CHAPTER IV

RESULTS

1. Synthesis of Deacetylipecoside and Deacetylisoipecoside Standards

Dopamine-secologanin condensing enzyme from *A. salviifolium* cell-free extract has been found recently to condense two molecules of the precursors dopamine and secologanin to form (*R*)-deacetylipecoside which is converted further to other alangiside-type glucosides (De-Eknamkul *et al.*, 1997). This finding was repeated in this study to confirm the results. In doing this, (*R*)-deacetylipecoside was first prepared for being used as standard in the identification of the enzymatic condensation product. The synthesis of deacetylipecoside (*R*-form) and deacetylisoipecoside (*S*-form) has been reported previously (Nagakura *et al.*, 1978) using a simple synthesis procedure (described in section 4, Experimental). The reaction involved the condensation of dopamine and secologanin under relatively mild acidic condition of pH 5.0 resulting in the formation of a mixture of two diastereoisomeric condensation products (Battersby *et al.*, 1971). This reaction occurs via a Pictet-Spengler mechanism (Figure 6) (Pictet and Spengler, 1911). The main feature is that after the condensation of amine with the aldehyde functional group, a Schiff base is formed, which is intramolecularly attacked by 1-position of catecholamine nucleus. The resulting isoquinoline isomers are subsequently rearranged to give a mixture of (1*R*)-deacetylipecoside and (1*S*)-deacetylisoipecoside (Figure 6). Then the condensation products of (*R*)-deacetylipecoside and (*S*)-deacetylisoipecoside could be undergone lactamization to form (*R*)-demethylalangiside and (*S*)-demethylisoangiside, respectively (Figure 7) (Itoh *et al.*,1995).

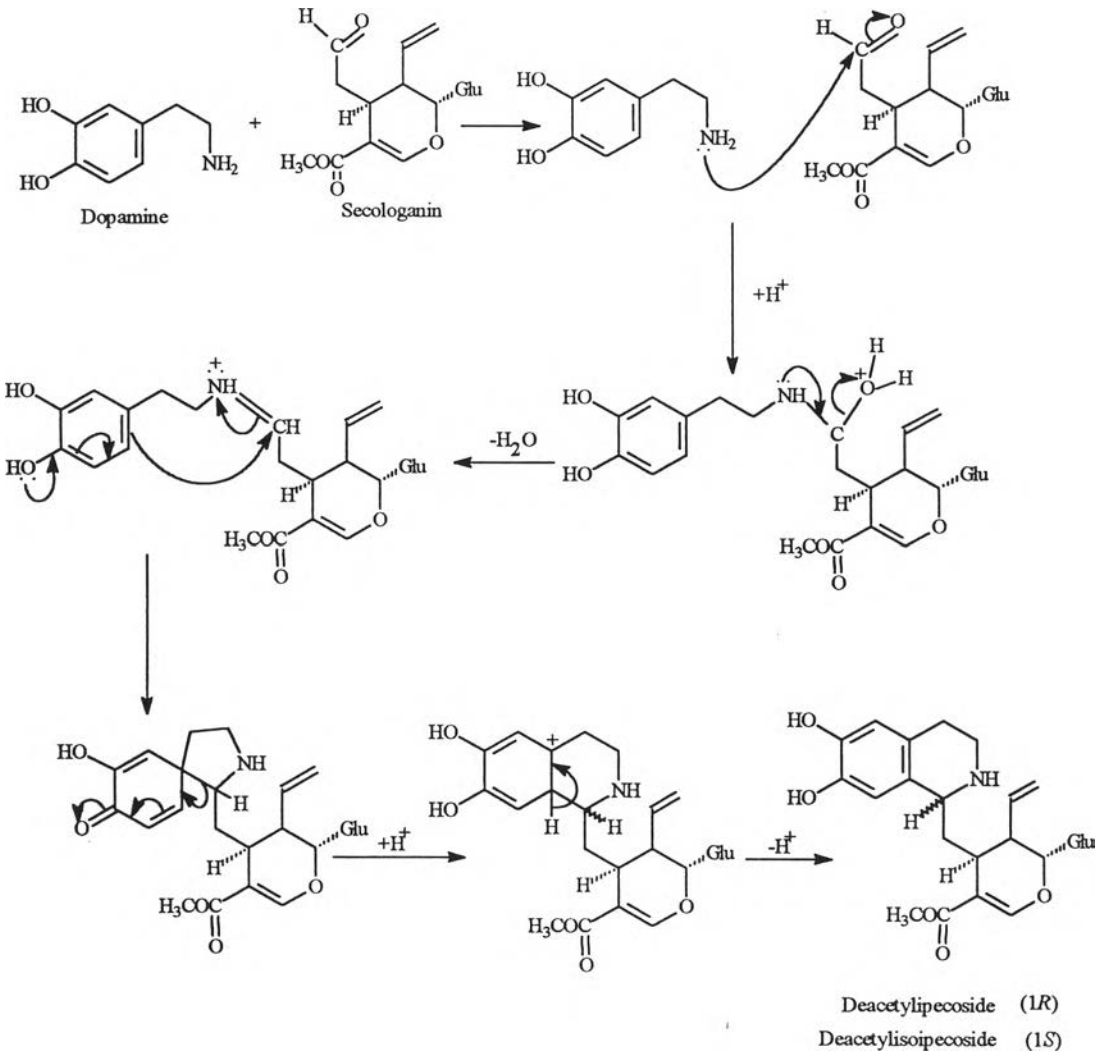


Figure 6 Pictet-Splenger manner condensation of dopamine and secologanin

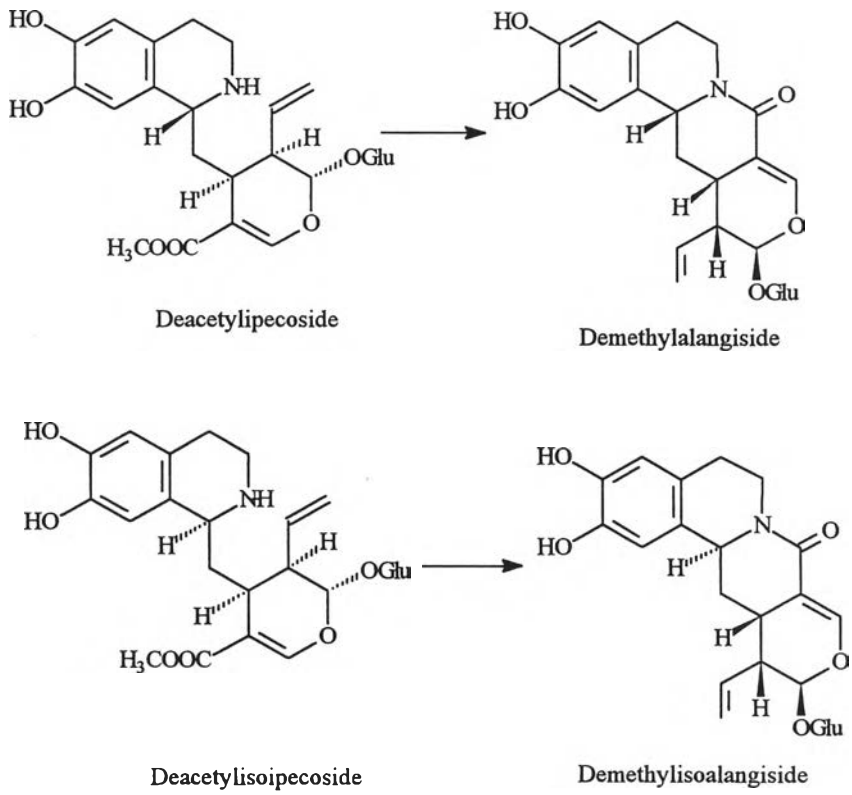


Figure 7 Spontaneous lactamization of dacetylipecoside and deacetylisoipecoside to form demethylalangiside and demethylisoalangiside

The lactamization products of (*R*)-demethylipecoside and (*S*)-demethyl-isoipecoside could be very well separated from each other by using Nova pak C_{18} (5 μm) column (300 x 3.9 mm) eluted with isocratic solution of H_2O-MeOH (1:1). Under these conditions, authentic demethylalangiside was eluted at R_f = 11.05 min and demethylisoalangiside at R_f = 17.83 min. Both diastereoisomers show λ_{max} at 240 nm and 290 nm in their UV-absorbtion spectra. Based on the peak areas of both products, it appeared that the formation ratio of (*S*)-demethylalangiside and (*R*)-demethylisoalangiside was approximately 9 : 1 (Figure 26A).

2. Detection of Dopamine-Secologanin Condensing Enzyme Activity in *Alangium salviifolium* Cell-Free Extracts

Previous phytochemical studies on many parts of this plant have shown that the plant contains a number of emetine alkaloids and nitrogenous glucosides, most of which are characterized by the presence of a tetrahydroisoquinoline monoterpene alkaloids (see references in Table 1). For this reason, this plant was selected to be used in this study. Detection of the activity of dopamine-secologanin condensing enzyme was first reported in the cell-free extracts prepared from the leaves of *A. salviifolium*. The crude enzyme extract was incubated in the presence of dopamine and secologanin at pH 7.5 for 60 min. The reaction product was detected by TLC densitometer. As shown in Figure 9, the TLC-chromatogram of the reaction mixture obtained after 60 min incubation period revealed the presence of a condensation product peak at $R_f = 0.57$. No apperance of peak detected at the same R_f value in the boiled control and 0 min incubation control. The peak at $R_f = 0.57$ was scanned by wavelength and the obtained UV-absorption spectrum showed combined UV-absorption characteristics of both dopamine (λ_{max} 290 nm) and secologanin (λ_{max} 240 nm) (Figure 10). Furthermore, when the TLC plate was sprayed with Dragendorff's alkaloid reagent, they showed the orange spots at alkaloid position (Figure 8). The results clearly suggested the existence of an enzyme activity in the crude protein extracts of *A. salviifolium* leaves that indicated the rapid catalyse the condensation of dopamine and secologanin into an alkaloidal products.

The presence of the condensing enzyme activity in the crude protein extracts also suggested the possibility to work further on the purification and characterization the enzyme by using *A. salviifolium* leaves as the enzyme source.

