CHAPTER III

EXPERIMENTAL

1. Chemicals

Dopamine, β-glucuronolactone, tricine, glycine, ethylenediamine tetraacetic acid (EDTA), 2-mercaptoethanol, trisma base, polyvinylpolypyroridone (PVPP), bovine serum albumin and dextran blue were obtained from Sigma Chemical Company, St. Louis, MO, USA. Radioactive [2,5,6-^{3}H] dopamine was obtained from Amersham Life Science, Buckinghamshire HP79NA, England. Potassium chloride and ammonium sulfate were purchased from Fermitalia, Milan, Italy. Organic solvents used for thin layer chromatography (TLC) were all analytical grade. Organic solvents used for HPLC were HPLC grade. Water was either double distilled or Milli-Q water grade. TLC plates of silica gel 60 F254 on aluminium sheet were purchased from E. Merck, Damstadt, Germany.

Secologanin was a gift from Prof. Meinhart H. Zenk of the Institute of Pharmaceutical Biology, University of Munich, Munich, Germany.

Authentic (*R*)-demethylalangiside and (*S*)-demethylisoalangiside were prepared from dopamine and secologanin as discribed previously (Nakagura *et al.*, 1978)

Chromatographic materials : Sephadex G25, Phenyl Sepharose CL-4B, DEAE-Sephacel, Superose 6 were purchased from Pharmacia Biotechnology, Uppsala, Sweden. Ultrafiltration materials, Centriprep 10 and Centricon were purchased from Amicon, Danver, USA.

Molecular weight standard proteins for gel filtration (thyroglobulin, 670 kD; gama globulin, 158 kD; ovalbumin, 44 kD; myoglobulin, 14 kD; vitamin B1, 1.35 kD, and for SDS-PAGE (phosphorylase b, 97.4 kD; bovine serum albumin, 66.2 kD; ovalbumin, 44 kD; carbonic anhydrase, 31 kD; soybean trypsin inhibitor; 21.5 kD, lysosyme, 14.4 kD were purchased from Bio-Rad Laboratories, Richmond, CA., USA. Chemical for gel electrophoresis, including acrylamide, bis-acrylamide, amonium persulfate, N, N, N′, N′ tetramethylethylenedianmine (TEMED), coomassie brilliant G_{250} (for protein determination), coomassie brillant R250 (for coomassie blue staining), silver staining kit and bromophenol blue were also purchased from Bio-Rad Laboratories, Richmond, CA., USA.

2. Plant Material

Fresh leaves of *Alangium salviifolium* Wang were collected from an 8-year old plant grown in the field of the Faculty of Pharmaceutical Sciences, Chulalongkorn University, Bangkok, Thailand.

3. Buffers

Extraction buffer was 0.1 M tricine-NaOH pH 7.5, containing 3 mM EDTA and 10 mM 2-mercaptoethanol.

Buffer A was 0.4 M $(NH_4)_2SO_4$ in 0.1 M tricine-NaOH pH 7.5, containing 3 mM EDTA and 10 mM 2-mercaptoethanol.

Buffer B was 1 M KCl in 0.1 M tricine-NaOH pH 7.5, containing 3 mM EDTA and 10 mM 2-mercaptoethanol.

4. Synthesis of Deacetylipecoside and Deacetylisoipecoside Standards

This procedure was modified from the method used previously (Nakagura, *et al.*, 1978). A solution of 1 mg dopamine and 1 mg secologanin in 500 µl 0.2 M

citrate-phosphate buffer pH 5.0 was mixed and allowed to react for 72 hr. in darkness at room temperature under a N_2 atmosphere. The reaction mixture containing both (*R*)-deacetylipecoside and (*S*)-deacetylisoipecoside was then extracted by 200 µl ethylacetate for 4 times and dried under nitrogen gas.

5. Preparation of Cell-Free Extracts of *A. salviifolium* Leaves

Fresh leave material (250 g) of *A. salviifolium* was fast frozen by liquid nitrogen and powdered by grinding in a pre-cooled motar. After addition of 75 g/kg of polyvinylpyrolidone (PVPP), the frozen powder was throwed and stirred for 20 min in 500 ml of 0.1 M tricine -NaOH buffer pH 7.5, containing 3 mM EDTA and 10 mM 2-mercaptoethanol (extraction buffer). The mixture was passed through four layers cheese-cloth. Brei cell passing through was separated by centrifuge at 8x1000 *g* for 20 min in High-Speed Refrigerated Centrifuge (Hitachi Model CR20B3, Japan). The supernatant was used as a crude enzyme extract.

