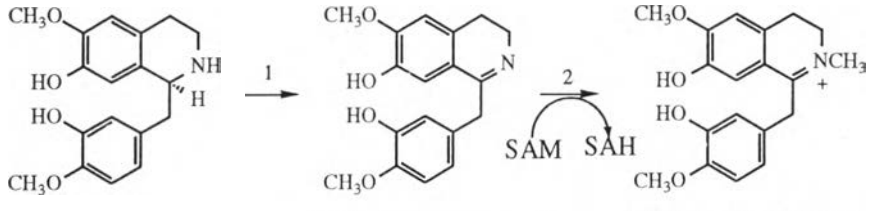
CHAPTER IV

RESULTS

1. Synthesis of [N-C^3H_3]-1,2-Dehydroreticuline

The enzyme 1,2-dehydroreticuline reductase has been found to exhibit high substrate specificity toward 1,2-dehydroreticuline since no reaction could be detected with closely related compounds like 1,2-dehydronorreticuline and 1,2-dehydrococlaurine (De-Eknamkul and Zenk, 1992). Therefore, only 1,2-dehydroreticuline must be used as substrate for the enzyme assay of the enzyme. Unfortunately, this compound is not commercially available and had to be synthesized in the laboratory. The synthesis of 1,2-dehydroreticulinium ion has been reported previously using a 7-stages chemical synthesis but had low yield and time consuming (Borkowski *et al.*, 1987). Attempts to synthesize this compound more effectively have been made (see Historical, section 8). In this study, we chose the method of preparing 1,2-dehydroreticuline from (S)-norreticuline by two consecutive enzymatic reactions, as shown below.



(S)-Norreticuline 1,2-Dehydronorreticuline 1,2-Dehydroreticuline

1= S-terahydroprotoberberine oxidase (STOX), 2= N-methyltransferase (NMT)

Since radioisotopic assay is the only method which is sensitive enough to detect the enzyme activity, the radioactively labelled substrate [N-C^3H_3]-1,2-dehydroreticuline was prepared using the two enzymatic steps as mentioned above. In the first step, (S)-norreticuline was dehydrogenated to 1,2-dehydronorreticuine by using *Berberis* (S)-tetrahydroprotoberberine oxidase (STOX). In the second step, 1,2-dehydronorreticuline was methylated with [C^3H_3]-SAM at N-position by using

Berberis N-methyltransferase (NMT). The detail of [N-C^3H_3]-1,2-dehydroreticuline synthesis is described below.

1.1 Preparation of STOX and NMT enzymes from *Berberis* cell cultures

STOX and NMT were prepared as described in Experimental, section 4.1. In the case of NMT preparation, it was subjected to one more step of column chromatography in order to get rid of some enzymes (eg. berberis bridge enzyme) which may cause side reactions. The partially purified NMT was obtained from DEAE-Sephacel chromatography (1x10 cm) and NMT activity was determined. Fig. 18 shows the profiles of absorbance at 280 nm (protein elution) and NMT activity. The NMT fraction was found in fractions 6-12 which did not bound to the column. The fractions of NMT activity were pooled and used as enzyme in the following methylation step (second reaction).

1.2 Enzymatic synthesis 1,2-dehydronorreticuline from (S)-norreticuline

The dehydro derivative of (S)-norreticuline was prepared by using *Berberis* STOX enzyme as a catalyst. The incubation mixture contained 150 mM borate buffer pH 8.9, 10 mM ascorbate and 500 µl of the partial purified STOX enzyme. Under these conditions, the reaction mixture turned to yellow gradually, compared to control (boiled enzyme). The complete of the reaction was monitored by TLC of sampling the mixture at 6 and 15 hr. The plate was developed with solvent system of CH_2Cl_2:MeOH:NH_4OH; 90:9:1. (S)-norreticuline and 1,2-dehydronorreticuline were found at Rf 0.40 and 0.52, respectively (Fig.19). The TLC-scanned chromatograms of the conversion of (S)-norreticuline to 1,2-dehydronorreticuline are shown in Fig.20. It can be seen that the conversion was almost completed within 15 hr at 30 °C. Therefore, after 15-hr incubation, 1,2-dehydronorreticuline was purified by preparative TLC, to give a yellow dried product at 45% yield.

