-MS, the reaction product was subjected to HPLC on a Nova-Pak C_{18} (5 μm) column (300 × 3.9 mm) using an isocratic solvent of H_2O–MeOH (11:9) at a flow rate of 0.8 ml min^{-1}. The mass spectral analyses were performed by using the atmos. pres. chemical ionization (APCI) method: vaporizer temp. 400°, nebulizer temp. 235°, drift voltage 40 V, total ion current *m/z* 488–498, at a flow rate of 0.8 ml min^{-1} and monitored by UV absorption at 254 nm. Under these conditions, authentic demethylalangiside (**3**) and demethylisoalangiside (**4**) were eluted at R_t values of 4.0 and 6.3 min, respectively, with the same pseudomolecular ion $[M+H]^+$ at *m/z* 492.

Enzyme assay. Mixture contained 0.5 mM dopamine, 0.5 mM secologanin, 90 mM tricine–NaOH buffer, pH 7.5 and enzyme, in a total vol. of 200 μl. The mixture was incubated for 30 min at 30°. The reaction was terminated by the addition of 1 ml EtOAc. Tubes were vortexed for 20 sec to extract reaction products into EtOAc and phase separation was achieved by centrifugation. The *A* of the organic layer was read against EtOAc (AR grade) at 290 nm. The *A* from boiled control or reagent blank was also read in each lot of the assay. The difference value was converted to the amount of the reaction products by using a calibration curve that showed linearity from 40 to 200 μM of demethylalangiside. Demethylalangiside was prepared from dopamine and secologanin according to the method described in ref [6].

Phenyl-Sepharose column chromatography of crude enzyme extract. The non-desalted crude enzyme extract (200 ml) prepd as described above was made up to 80% satn with $(NH_4)_2SO_4$ and the pellet was collected by centrifugation (10000 *g*, 10 min, 4°). The

pellet was dissolved in a minimal vol. of extraction buffer containing 0.2 M $(NH_4)_2SO_4$. The concd enzyme soln (10 ml) was then applied to a Phenyl-Sepharose CL-4B column (20 × 2.5 cm) pre-equilibrated with the same buffer. After washing with 200 ml of the buffer at a flow rate of 1 ml min^{-1}, the column was eluted with 150 ml of extraction buffer and 10 ml frs were collected. Each fr. was then assayed for the activity of dopamine–secologanin condensing enzyme.

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