6. Preliminary Study on the Activity of Dopamine-Secologanin Condensing Enzyme in Cell-Free Extracts

Dopamine-secologanin condensing enzyme from the cell-free extract of *A. salviifolium* leaves was partially purified by precipitation with ammonium sulfate (40-60% saturation) as described in section 9.1. The pellet was dissolved with extraction buffer and desalted by using sephadex G-25 (PD 10 column, Pharmacia, Uppsala, Sweden). This enzyme preparation (ca~ 1 mg of protein/ml) was incubated for 60 min at 37°C with 5 mM dopamine, 5 mM secologanin, 50 mM β-glucuronolactone under the conditions of 0.1 M tricine-NaOH pH 7.5 in a total volume of 5 ml. The reaction mixture was extracted by 2 ml ethylacetate for 3 times. The organic solvent was then removed by vacuum drier (Speed Vac100, Savant, USA). The formation of the condensing product was followed by TLC-densitometry. By this technique, the evaporated fraction was dissolved with 20 µl ethylacetate and applied on a silica gel 60 F254 plate. Using the solvent system of $CHCl_3$-nPrOH-

MeOH-H_2O, 45 : 15 : 60 : 40 ($CHCl_3$ phase), the mixture of the two epimers (*R*)-deacetylipecoside and (*S*)-deacetylisoipecoside were separated with the $R_f \sim 0.6$. The TLC plate was then scanned to obtain chromatogram by a TLC-densitomter (Shimazu Dual-Wavelength Thin Layer Chromo Scan Model CS 930, Shimazu Cooperation, Kyoto, Japan) using a wavelength of 290 nm. At the TLC spot position of the putative reaction product, it was scanned from 200-370 nm in order to obtain a UV absorption spectrum compared with chemical synthetic standard.

7. Enzyme Assay

The activity of dopamine-secologanin condensing enzyme was measured in the protein fractions obtained from various column chromatographic separations. A specific activity of the enzyme was expressed in the unit of pkat/mg of protein. The enzyme assay was performed in a total volume of 200 µl which contained 5 mM dopamine, 5 mM secologanin, 50 mM β-glucuronolactone and 0.45 µCi [2,5,6-^{3}H] dopamine in 0.1 M tricine-NaOH buffer pH 7.5. The reaction mixture was incubated at 37°C for 30 min in Multi-Block Heater, Lab Line. Boiled control and reagent blank were also run concurrently. The reaction mixture was terminated by the addition of 500 µl ethylacetate and 20 µl of 3.5 M NH_4OH. Tubes were vortexed for 30 sec to extract reaction product into ethylacetate and phase separation was achieved by centrifugation in Microcentrifugation (Fisher Model 235C). The radioactive of 200 µl of organic phase was pipetted into Eppendorf tube which contained 1 ml of scintillation fluid (Hisafes, Wallac Oy, Finland). The radioactive mixture was mixed well and measured by Wallac 1409 Liquid Scintillation Counter (Wallac Oy, Finland). The activity of dopamine-secologanin condensing enzyme was determined by the following formula.

$$\text{Enzyme activity (pkat/ml)} = \frac{50 \times \text{Total count (dpm/assay)}}{\text{Sp.act of [2,5,6-}^3\text{H] dopamine (dpm/mol)} \times \text{incubation time (sec)} \times \text{volume (ml)}}$$

And the specific activity was determined by formula expressed below

$$\text{Specific activity (pkat/mg protein)} = \frac{\text{Enzyme Activity (pkat/ml)}}{\text{total protein (mg)}}$$

8. Protein Determination

During chromatographic separations, the protein profiles were monitored by UV detector which was set up at 280 nm. For each step of protein purifications, the total proteins of those active enzyme fractions was determined by the method of Bradford (1976) using bovine serum albumin (BSA) as protein standard. The linear range of microtiter plate assay was achieved by vary concentration of BSA from 5.0 μg/ml to 30.0 μg/ml. For each protein determination : a sample solution (160 μl) was pipetted into each well of 96 well plate, followed by 40 μl of the concentrated dye solution (Bio-Rad Protein Assay, Bio-Rad). The solution was mixed throughly by microplate shaker and incubated at room temperature for 5 min. The absorbance was then measured at 595 nm by using Microtiter Plate Reader Model 450 (Bio-Rad).