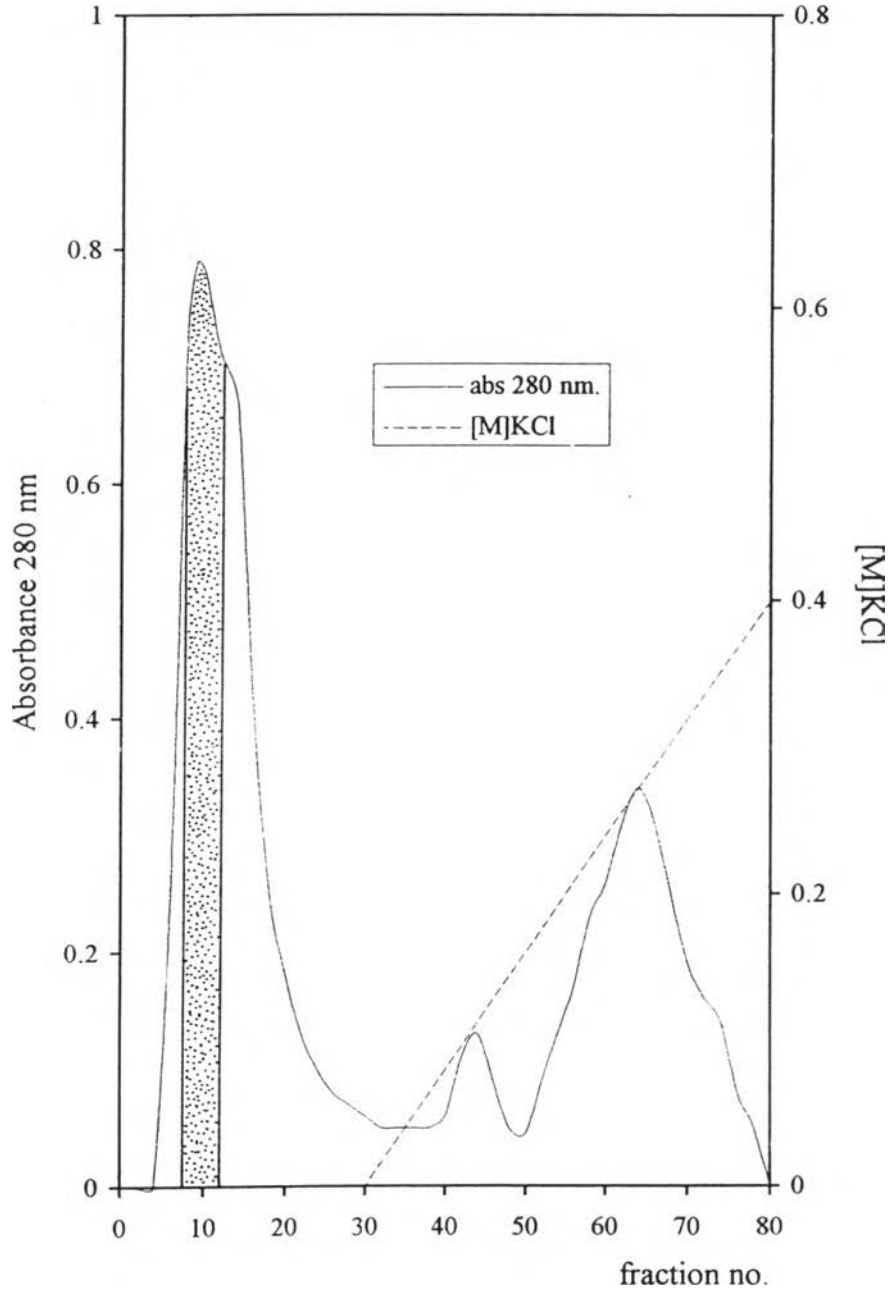


Figure 18 DEAE-Sephacel anion exchange chromatography of partially purified of N-methyltransferase from *Berberis stolonifera* cell culture;

Condition: Sample was desalted on Sephadex G-25M column, buffer A, 50 mM phosphate pH 7.5, with 10 mM β-mercaptoethanol, buffer B, 0.4 M KCl in buffer A; gradient 0-100%B in 50 min; flow rate 1 ml/min; fraction size 1 ml, UV detection, 280 nm at 1.0AUFS = N-methyltransferase