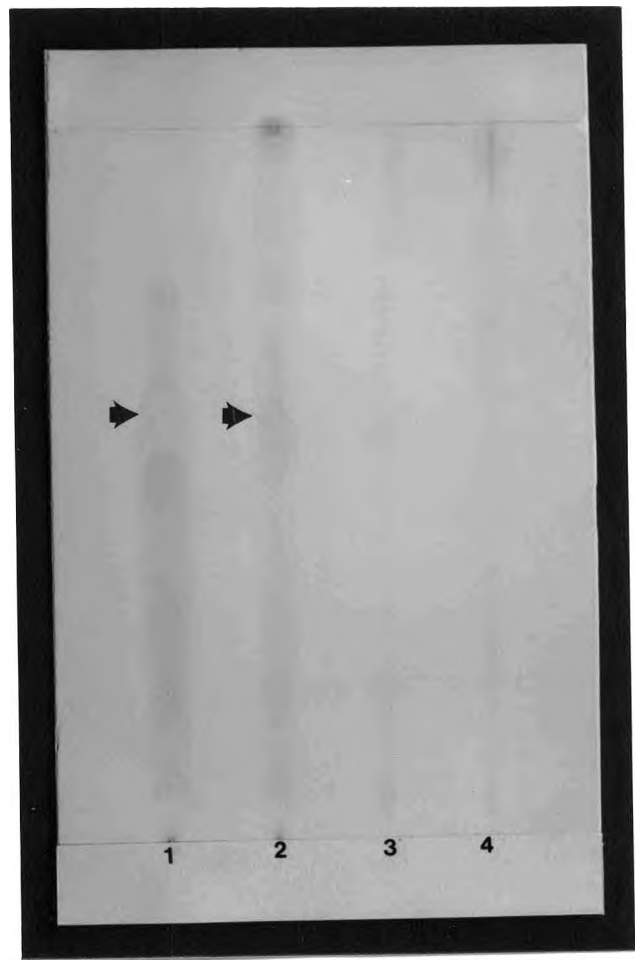


Figure 8 TLC patterns of the reaction mixtures under various conditions showing the condensation of dopamine and secologanin after 60 min incubation period. The patterns was observed after the plate was sprayed with Dragendorff's reagent (1) Authentic mixture of deacetylipecoside and deacetylisoipecoside (2) Sample reaction of *A. salviifolium* crude extracts (3) Boiled control (4) 0 min incubation control

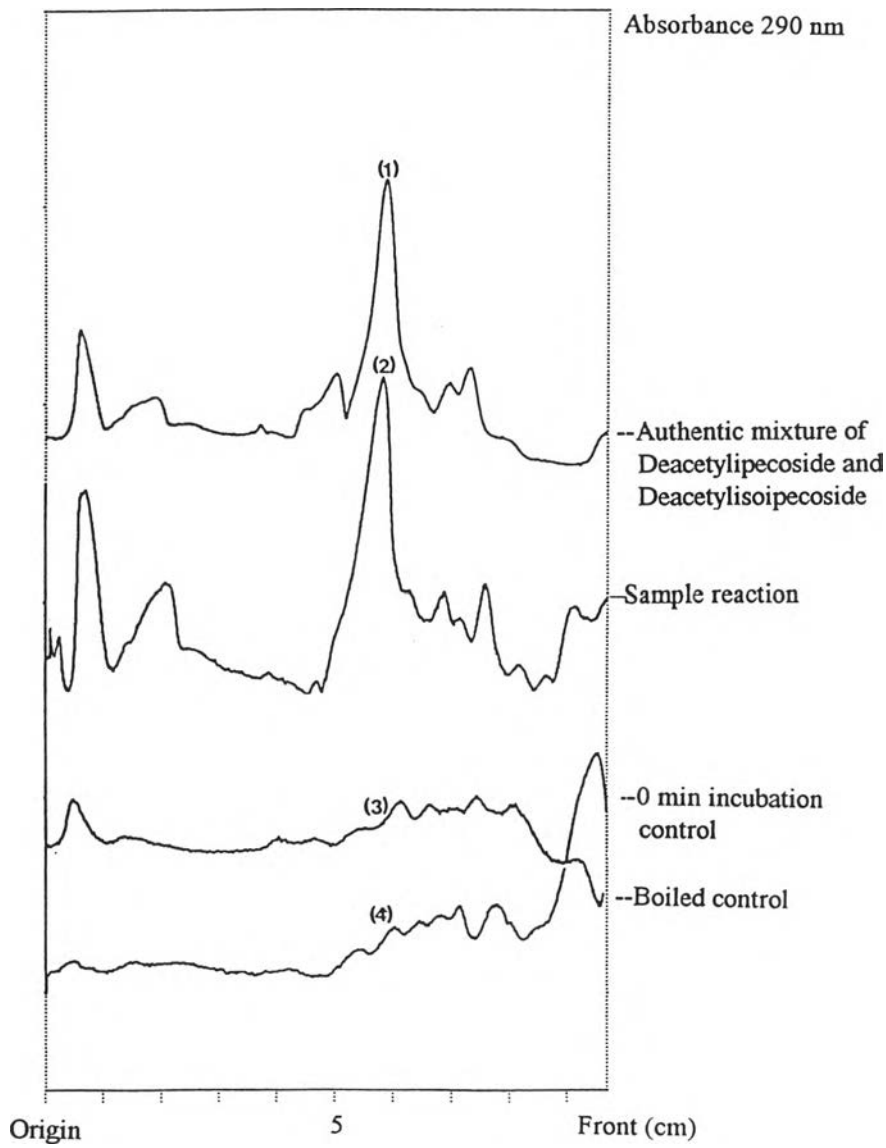


Figure 9 TLC-densitometric chromatograms of the TLC plate in Figure 8 The chromatograms were obtained by using the wavelength of 290 nm. (1) Authentic mixture of deacetylipecoside and deacetylisoipecoside (2) Sample reaction of *A. salviifolium* crude enzyme extracts (3) Boiled control (4) 0 min incubation control of sample reaction

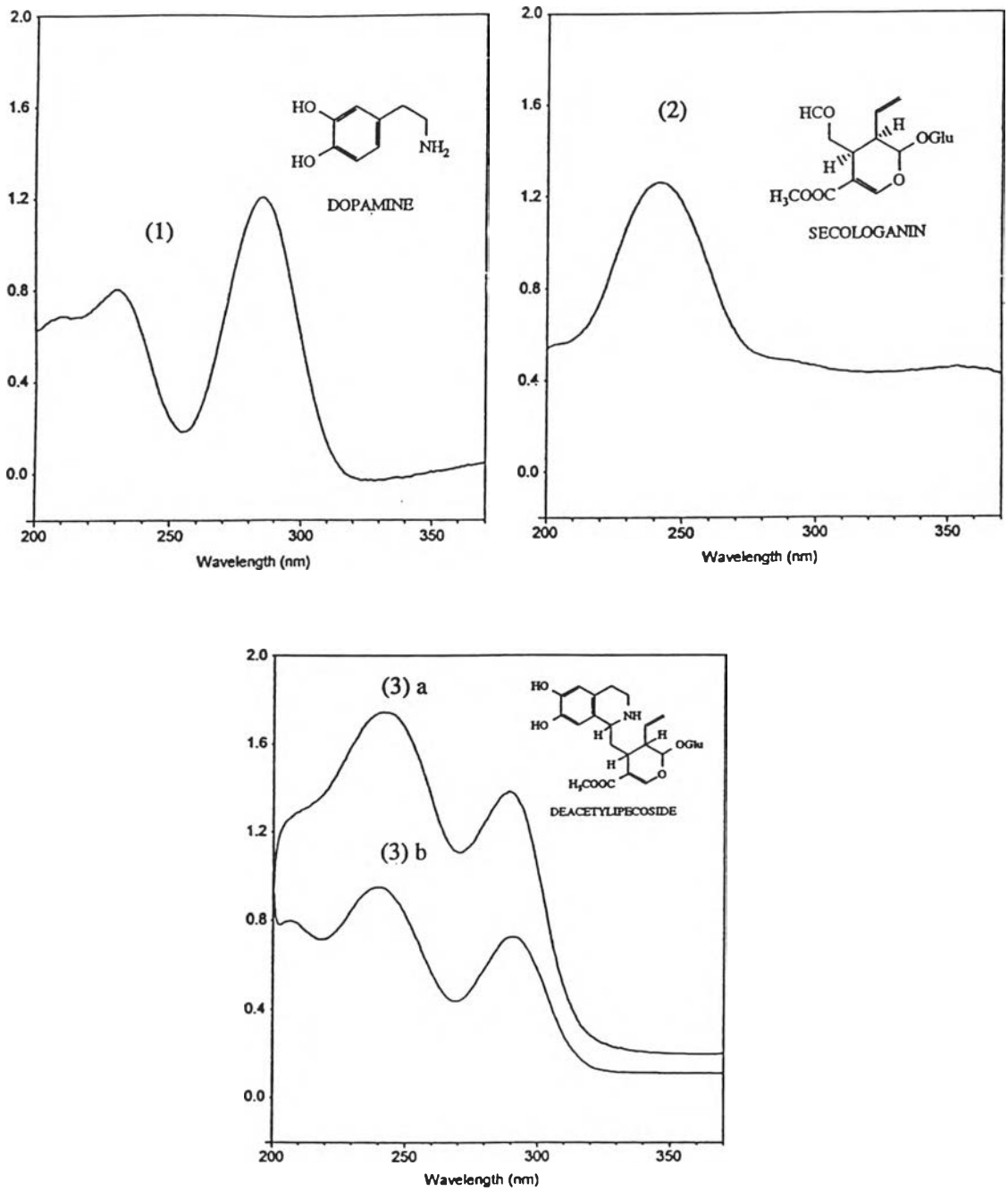


Figure 10 UV-spectra of (1) dopamine (2) secologanin (3a) Authentic mixture of deacetylipecoside and deacetylisoipecoside (3b) Product from the enzymatic reaction appearing on the TLC plate of Figure 8 and Figure 9

3. Purification of Dopamine-Secologanin Condensing Enzyme

The leaves of *A. salviifolium* used for enzyme isolation and purification were the fresh leaves with dark green color. Very young or old leaves were found to have low enzyme activity. After obtaining the crude enzyme extracts as described in Experimental, section 5, the condensing enzyme was purified by using a combination of various steps, including ammonium sulfate precipitation, ultrafiltration and column chromatography using Phenyl Sepharose CL-4B (hydrophobic interaction chromatography), DEAE-Sephacel (anion exchanger chromatography), Superose 6 HR 16/50 (size exclusion chromatography). By these procedures, the dopamine-secologanin condensing enzyme was purified 573-fold with an over all yield of 6%. The specific activity of the purified preparation varied from 650 nkat/mg protein to 750 nkat/mg protein. The results are summarized in Table 3.

Although considerable amount of enzyme activity was found in the dark green young leaves, the crude enzyme extract obtained also appeared to have relatively high amount of secondary metabolism products which could cause loosing the enzyme activity. Many studies have suggested that the phenolic products present abundant in plants have a high possibility to destabilize proteins by forming strong hydrogen bonding complex with proteins (Loomis *et al.*, 1974) and non-specific binding with others secondary metabolites. The polyvinylpolypyrrolidone (PVPP) was added during extraction procedure to remove the phenolic compounds present in leaves.

In the initial step of enzyme purification, a large amount of undesired proteins were removed by ammonium sulfate precipitation between 40-60% saturation. In this saturation range, the enzyme was concentrated into a small volume. This step could reduce major portion of proteins (92%) in the crude extracts although 75% of the enzyme activity was also lost in the process. This step resulted in a 3-fold purification.

The 40-60% saturation of ammonium sulfate enzyme solution was then loaded directly into a Phenyl Sepharose CL-4B column. This hydrophobic interaction chromatography appeared to be an excellent technique for removing the interfering secondary compounds and also resulted in a resonable separation of dopamine-secologanin condensing enzyme from the bulk of proteins. The enzyme appeared to have more hydrophobic nature when compared to the rest of the proteins. Figure 11 shows the profile of proteins eluted from the column detected absorbance at weavelength 280 nm and the enzyme activity. The proteins was eluted in two peaks and the enzyme activity was detected mostly in the second peak. In this purification step, the yield of protein was reduced to 8% with 27-fold purification. These fractions containing the enzyme activity were pooled and passed through the anion exchange column of DEAE-Sephacel.