9. Purification of Dopamine-Secologanin Condensing Enzyme from *A. salviifolium* Wang Leaves

9.1 Amonium Sulfate Fractionation

The solution of the crude enzyme extract (prepared as described in section 5) was fractionated with 40-60% saturation of $(NH_4)_2SO_4$. Finely ground powder of $(NH_4)_2SO_4$ was added slowly into the stirred crude enzyme solution to obtain 40% saturation for 30 min, at 4°C. The precipitate was separated by centrifugation at 10 x1000 *g* for 20 min. The precipitant was discarded, whereas the supernatant was added with $(NH_4)_2SO_4$ to obtain 60% saturation and stirred for 30 min. The precipitant was separated by centrifugation at 10x1000 *g* for 20 min, and dissolved with 0.4 M $(NH_4)_2SO_4$ in extraction buffer to obtain relatively clear solution. The concentrated enzyme solution was centrifuged at 10x1000 *g* for 20 min. The pellet was again resuspended with minimal volume of extraction buffer and used for further purification.

9.2 Purification of Dopamine-Secologanin Condensing Enzyme by the Column Chromatography

The 40-60% $(NH_4)_2SO_4$ fractionation was obtained from crude enzyme extraction was applied on to a hydrophobic column of Phenyl Sepharose CL-4B (2.6 i.d. x 26 cm) pre-equilibrated with extraction buffer containing 0.4 M $(NH_4)_2SO_4$ (Buffer A). The column was washed with buffer A at flow rate of 1.5 ml/min until getting the constant baseline. The column was then eluted the bound protein with isocratic elution of 300 ml of extraction buffer at flow rate 1 ml/min. The enzyme activity was detected after 90 ml of extraction buffer as broad peak in a total volume of 80 ml. The fractions containing enzyme activity were pooled and then applied on to the DEAE-Sephacel (2.6 i.d. x 15 cm) anionic exchanger which has been equilibrated before used with extraction buffer. After washing at flow rate of 1 ml/min with 400 ml of extraction buffer or until constant baseline, by mean of 0-40, 40-70 % of 2-steps linear gradient elution of 1 M KCl in extraction buffer (buffer B) at flow rate of 0.5 ml/min, the enzyme was was eluted out during the concentration of KCl increasing from 40% to 70% in buffer B. The fractions containing enzyme activity were pooled and concentrated to 1.5 ml by Centriprep 10 ultrafiltration. Then, the protein was injected into Superose 6 (1.6 i.d.x 50 cm) size exclusion which was previously equilibrated with extraction buffer. With the flow rate of 0.4 ml/min, the enzyme activity was obtained at Ve ~ 75 ml. The fraction with the highest activity was kept as the purified enzyme. All steps were performed at 4 ^{0}C to maintain the activity of enzyme.

10. Molecular Weight Determination

10.1 Determination of the Molecular Weight by SDS-PAGE

This was performed by Bio-Rad Mini Protein II Apparatus with SDS-polyacrylamide gel electrophoresis (PAGE) on Mini-slab gel (70 x 80 x 7.5 mm). The separating gel (50 x 80 x 0.75 mm) contained 12% polyacrylamide (prepared from a stock solution of 30% w/v acrylamide and 0.8% w/v N,N'-methylene-bis-acrylamide,

2.67%C), 0.375 M tris-HCl pH 8.8 and 0.1% w/v sodium dodecyl sulphate (SDS). The separating gel was polymerized chemically by addition of 0.05% w/v ammonium persulfate (APS) and 0.05% N,N,N′,N′-tetramethylethylenediamine (TEMED), polymerizing agent (Table 12, Appendix). The monomer solution was mixed well by swirling and poured into an assembled gel sandwich using glass pipet. The gel solution was intermediately overlayed with water and allowed to polymerized for 45 min to 1 hr. The overlay solution was then rinsed off completely with distilled water. For the stacking gel, the stacking gel monomer solution containing 4% polyacrylamide (Combine reagent was prepared from the same stock of separating gel as mention in Table 2) in 0.125 M tris-HCl pH 6.7, 1.0% of 10% w/v SDS. 0.5% of 10% w/v APS and 0.1% v/v TEMED were added to polymerized the gel. A comb was placed in the gel sandwich and the stacking monomer soltuion was poured down to the upper part of separating gel until all the teeth were covered by solution. The stacking gel was allowed to polymerize for 30-45 min. The comb was removed by pulling it straight up slowly and gently. The wells were rinsed completly with distilled water. The gel was then ready to be used.