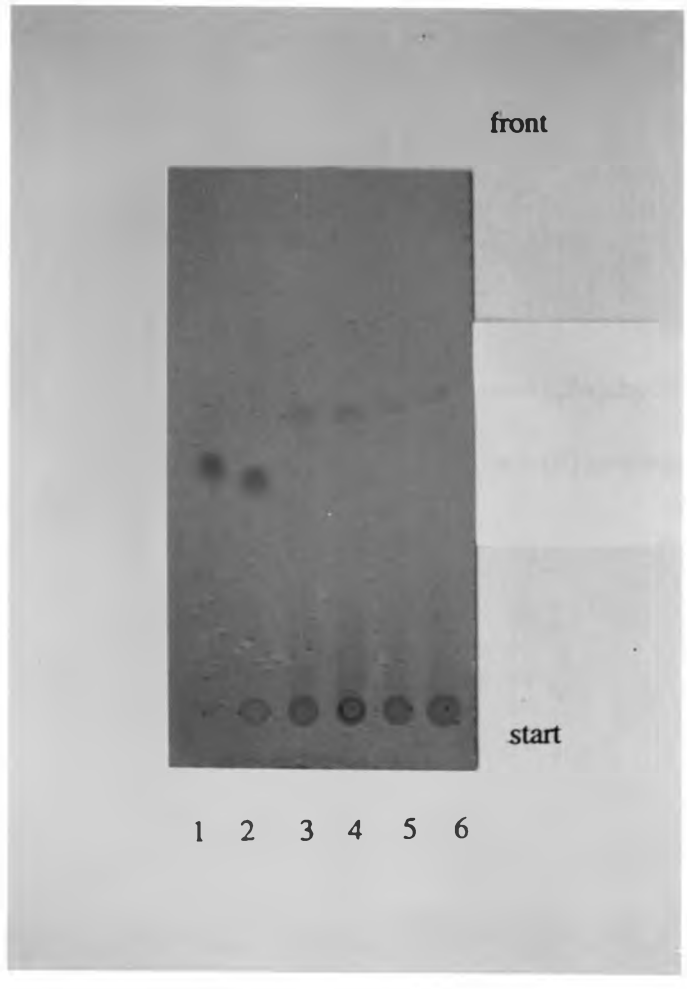


Figure 19 TLC pattern of the reaction mixture: the conversion of (S)-norreticuline to 1,2-dehydronorreticuline after 15 hr-incubation. The pattern was observed after sprayed with dragendorff's reagent
1) standard (S)-norreticuline 2) blank (boiled enzyme)
3-6) sampling reactions

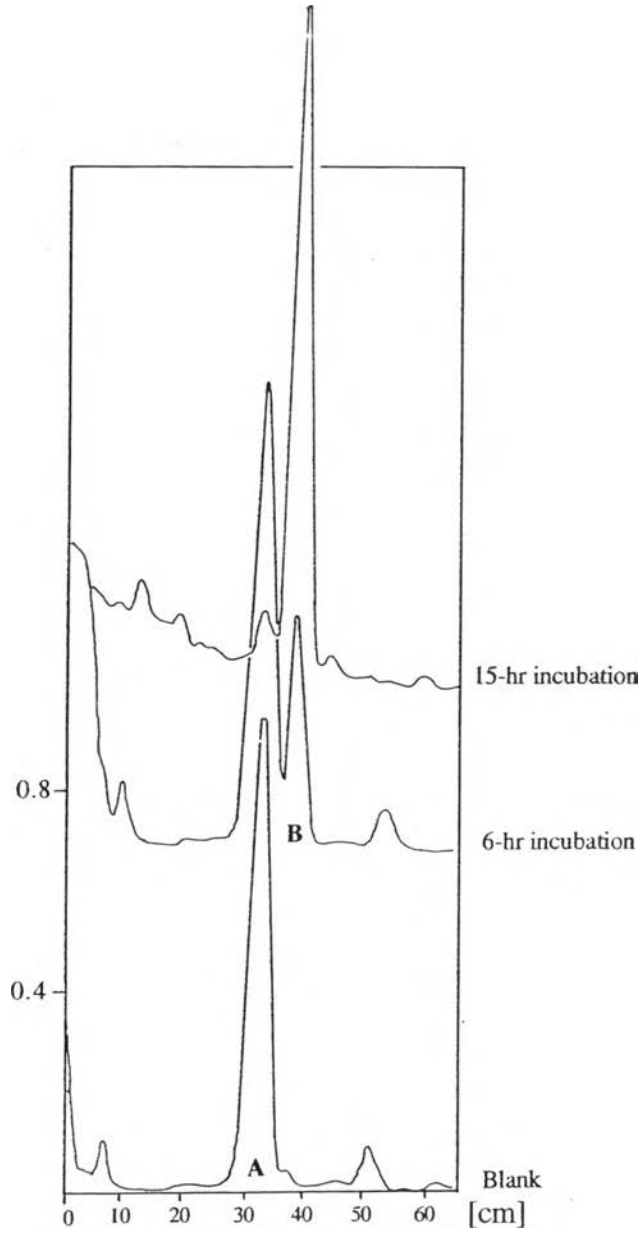


Figure 20 TLC chromatogram (wavelength 285 nm): the conversion of (S)-norreticuline to 1,2-dehydronorreticuline by STOX from *Berberis stolonifera* cell culture; solvent system; CH_2Cl_2: MeOH: NH_4OH, 90:9:1