When the enzyme preparation was subjected to DEAE-Sephacel chromatography, the dopamine-secologanin condensing enzyme showed binding interaction under 0.1 M tricine-NaOH buffer pH 7.5 and elution by 2-step gradient of 1 M KCl in tricine-NaOH buffer. Although the eluted proteins did not separated completely, the main of enzyme activity showed in the third peak occurring during an increase of the salt concentration from 45 mM to 70 mM (Figure 12). This step gave a 108-fold of purification with 11 % yield of proteins.

The activity protein fractions were pooled and then subjected to ultrafiltration. This step remove proteins with M_r under 10 kD and KCl salt and concentrated the protein preparation at the same time.

The final purification step was on Superose 6 size exclusion chromatography. By this step, the enzyme was separated and purified to homogeneity. As shown in Figure 13, enzyme activity was found in the begining of the chromatogram. The final preparation of dopamine-secologanin condensing enzyme was obtained with 573-fold purification and specific activity of 717 nkat/mg protein.

The entire purification procedures could be completed within four days. The specific activity of the purified preparation was 717 nkat/mg protein. The purity of dopamine-secologanin condensing enzyme in each step of purification procedure was checked by 12% SDS-PAGE (Figure 14). The purified enzyme preparation was then kept at -20°C for further studies.

Table 3 Summary of the purification steps of dopamine-secologanin condensing enzyme from *Alangium salviifolium* Wang. leaves.

Purification step	Volume (ml)	Total protein (mg)	Total activity (nkat)	Specific activity (nkat/mg)	Yield (%)[a]	Purification factor (-fold)
Crude extracts	550	462	575	1.3	100	1
40-60% $(NH_4)_2SO_4$	105	39	125	3.1	22	3
Phenyl Sepharose CL-4B	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

a = % yield calculated from total activity

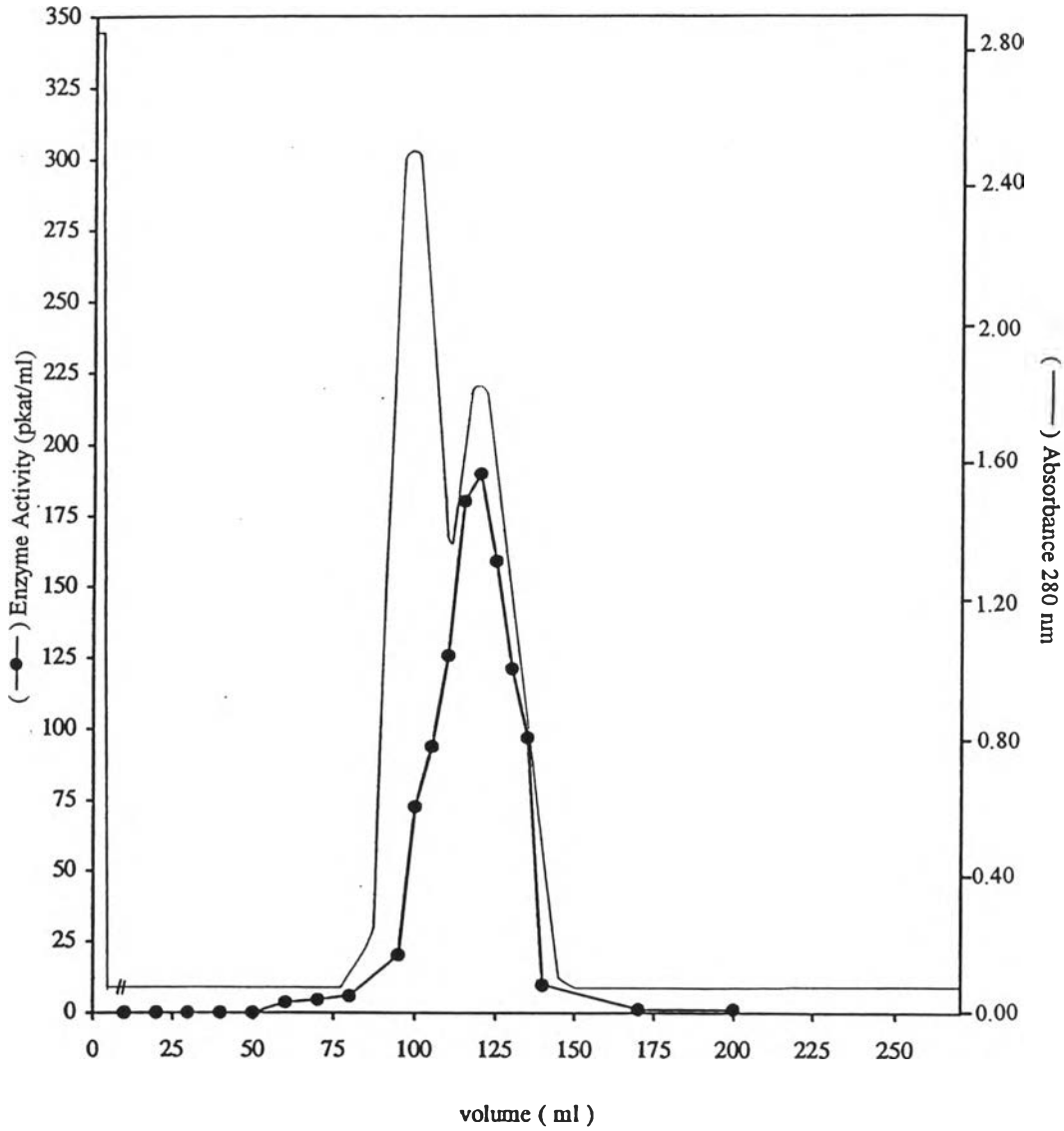


Figure 11 Phenyl Sepharose CL-4B hydrophobic column chromatography of dopamine-secologanin condensing enzyme obtained after 40-60 % ammonium sulfate precipitation.

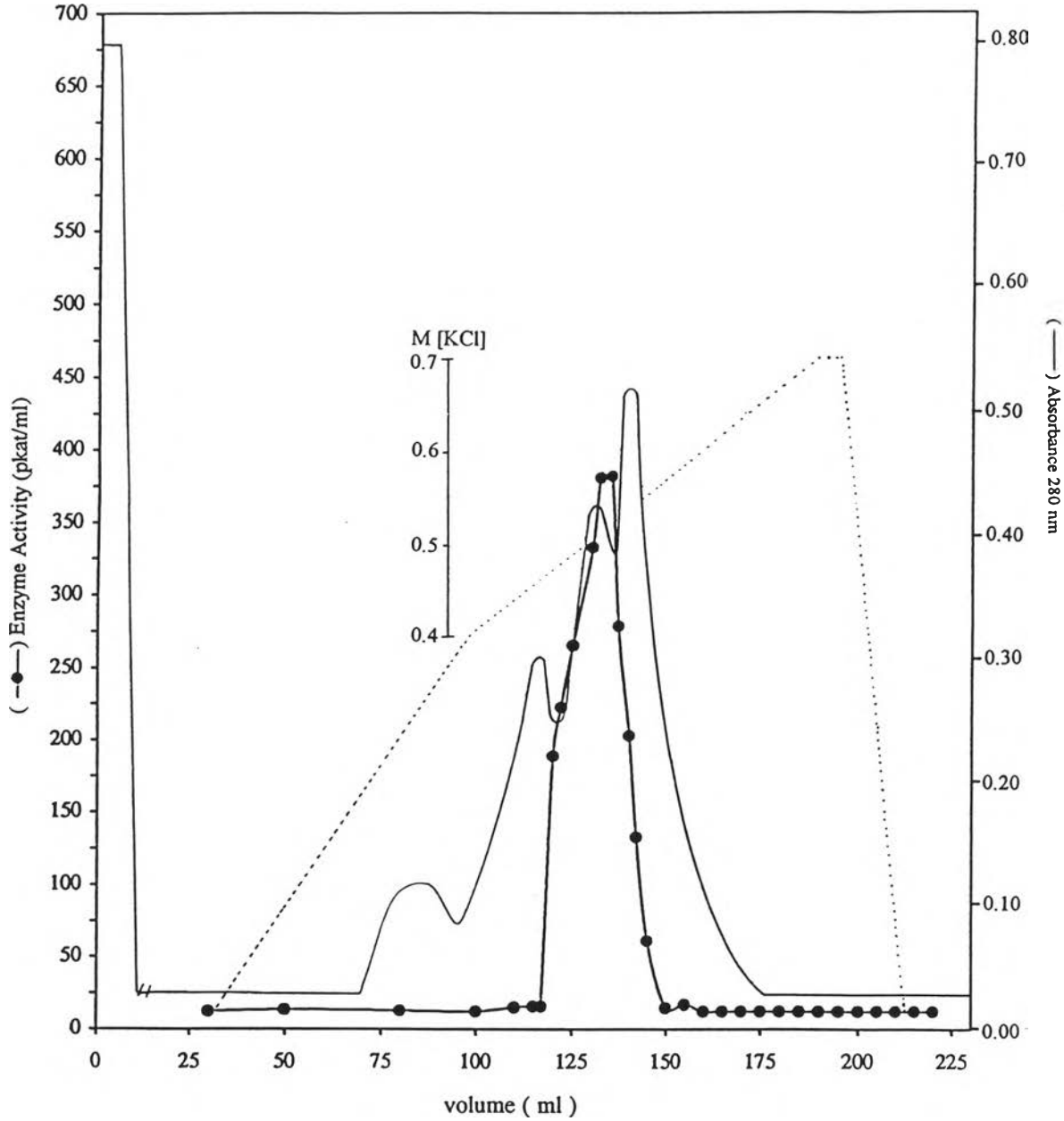


Figure 12 DEAE-Sephacel anion exchange column chromatography of the pooled of active fractions from Phenyl Sepharose CL-4B