For protein sample preparation, the protein solution was first diluted with sample buffer 1:4 in ratio, and heated for 5 min at 95°C. The protein samples were accurately pipetted into each well, and run approximately for 3 hr at 45 mA constant current for two gels. The voltage should started at about 70 V, but it increased during run. When the dye reached the bottom of the gel, the power supply was turned off and disconected. The gel was removed from the electrophoresis cell and put into the staining solution. The procedure of staining was described in Appendix.

Table 2 SDS polyacrylamide gel composition

Component	Separating gel 0.375 M Tris, pH 8.8 (12% AA)		Stacking gel 125 M Tris pH 6.8 (4% AA)	
Distilled water	3.35	ml	6.1	ml
1.5 M Tris-HCl, pH 8.8	2.5	ml	-	
0.5 M Tris-HCl, pH 6.8	-		2.5	ml
10% w/v SDS stock	100	µl	100	µl
Acrylamide / Bis (30% stock)[a]	40	µl	1.3	µl
10% Ammonium persulfate[b]	50	µl	50	µl
TEMED	5	µl	10	µl
Total monomer	10	ml	10	ml

a = Degas at least 18 min at room temperature before used

b = freshly prepared daily

The stained gel was then destained by silver staining or coomassei blue staining before being stored as wet gel at 4°C. The wet gel was simple wrapped with a sheet of plastic-wrap. This permitted handling the gel without the risk of breakage. The wraped gel was inserted into sealable bag to store up to one month.

For gel drying, it was performed according to the dryer instruction (LKB 2003 Slab Gel Dryer Unit, LKB, Uppsala, Sweden). The stained gel was placed between two sheets of soaked porous cellophane. The bubbles were complete smoothed out the gel support material was laid on the filter paper and closed with the cover sealing. Then, the vacuum pump was turned on for 90 min, the gel was completely dried. The dry gel should be stored in a book to prevent curling.

The gel was calibrated by using the low range of SDS-PAGE protein standard : phosphorylase b (97.4 kD), bovine serum albumin (66.2 kD), ovalbumin (45 kD), carbonic anhydrase (31 kD), soybean trypsin inhibitor (21.5 kD), lysozyme (14.4 kD). Standard curve was plotted on the log protein size (Y-axis) of standard protein versus relative mobility (R_f) (X-axis), described as followed

$$\text{Relative mobility } (R_f) = \frac{\text{Distance Migrated by Protein}}{\text{Distance Migrated by Marker}}$$

The calibration curve of log protein size versus relative mobility (R_f) generated the equation of line was $y = mx + b$ where m was the slope and b was the y intercept. In this case, the equation became

$$\text{Log Molecular Weight} = (\text{Slope})(\text{Mobility}) + \text{y-intercept}$$

10.2 Molecular Weight Determination Gel Filtration

To determine the native molecular weight of dopamine-secologanin condensing enzyme, the purified enzyme was prepared as described in section 7. The sample of 200 µl was injected to FPLC-Superose 6 HR 16/50 (1.6 id x 50 cm) pre-calibrating column which was pre-equilibrated with 0.1 M tricine-NaOH buffer pH 7.5. The column was eluted with the same buffer at the constant flow rate of 0.5 ml/min. The protein content was measured by measuring absorbance at 280 nm. The activity of dopamine-secologanin condensing enzyme was determined as described in section 7.