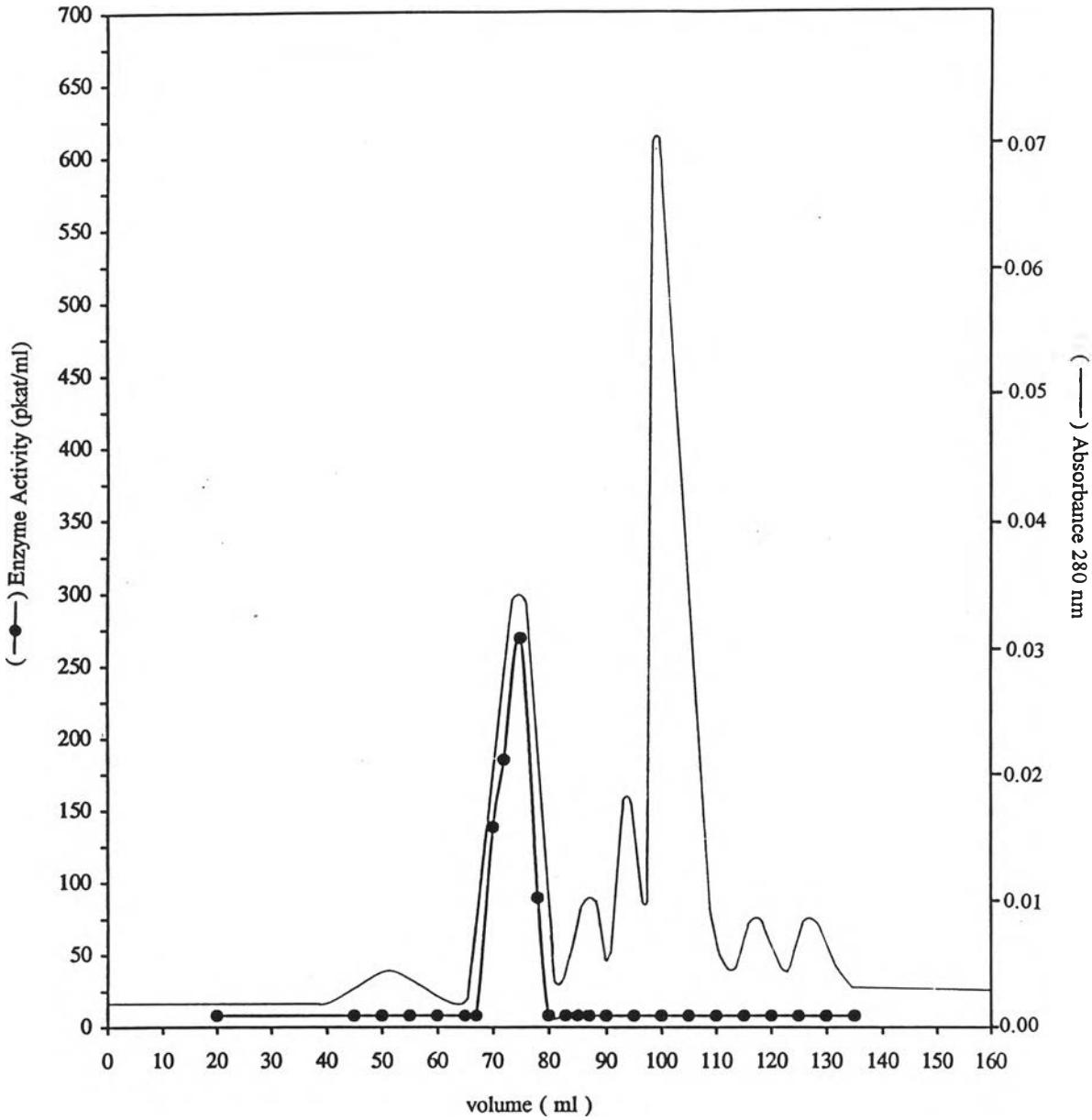


Figure 13 Superose 6 size exclusion column chromatography of the pooled of active fractions from DEAE-Sephcel after concentrated by ultrafiltration

4. Purity Check of Purified Dopamine-Secologanin Condensing Enzyme

SDS-PAGE was utilized in order to determine the purity of the purified enzyme. This was carried out by comparing protein components present in each purification step. Experimentally, the active fractions were pooled and a small volume of each enzyme solution was loaded onto a 12% acrylamide slab gel. Electrophoresis was carried out with a constant current of 45 mA. After operation, the gel was stained using silver staining. Figure 14 shows the SDS-PAGE pattern which indicated that dopamine-secologanin condensing enzyme was purified in each step of purification. The purified enzyme migrated as a single band showing major protein concentration. Furthermore, the gel also showed an increasing concentration of the protein bands along step of chromatography, confirming that purified enzyme was the one with the dopamine-secologanin condensing enzyme activity.

5. Molecular Weight of Dopamine-Secologanin Condensing Enzyme

5.1 Determination by SDS-PAGE

The purified dopamine-secologanin condensing enzyme preparation was loaded onto an electrophoresis gel. After electrophoresis, the gel was developed with silver staining. The molecular weight of the enzyme was estimated by comparing its relative mobility (R_f) to those of known molecular weight proteins. In this case, the following protein standards were used : phosphorylase b (97.4 kD, R_f 0.3), bovine serum albumin (66.2 kD, R_f 0.42), ovalbumin (45 kD, R_f 0.54), carbonic anhydrase (31 kD, R_f 0.66), soybean trypsin inhibitor (21.5 kD, R_f 0.78) and lysozyme (14.4 kD, R_f 0.9). The calibration curve of proteins was plotted on the Log protein size (y-axis) versus relative mobility (R_f) (x-axis) (Figure 15). The calibration curve of Log protein size versus relative mobility (R_f) generated the equation of line as follows

$$\text{Log Molecular Weight} = -1.37 \times (R_f) + 5.40$$

The relative mobility of dopamine-secologanin condensing enzyme was 3.35. The molecular weight of the enzyme calculated from this equation gave the molecular weight values of 30.05 kD.

5.2 Determination by Gel Filtration

The calibrated column of Superose 6 HR 16/50 provided a simple and well documented way for determining the molecular weight of enzyme during a natural stage in purification processes and thus giving information on the molecular weight or size of native protein. The profile of the dopamine-secologanin condensing enzyme activity versus the volume of elution buffer is shown in Figure 13. Superose 6 HR 16/50 column calibrated by the following standard proteins; thyroglobulin (670 kD), gamma globulin (158 kD), ovalbumin (44 kD), myoglobulin (17 kD), vitamin B-12 (1.35 kD) (Figure 16). The void volume (Vo) of Superose 6 HR 16/50 was found to be 38 ml. Figure 17 shows the calibration curve of proteins which was plotted against Log molecular weight (x-axis) versus Vo/Ve (y-axis) of standard proteins : thyroglobulin (0.61), gamma globulin (0.56), ovalbumin (0.52), myoglobulin (0.49) and vitamin B12 (0.40). The equation was generated from the calibration curve is as follow :

$$\text{Log Molecular Weight} = 12.22 \times (Vo/Ve) - 1.70$$

Using this calibrated column, the dopamine-secologanin condensing enzyme was eluted out at 75 ml, with the Vo/Ve ratio of this enzyme was 0.51, corresponding to a molecular mass about 30.38 kD.

Based on the results of both SDS-PAGE and gel filtration techniques, it was suggested that the dopamine-secologanin condensing enzyme was a monomeric protein with molecular weight of approximately 30 kD.

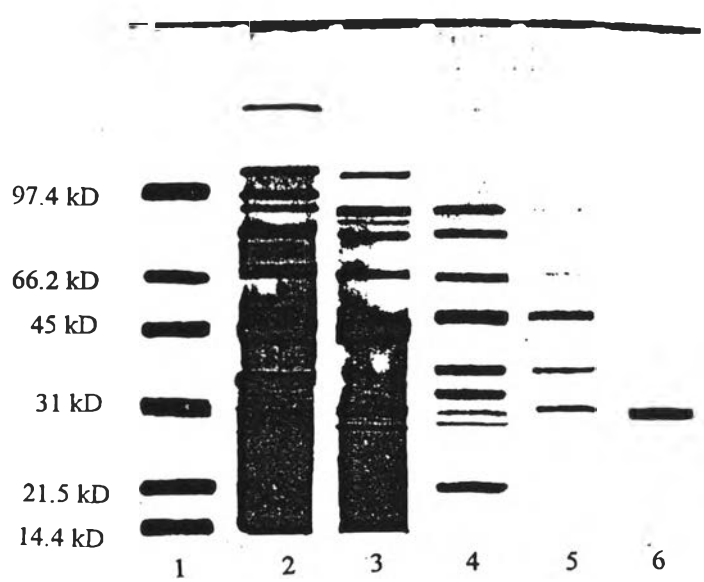


Figure 14 SDS-PAGE pattern of purified proteins in each step of purification
Lane 1. : Low molecular weight marker
Lane 2. : Crude enzyme extracts
Lane 3. : 40-60 % ammonium sulfate precipitation
Lane 4. : Pooled active fractions from Phenyl Sepharose CL-4B
Lane 5. : Pooled active fractions from DEAE-Sephacel
Lane 6. : Pooled active fraction from Superose 6

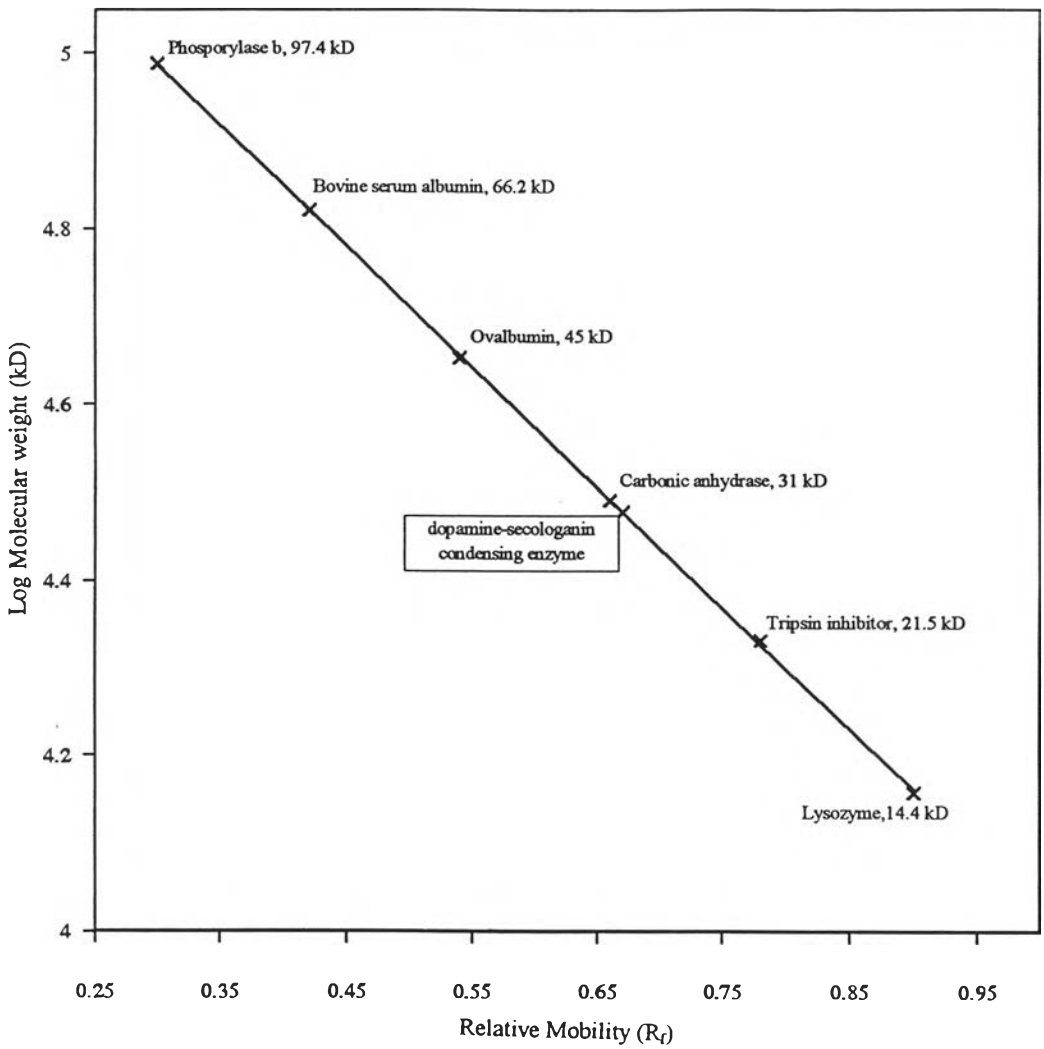


Figure 15 Standard calibration curve of Log molecular weight plotted against R_f values of standard proteins from 12% SDS-PAGE

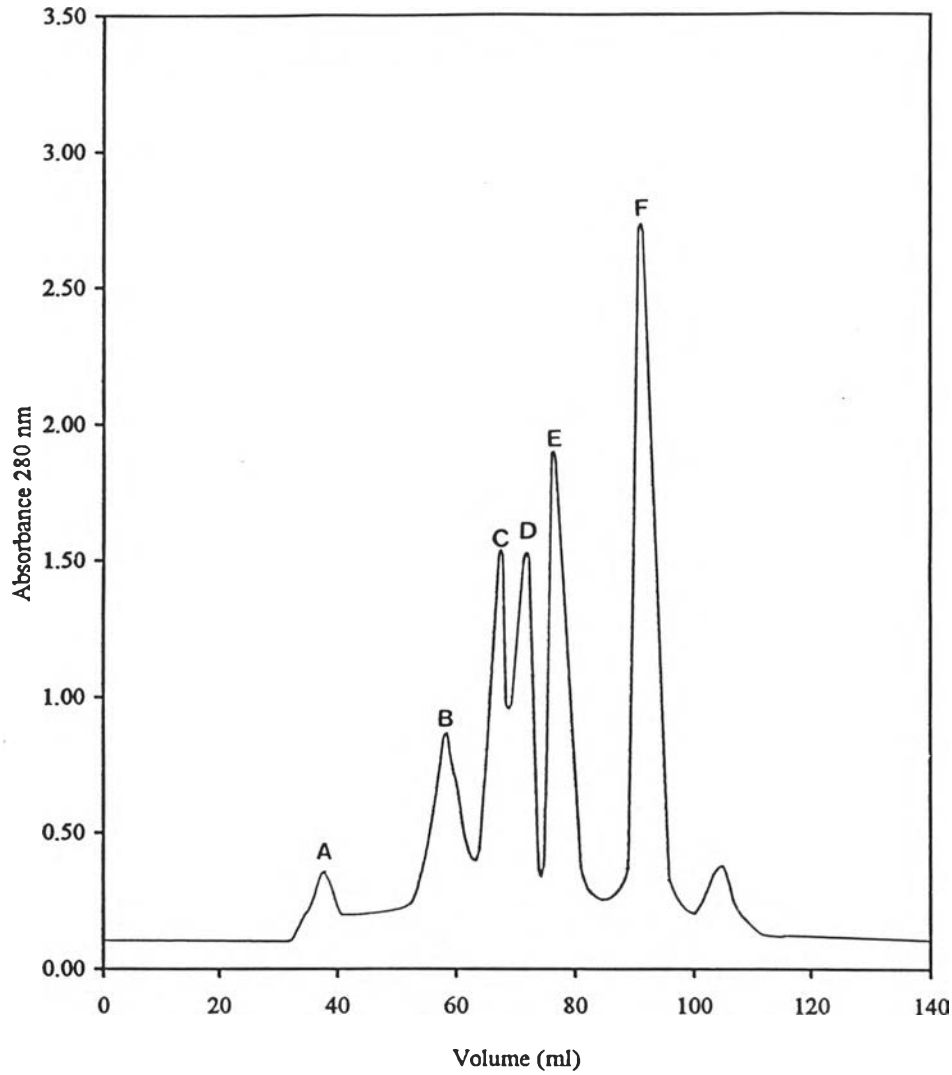


Figure 16 Elution profile of Bio-Rad molecular weight standards on Superose 6 HR16/50 at a flow rate 0.5 ml/min in 0.1 M tricine-NaOH buffer pH7.5. A = void peak; B = thyroglobulin, 670 kD; C = gamma globulin, 158 kD; D = ovalbumin, 44 kD; E = myoglobulin, 17 kD; F = vitamin B 12, 1.35 kD

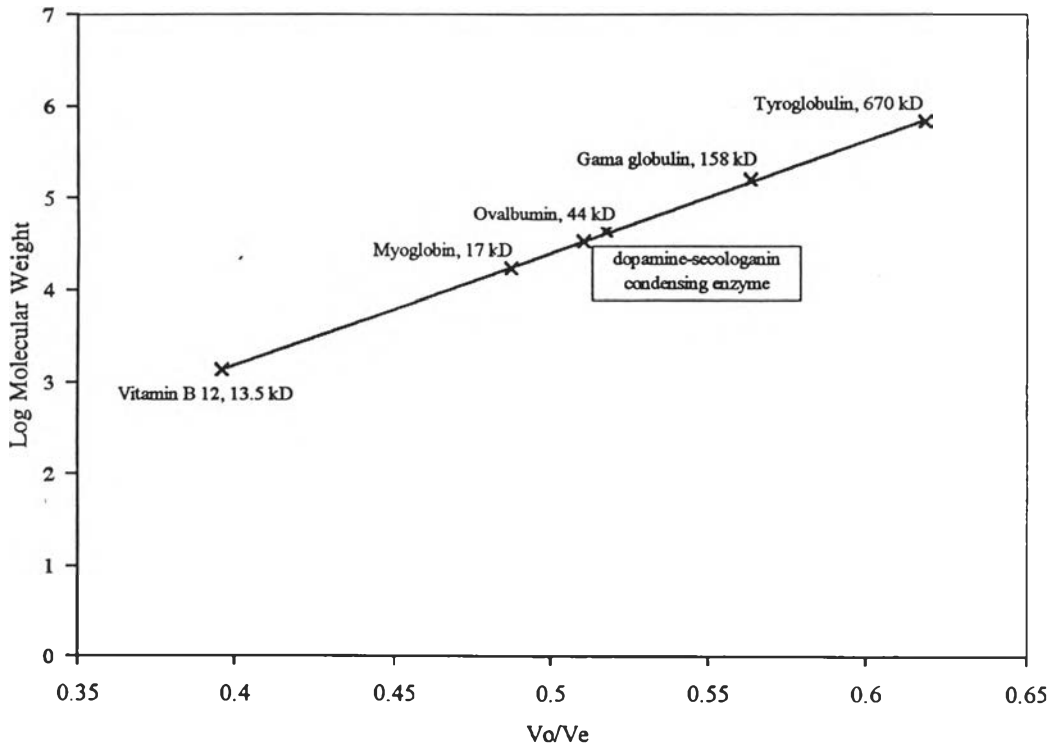


Figure 17 Standard calibration curve of Log molecular weight plotted against Vo/Ve of Superose 6 HR16/50 size exclusion column

6. Enzyme Stability

The stability of the enzyme activity in the crude enzyme extracts in extraction buffer pH 7.5 kept at 4°C and -20°C was determined during period of two weeks. It was found that enzyme activity stored at 4°C tended to decrease rapidly after 3 days of storage. The activity reduced to half of the maximal activity in 5 days and lost activity completely in 7 days. The enzyme preparations stored at -20°C for 3 and 6 days were also found to the reduce in enzyme activity to 50% and 5% of maximal activity respectively (Figure 18). It was cleared that the enzyme stored at -20°C did not maintain the activity of the condensing enzyme. Therefore, the purification process and the enzyme characterization must be finished in short period of time less than 5 days.

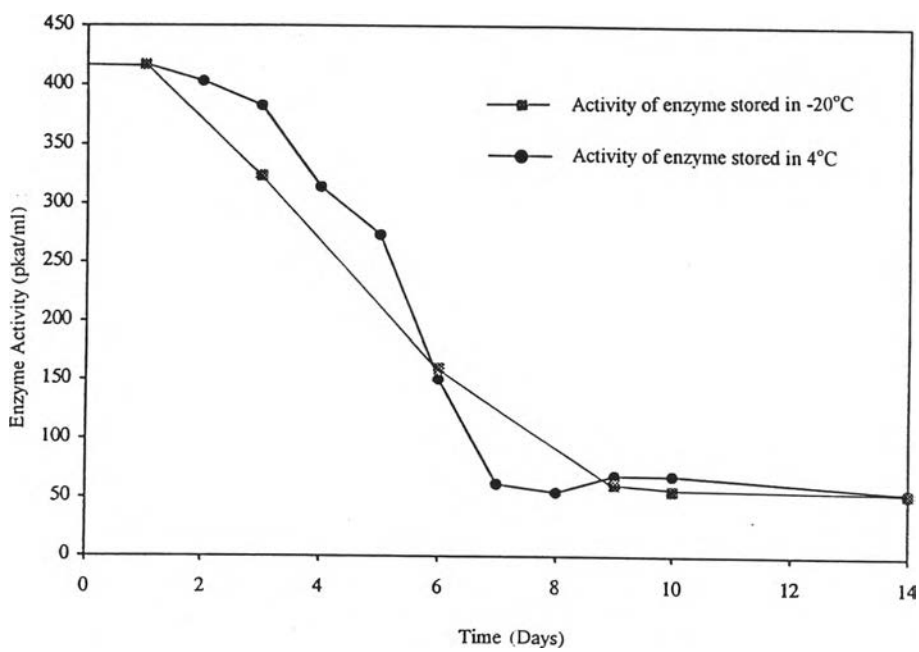


Figure 18 The stability of enzyme activity at 0°C and -20°C

7. Temperature Optimum for the Enzyme Activity

The optimum temperature of enzyme activity was determined by separated incubation of dopamine-secologanin condensing enzyme at various temperatures : 27, 37, 50, 60, 70, and 100°C ,respectively. As shown in Figure 19, the enzyme activity could be observed at room temperature incubation (27°C) and reached to its maximum at temperature between 37°C and 50°C. At higher temperature, the enzyme activity decrease and completely lost at 60°C. At 70°C, the enzyme solution was turbid and the proteins began to aggregrate. The clotted proteins dispersed in the solution when the temperature reached to 100°C. From this experiment, the optimum temperature for the enzyme catalytic activity could be indicated at 37°C.

8. pH Dependency of the Enzyme Activity

The relationship between pH and enzyme activity was determined by assaying the activity of dopamine-secologanin condensing enzyme in various pH range. The buffers and their respective pH were 0.1 M glycine (pH 2), 0.1 M citrate-phosphate (pH 4.0, pH 4.5), 0.1 M sodium-phosphate (pH 6.0), 0.1 M tricine-NaOH (pH 7.0, pH 7.5, pH 8.0 and pH 8.5), 0.1 M glycine-NaOH (pH 9.0, pH 10.0). As shown in Figure 20, the highest enzyme activity was found at pH 7.5. The 50% of maximal activity was at pH 5.5 and pH 8.5 (Figure 20). The enzyme activity was lost at pH 9.0-10.0 and pH 2.0-4.0. It was likely that strong acid and strong basic conditions may caused denaturing of the enzyme. The pH optimum of enzyme was therefore concluded to be 7.5.