The FPLC Superose 6 HR 16/50 column was calibrated by using the following standard proteins for gel filtration standard protein (125 µl) : thyroglobulin (670 kD), gamma globulin (158 kD), ovalbumin (44 kD), myoglobulin (17 kD), vitamin B-12 (1.35 kD). The position of void volume (Vo) was determined by blue dextran. The relative molecular weight of enzyme was calculated from a calibration curve between log molecular weight and Vo/Ve (Ve = elution volume) of standard protein plotted. The equation of line also $y = mx + b$ where m was the slope and b was the y intercept. The equation performed

$$\text{Log Molecular weight} = (\text{Slope})(\text{Vo/Ve}) + \text{y-intercept}$$

11. The Properties of Dopamine-Secologanin Condensing Enzyme

11.1 Enzyme Stability

The stability of dopamine-secologanin condensing enzyme was studied by using enzyme preparation obtained from partial purification by gel filtration on Sephadex G25 (PD 10 column). This enzyme preperation (1 mg protein/ml) was stored in various conditions (4°C and shock frozen by liquid nitrogen before stored in -20°C). The enzyme activity of the resulting preparation kept in 4^0C was determine at 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 14 days and the stability of frozen enzyme was investigated for 1, 3, 6, 9, 14 days. The frozen enzyme was throwed before using and the condensation activity also detected in the reaction mixture containing 5 mM of dopamine, 5 mM of secologanin and 50 mM of β-glucuronolactone in 0.1 M tricine-NaOH pH 7.5 and the crude enzyme extract in the total volume 200 µl. After 60 min incubation, the reaction was terminated by adding 1 ml ethylacetate. Each tube was vortexed for 10 min to extract reaction products into organic phase and phase separation was achieved by centrifugation. The absorbance of organic phase was read against ethylacetate at 290 nm. The boiled control was the blank of reaction. The difference value was converted to the amount of reaction products by using a calibration curve of standard demethyl(iso)alangiside which was prepared from chemical reaction between dopamine and secologanin according to method described in section 4.

11.2 Temperature Optimum for the Enzyme Activity

The optimal temperature of enzyme activity was determined by separated incubation of dopamine-secologanin condensing enzyme with the substrates; dopamine, secologanin, [2,5,6-^{3}H] dopamine and β-glucuronolactone as described in enzyme assay section 7 at 27°C, 37°C, 50°C, 60°C, 70°C, 100°C, respectively. The enzyme was purified to the step of DEAE-Sephacel column chromatography. The reaction mixture was incubated for 60 min and stopped reaction by adding 3.5 M

NH_4OH. The process of product determination was the same as the enzyme assay described in section 7.

11.3 pH Dependency of Enzyme Activity

The influence of pH on the activity of dopamine-secologanin condensing enzyme was tested between the pH range from 2 to 10. The buffers used for these pH, were as followed : 0.1 M glycine-HCl (pH 2), 0.1 M citrate-phosphate, (pH 4.0), 0.1 M citrate-phosphate (pH 4.5), 0.1 M sodium-phosphate (pH 6.0), 0.1 M tricine-NaOH (pH 7.0), 0.1 M tricine-NaOH (pH 7.5), 0.1 M tricine-NaOH (pH 8.0), 0.1 M tricine-NaOH (pH 8.5), 0.1 M glycine-NaOH (pH 9.0), 0.1 M glycine-NaOH (pH 10). The solution contained 5 mM of dopamine, 5 mM of secologanin, 50 mM β-glucuronolactone, 140,000 dpm of [2,5,6-^{3}H] dopamine and ~ 40 μg/ml 108-fold purified enzyme preparation in DEAE-Sephacel column, was incubated in different pH in a total volume of 200 μl. After incubation, the reaction was stopped by addition of 10 μl 3.5 M NH_4OH and the reaction products were extracted with 500 μl of ethylacetate. Then the radioactivity of 200 μl of the organic phase was measured by scintillation counter (the routine enzyme activity was described as enzyme assay in section 7)

11.4 Influence of Metal ions on the Enzyme Activity

The effect of metal ions was tested by adding 1 mM of each metal ion solutions ($MnSO_4$, $MgCl_2$, $ZnSO_4$, $FeSO_4$, $CoCl_2$, $CuSO_4$, $FeCl_3$) into reaction mixture. The test was carried out in the standard reaction mixture of enzyme assay described in section 7. The enzyme was purified until the Phenyl Sepharose CL-4B column chromatography (27-fold purification). After 60 min incubation at 37°C, the reaction was stopped by adding 3.5 M NH_4OH and followed extraction with 500 μl ethylacetate. The procedure of product determination was the same as the enzyme assay described in section 7.