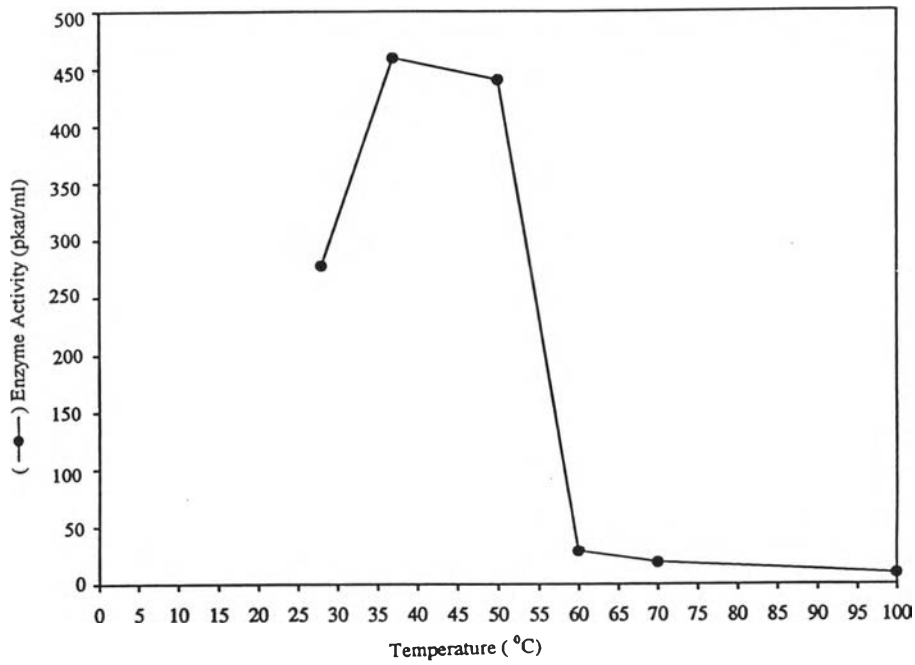


Figure 19 The optimum temperature for the enzyme activity

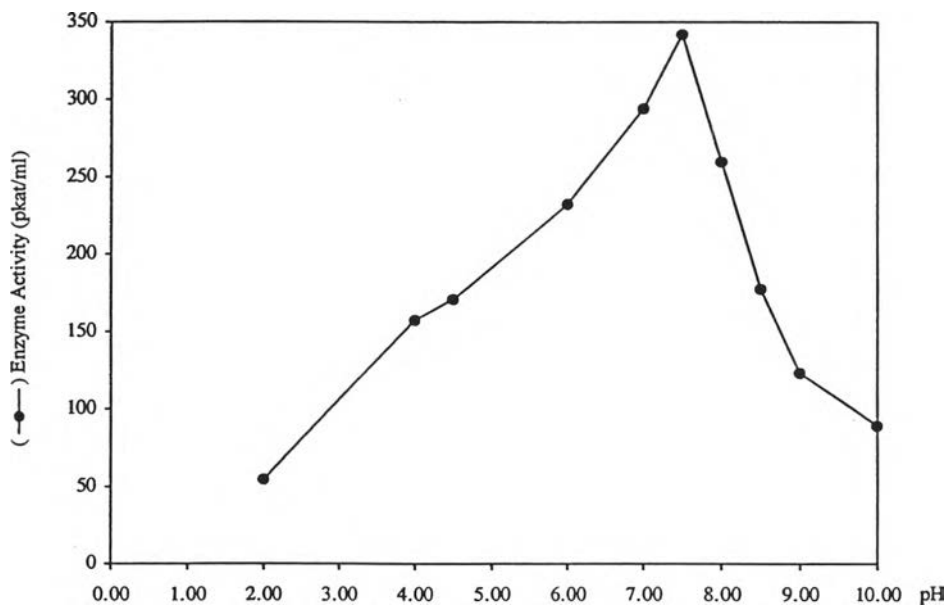


Figure 20 The pH dependency of enzyme activity. The concentration of buffer was 0.1 M

9. Influence of Metal Ions on Enzyme Activity

The influence of metal ions on the enzyme activity was determined in the presence and absence of 1 mM of $MnSO_4$, $MnCl_2$, $ZnSO_4$, $FeSO_4$, $FeCl_3$ and $CoCl_2$. As shown in Table 4, no metal ions tested were found to have significant effect on the enzyme activity.

Table 4 The influence of metal ions on the enzyme activity

Metal ions	Enzyme activity (pkat/ml)[a]	
	Presence of metal ion	Absence of metal ion
1 mM $MnSO_4$	200.93	227.46
1 mM $MnCl_2$	211.45	227.46
1 mM $ZnSO_4$	231.42	227.46
1 mM $FeSO_4$	212.44	227.46
1 mM $FeCl_3$	225.91	227.46
1 mM $CoCl_2$	234.36	227.46

a = The enzyme was purified to 27-fold.

10. Substrate Specificity

Substrate specificity of the dopamine-secologanin condensing enzyme was tested by assay the enzyme activity in the presence of various compounds including dopamine, tyramine or indole derivative tryptamine. As shown in Table 5, the enzyme exhibited its activity only with dopamine. The enzyme activity was not detected with tyramine, whereas the enzyme activity for condensation of secologanin and 5 mM of tryptamine was 50% of relative activity compared with that of dopamine and secologanin condensation. In the lower concentrations 1 mM and 0.1 mM tryptamine, no enzyme activity was observed. Furthermore, addition each of these compounds in the assay reaction mixture appeared to have no interference on the enzyme activity utilizing dopamine although 5 mM of tryptamine could reduced enzyme activity.

Table 5 Effect of some substrate analogs on the enzyme activity

Compounds	Relative activity (%)		
	0.1 mM	1.0 mM	5 mM
Dopamine	15	61	100
Tyramine	0	0	4
Tryptamine	0	0	57

nd = not determined

11. Inhibition of Enzyme Activity by Some of Emetine Alkaloids

The possibility of enzyme inhibition by the alkaloidal products was studied by incubating partial purification enzyme (108-fold purification) in the presence of various concentrations (0.01-0.1 mM) of alangimarckine, dehydroalangimarckine, cephaeline, emetine and tubulosine. The results were summarized in Table 6. The concentration of 0.1 and 1.0 mM alangimarckine and dehydroalangimarckine appeared to effective inhibit the enzyme activity (ca ~ 70-100% inhibition). The 50% inhibition of both compounds were approximately 10 μM. Interestingly, these alkaloids could be the inhibitors of dopamine-secologanin condensing enzyme. The 1 mM of emetine, tubulosine, cephaeline reduced the enzyme activity to 45%, 50% and 52%, respectively, whereas 0.01 mM of tubulosine and cephaeline had a little inhibitory effect. There was no inhibitory activity observed from 0.1 mM of cephaeline.

Table 6 Inhibition of dopamine-secologanin condensing enzyme by some emetine related alkaloids

Compounds	Inhibition (%)		
	0.01 mM	0.1 mM	1.0 mM
Alangimarckine	40	73	100
Dehydroalangimarckine	51	84	100
Cephaeline	0	0	50
Emetine	0	24	45
Tubulosine	0	11	52

12. Kinetic Studies of Dopamine-Secologanin Condensing Enzyme

12.1 Time Course of Reaction Product Formation

A 200 µl reaction mixture containing 100 mM β-glucuronolactone, 5 mM dopamine, 5 mM secologanin, 140,000 dpm of [2,5,6-^{3}H]dopamine in 0.1 M tricine-NaOH buffer, pH 7.5 was assayed at 37°C. The reaction mixtures were performed at various times from 0 min to 90min. The results in Figure 21 showed that the reaction occurred linearly for 30 min, showing the constant rate of product formation. After 30 min, the rate of product formation was decrease gradually until constant. A time of 30 minutes was therefore selected for subsequence kinetic studies.

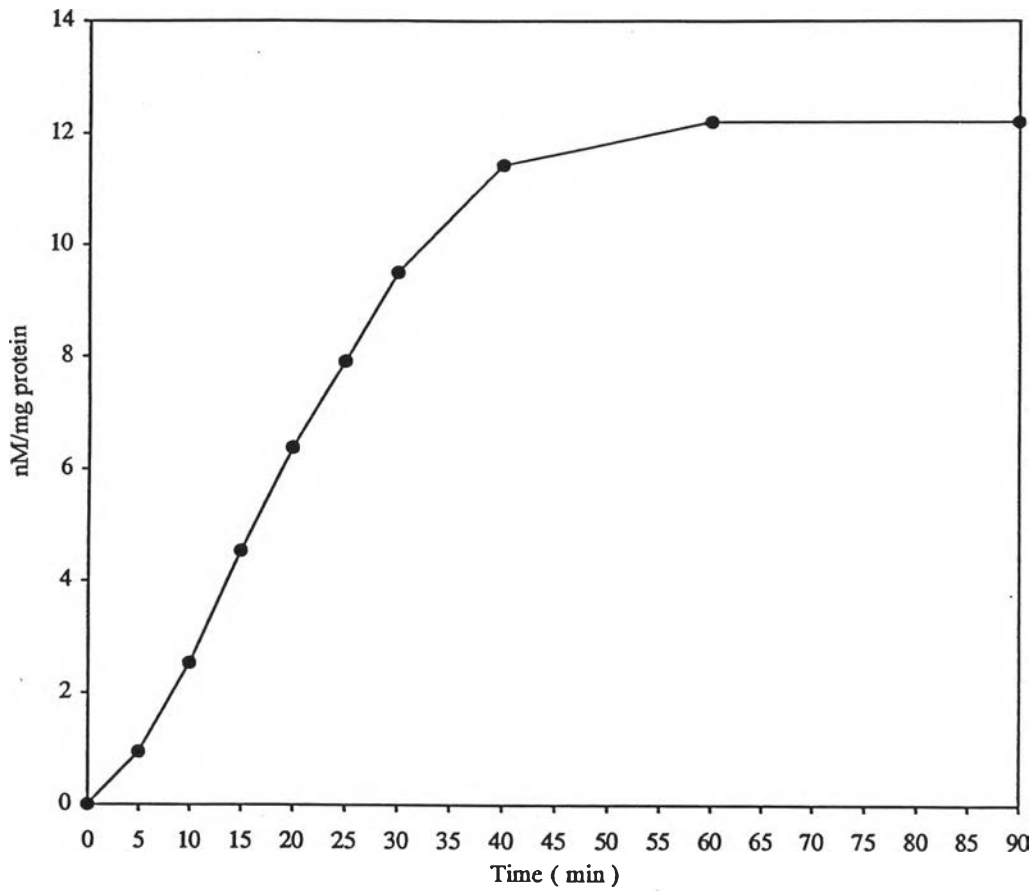


Figure 21 The relationship between time and product formation of the reaction catalyzed by dopamine-secologanin condensing enzyme.

12.2 Determination of K_m and V_{max} Values for Dopamine Substrate

The determination of K_m and V_{max} value for the substrate dopamine were carried out according to the method of Michealis-Menten (as described in Chapter III). The primary plot curve (Figure 22) between substrate concentration and initial velocity was converted to Lineweaver-Burke plot (Figure 23) to obtain K_m and V_{max} values. To obtain these results, the enzyme in the presence of bovine serum albumin ca~20 μg/ml was incubated at 37°C with 6 different concentrations of dopamine vary from 0.25 mM to 7.5 mM. The results showed that the K_m value for the dopamine was 0.69 mM and the V_{max} value for dopamine was 7.09 pkat/mg protein (Table 7). The Michealis-Menten curve was established by using various concentrations of dopamine in the presence of excess secologanin with constant concentration. No substrate inhibition was observed.

12.3 Determination of K_m and V_{max} Values for Secologanin Substrate

The K_m value for secologanin was determined as described in Chapter III. The primary plot between concentrations of secologanin and initial velocity (Figure 24) was converted to Lineweaver-Burk plot (Figure 25) to obtain K_m and V_{max} values. In doing this experiment, the enzyme in the presence of bovine serum albumin ca~20 μg/ml was incubated at 37°C with 6 different concentrations of secologanin between 0.1 mM and 3.0 mM. The result showed that the K_m value for secologanin was 0.92 mM and the V_{max} value for secologanin was 8.33 pkat/mg protein (Table 7). The Michealis-Menten plot was established by using various concentrations of secologanin in the presence of constant concentration of dopamine (excess amount). No substrate inhibition was detected.

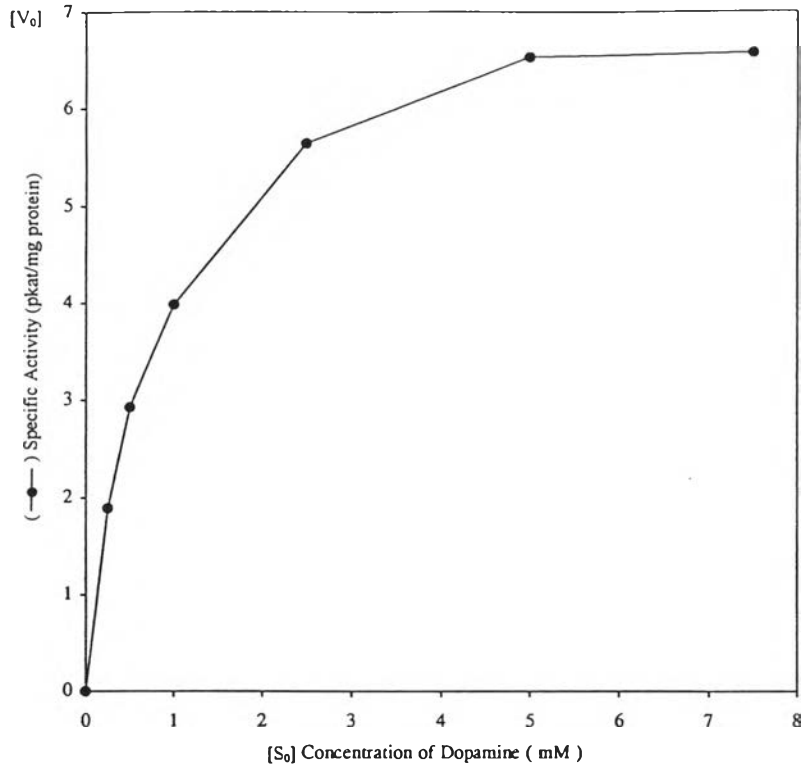


Figure 22 The effect of dopamine concentrations on the enzyme activity (Michealis-Menten plot)

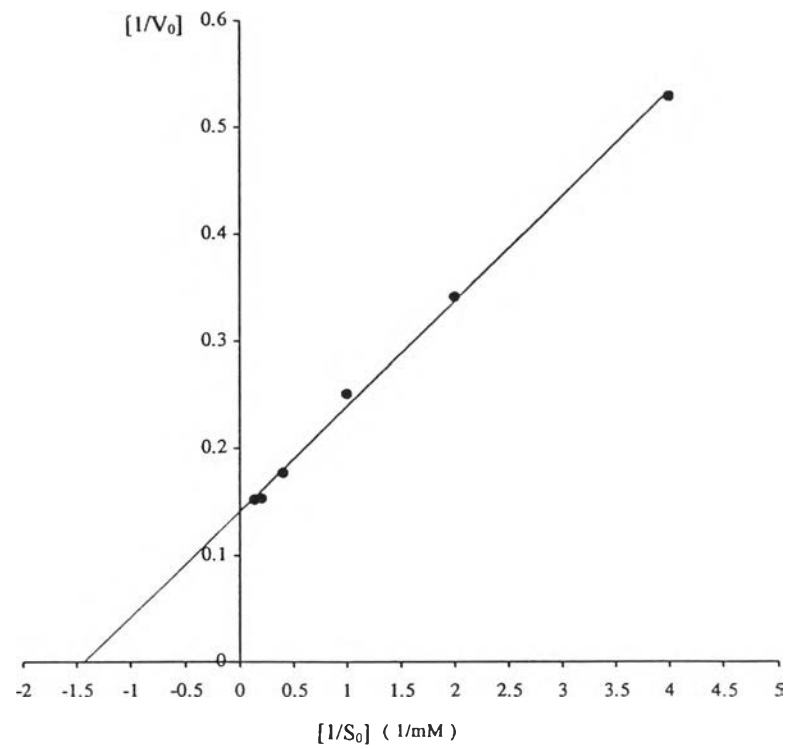
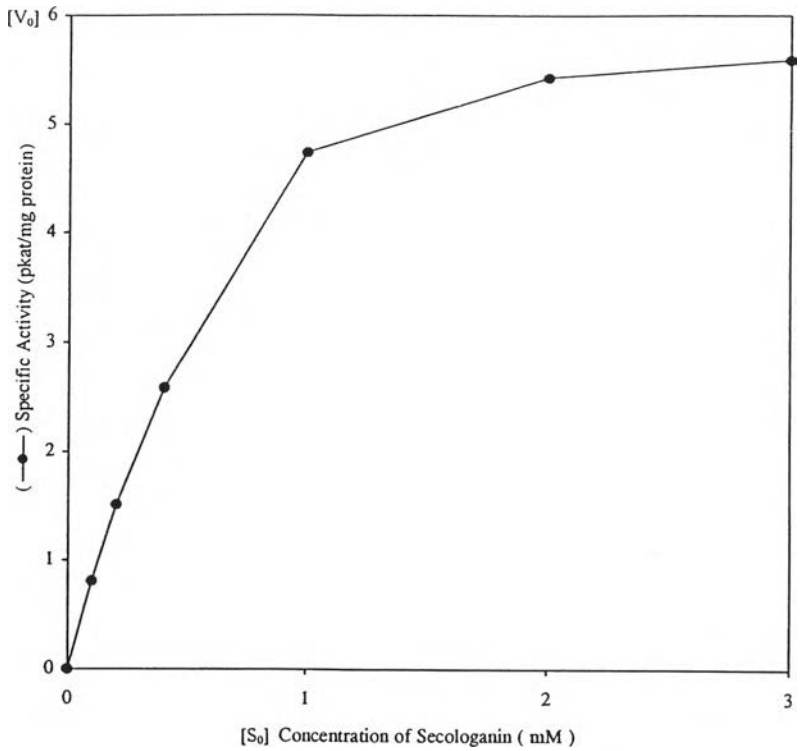


Figure 23 The double-reciprocal plot (Lineweaver-Burk plot) of data from Figure 22 which yield the K_m and V_{max} values of 0.69 mM and 7.09 pkat/mg protein, respectively (for dopamine substrate)



igure 24 The effect of secologanin concentrations on the enzyme activity (Michealis-Menten plot)

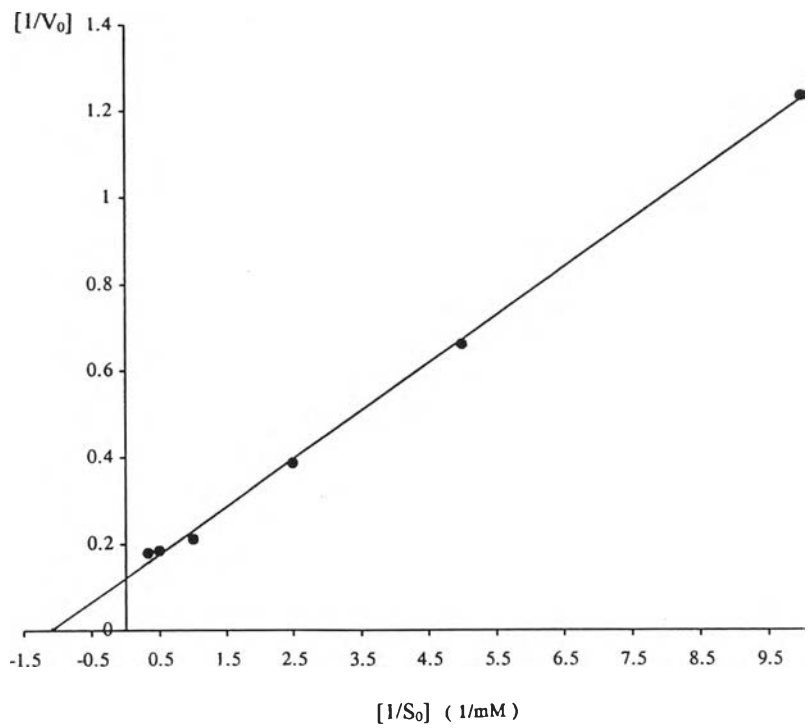


Figure 25 The double-reciprocal plot (Lineweaver-Burk plot) of data from Figure 24 which yield the K_m and V_{max} values of 0.92 mM and 8.33 pkat/mg protein, respectively (for secologanin substrate)

Table 7 Kinetic parameters of the dopamine-secologanin condensing enzyme from *A. salviifoium*

Substrate	K_m (mM)	V_{max} (pkat/mg protein)	Substrate inhibition
Dopamine	0.69	7.09	no
Secologanin	0.92	8.33	no

13. Analysis of [*R*]-Deacetylipecoside Product

In order to confirm the enzymatic reaction product obtained from the condensing enzyme was (R)-deacetylipecoside, the partial purification enzyme (108-fold purification) was incubated with [2,5,6-^{3}H] dopamine and secologanin. The resulting incubation mixture was worked up by ethylacetate and injected to HPLC equipped with a photodiode array detector. As shown in the HPLC chromatogram (Figure 26A), the chemical reaction mixture showed the presence of three major peaks at R_f = 7.40 min, 9.53 min and 11.05 min which corresponded to dopamine, secologanin, demethylalangiside, respectively and one minor peak at R_f =17.83 min of demethylisoalangiside.

The enzymatic condensation product was identified by HPLC with its retention time of 10.80 min. This was also confirmed by the presence of radioactivity of the peaks which showed that the elution peaks at R_f = 7.40 min (dopamine) and 10.80 min contained the radioactivity. These results suggested that the peak at R_f = 7.40 min should be the radioactivity of the excess or unutilized of dopamine substrate and the peak at R_f = 10.8 min was the enzymatic condensation of dopamine and secologanin which is (*R*)-demethylalangiside).

Furthermore, each peak in the chromatograms was also analyzed by its UV-absorption spectrum (using photodiode array detector). Figure 27 showed the UV-absorption spectra of demethylalangiside (R_f = 11.05) and demethylisoalangiside

(R_f = 17.83) obtained from the chromatogram of Figure 26. It can be seen that both peaks have the combination of λ_{max} of both dopamine (240 nm) and secologanin (290 nm). The enzymatic product obtained from the assay reaction showed very clearly the presence of demethylalangiside (R_f = 10.80) with identical UV-absortion spectrum (Figure 28C) to the chemical reaction product (Figure 27A). These results showed that there was an enzyme-catalysed condensation of dopamine and secologanin occuring in the reaction mixture. The intermediate condensation product were likely to be (*R*)-deacetylipecoside which were converted spontanously to (*R*)-demethyl-alangiside during extraction and purification processes.