11.5 Substrate Specificity

The substrate specificity was determined by incubating of the condensing enzyme with 5 mM of dopamine or 0.1 mM, 1mM and 5 mM of tyramine and tryptamine, under the conditions of standard enzyme assay, the enzyme preparation was obtained from step of DEAE-Sephacel. The reaction mixture was incubated at 37°C for 60 min and terminated by adding 10 µl of 3.5 M NH_4OH. The reaction product was extracted by 500 µl ethylacetate. After separation , 200 µl of organic phase was measured for radioactivity by liquid scintillation counter. (The process of analysis was the same as for enzyme assay, described in section 7). The boil control of each tube and the tube contained 5 mM of dopamine was used as the blank of reaction.

11.6 Inhibition of Enzyme Activity by Some Emetine Alkaloids

The inhibitory effect of some alkaloids present in *A. salviifolium* was also determined using the same conditions as the standard enzyme assay using 108-fold purified enzyme. The concentrations of the inhibitors including were 0.01, 0.1 and 1 mM of alangimarckine, dehydroalangimarckine, cephaeline, emetine and tubulosine. In this experiment, the boiled control and reagent blank were used as the blank of experiment.

12. Enzyme Kinetics

12.1 Time Course of Reaction Product Formation

The time course of reaction product formation was detected in the standard enzyme assay using 108-fold puified enzyme. The time interval used for this study was0, 5, 10, 15, 20, 25, 30, 35, 40, 60, 90 min. The boiled control was run concurrently with the procedure for product determination. The different value was converted to enzyme activity. To determine the initial velocity and time course of

product formation of enzyme, the activity of each time point was plotted against the incubation time.

12.2 K_m and V_{max} Determination

In this study, the kinetics of dopamine-secologanin condensing enzyme was considered as pseudo-single substrate kinetic with respect to one substrate at each time, where there is only one-substrate binding site per enzyme and no interaction between site. This kinetic experiment can be used instead of a multi-substrates reaction of dopamine-secologanin condensing enzyme. The experiment performed by varied the concentration of one substrate and maintaining the other. The initial velocity (v_0) was ploted against various concentrations of substrate to obtain the rectangular hyperbolar curve of Michaelis-Menten equation. Then the graph was tranformed to linear by the Lineweaver-Burk Plot. By this method, the K_m and V_{max} values can be obtained from the intercept of x-axis and y-axis respectively.

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

The K_m and V_{max} determination for dopamine as performed by incubating 20 μg/ml of enzyme purified from DEAE-Sephacel with six different concentrations of dopamine (0.25-7.5 mM) under standard assay condition. The boiled control of each dopamine concentration was used as blank of system (the process of product determination was the same as enzyme assay). The different value was converted to enzyme activity. In the suitable reaction time (30 min), the rate of enzymatic reaction were calculated and plotted against various concentrations of dopamine substrate to obtain the rectangular hyperbolar graph of Michaelis-Menten equation. The K_m and V_{max} values of dopamine-secologanin condensing enzyme with respect to dopamine as substrate were determined using Lineweaver-Burk plot.

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

As described previously in the K_m and V_{max} determination for dopamine, the determination of K_m and V_{max} values for secologanin was determined by using six different concentrations of secologanin (0.1-3 mM). The process of product determination was the same as described in enzyme assay (section 7). The boiled contol of each concentration of secologanin was run concurrently. The different value was converted to enzyme activity. In the suitable reaction time (30 min), the rate of enzymatic reaction were calculated and plotted against various concentrations of secologanin substrate to obtain the rectangular hyperbolar graph of Michaelis-Menten equation. The K_m and V_{max} parameters can be determined from a secondary plot of Lineweaver-Burk.

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

The identification of the enzymatic product was performed by HPLC conected to a photodiode array detector. The reaction product was prepared from 5-times of amount of incubation mixture containing the same composition as in enzyme assay described in section 7. The reaction obtained for 60 min was extracted with 2 ml (500 µl for each extraction). The pooled ethylacetate fractions were evaporated in vacuum dryer. Then the dry extraction was resuspended with 20 µl of ethylacetate and 5 µl of product was injected to HPLC on a Nova pak C_{18} (5 µm) column (300 x 3.9 mm) using an isocratic solvent system of H_2O-MeOH (1:1) at 0.5 ml/min. The chromatogram of enzymatic product was also detected at wave length 290 nm and the peak spectrum was analysed by the photodiode array. The peak elution from HPLC was collected and dried to measure the the radioactivity of enzymatic product.