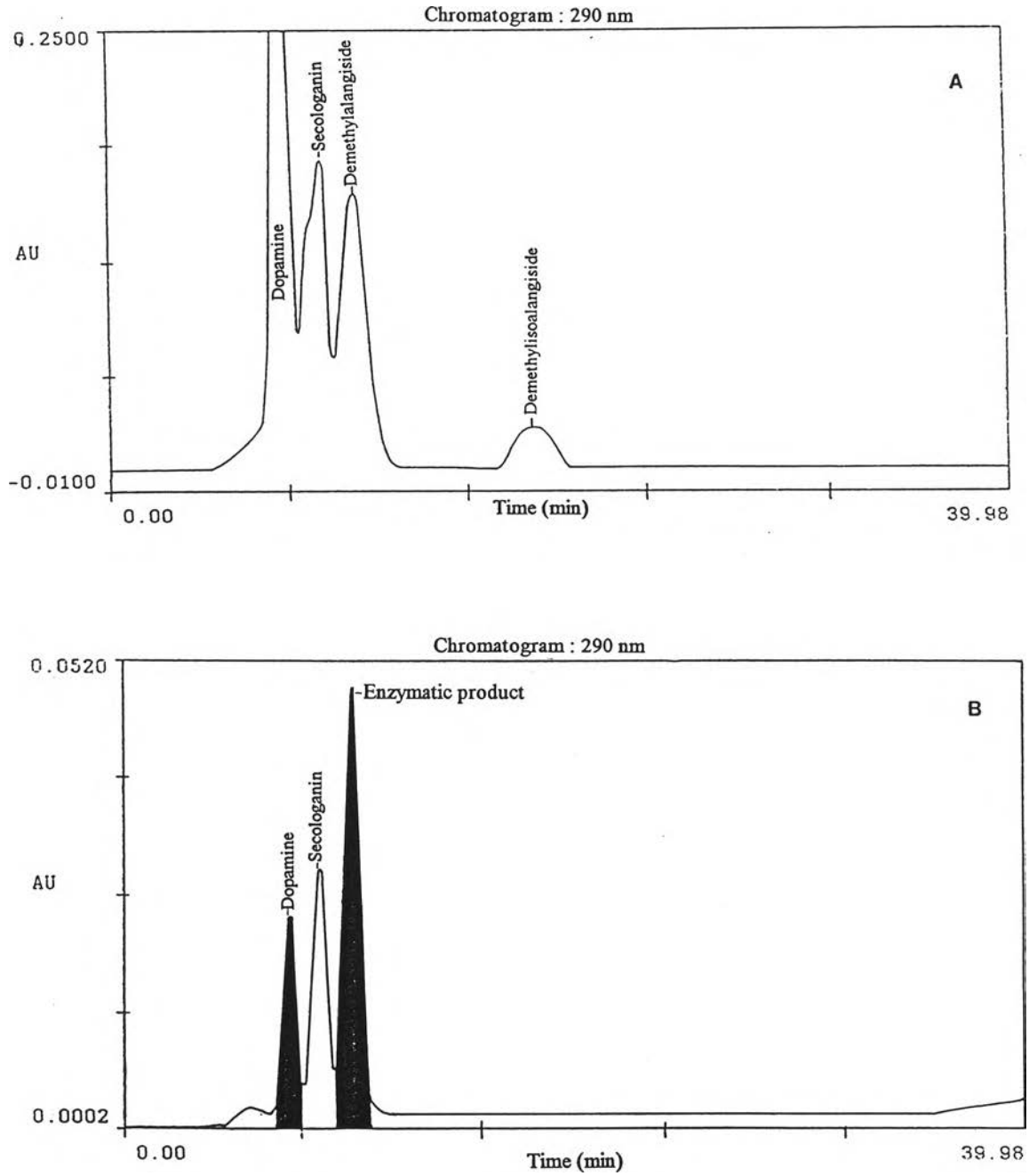
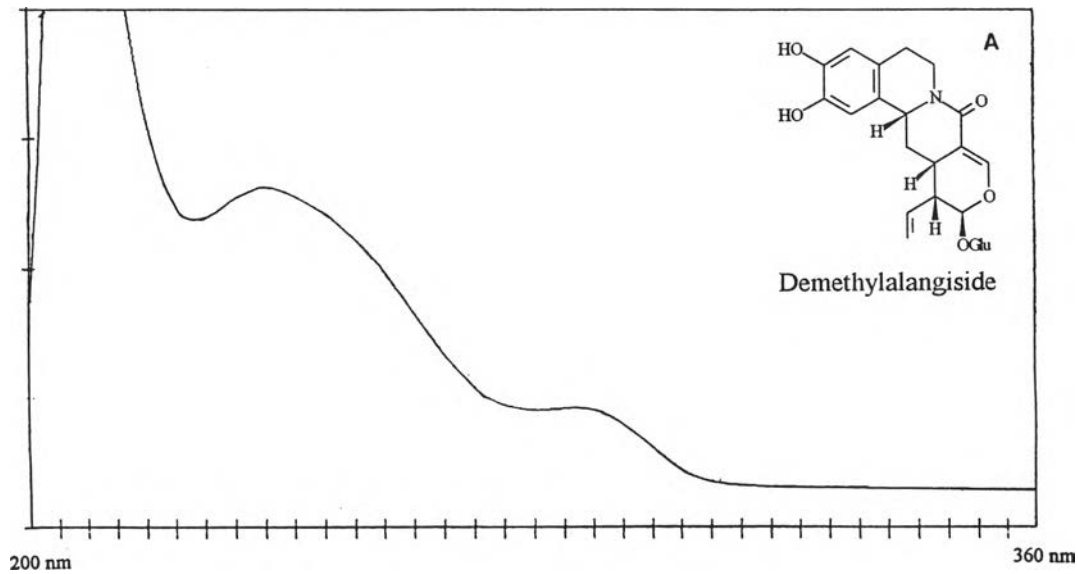


Figure 26 HPLC chromatograms of ethylacetate phase obtained from the extraction of (A) chemical reaction mixture of dopamine and secologanin (B) enzymatic reaction mixtures. The shade peaks show the presence of radioactivity.
Separation condition : Nova pak C_{18} (5μm) column (300 x3.9 mm) / H_2O-MeOH (1:1), flow rate 0.5 ml/min



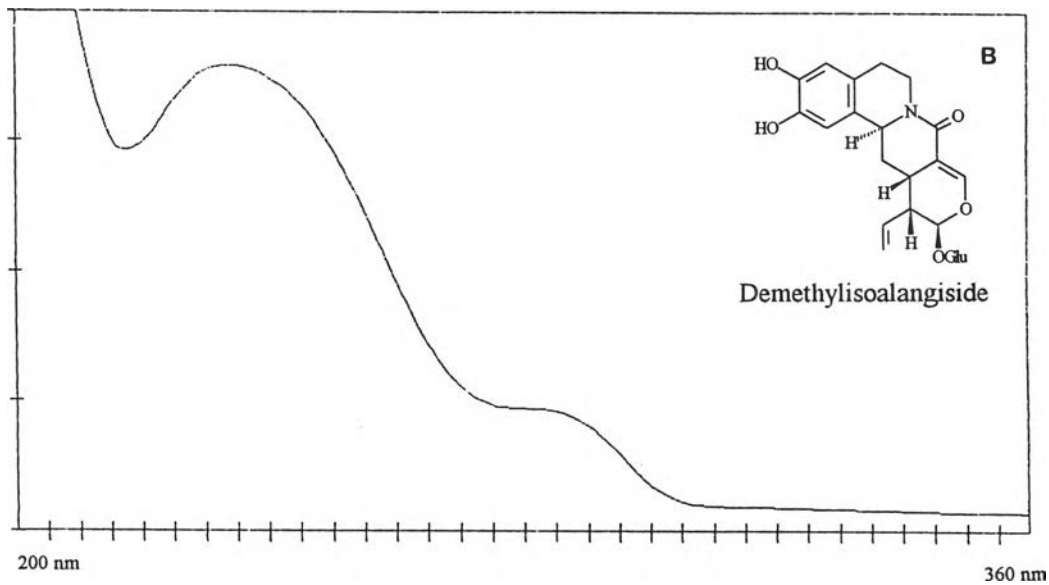


Figure 27 UV-absorbtion spectra of (A) demethylalangiside standard
(B) demethylisoalangiside standard

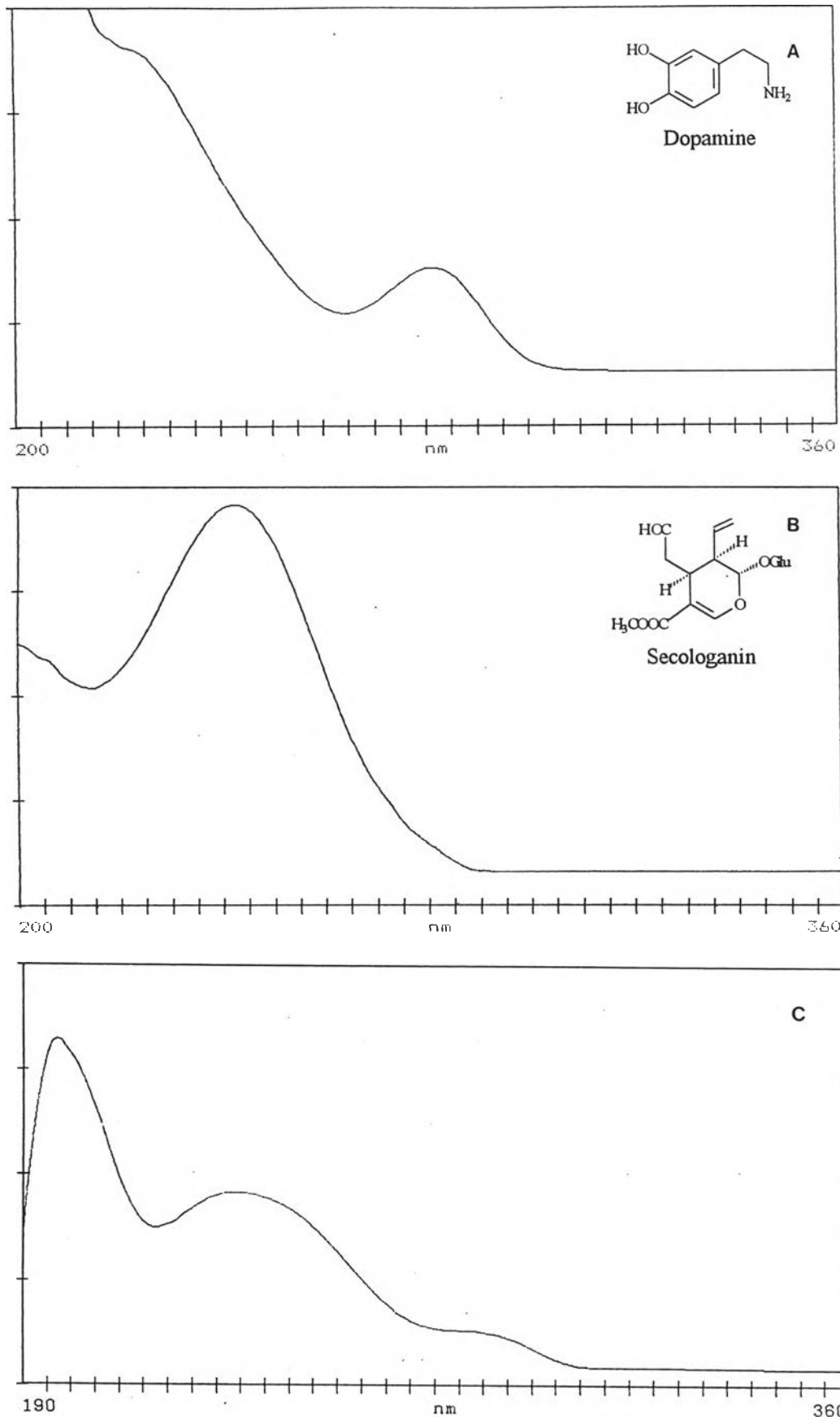


Figure 28 UV-absorbtion spectra of (A) dopamine (B) secologanin (C) enzymatic product