A) (S)-Norreticuline B) 1,2-Dehydronorreticuline

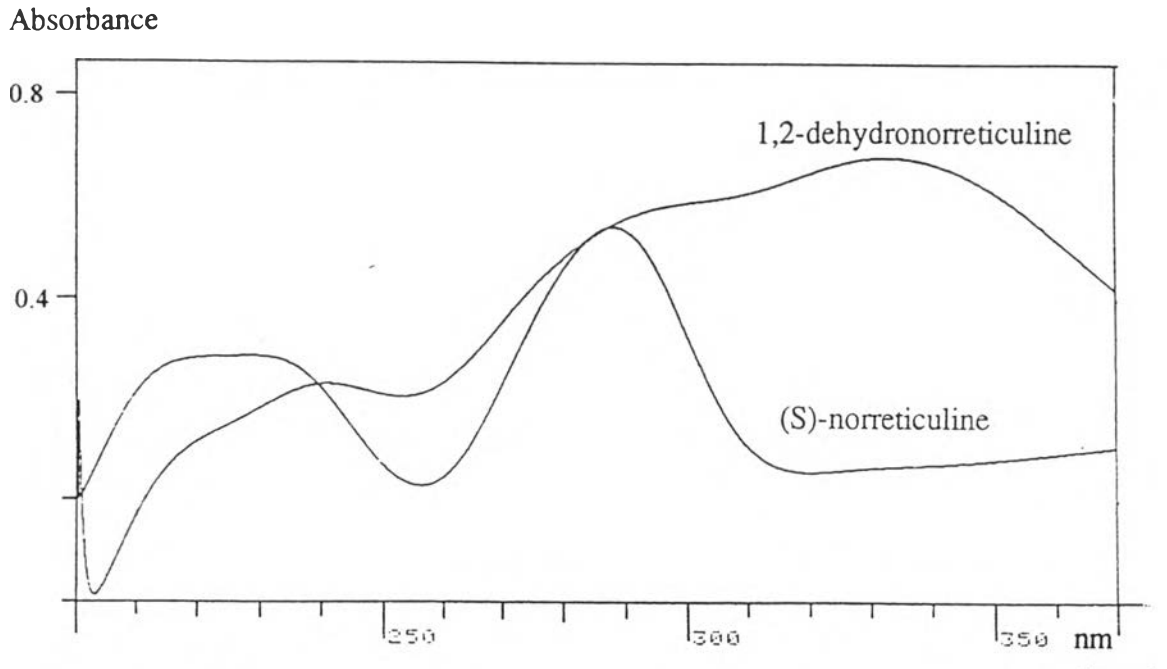


Figure 21 UV-absorption spectrum of 1,2-dehydronorreticuline

The purified 1,2-dehydronorreticuline showed characteristic yellow spot on TLC plate, visible at λ_{max} at 285 and 330 nm(Fig. 21). These characteristics were consistent with dehydro-compound as described by Battersby, *et al.* (1965b). Furthermore the mass spectral analysis by LC-MS (APCI) showed the molecular ion peak $[M+H]^+$ at 314 and another at 328, which are a peak of 1,2-dehydronorreticuline and unspecific methylated of 1,2-dehydronorreticuline, respectively (Fig.22)

1.3 Synthesis of [N-C^3H_3]-1,2-dehydroreticuline

The second step of [N-C^3H_3]-1,2-dehydroreticuline synthesis involved enzymatic methylation of 1,2-dehydronorreticuline using [C^3H_3]-SAM as a radio-labelled methyl donor and *Berberis* NMT as a biocatalyst. The incubation mixture was taken at interval time 1, 12 and 24 hr (at 30 °C) in order to monitor the reaction. Fig. 23 shows the TLC-radioscanned chromatogram of the conversion of 1,2-dehydronorreticuline to [N-C^3H_3]-1,2-dehydroreticuline. After 24-hr incubation, [C^3H_3]-SAM was found to be converted to two labelled peaks with Rf values of 0.4 and 0.7 ($CO(CH_3)_2$: H_2O: Et_2NH,8:1:1). The 24 hours incubation appeared to be a suitable time for the reaction. The peak with Rf value 0.7 was eluted and concentrated to give [N-C^3H_3]-1,2-dehydroreticuline with a yield of 16% (0.44μCi, specific activity 194 μCi/μmol).

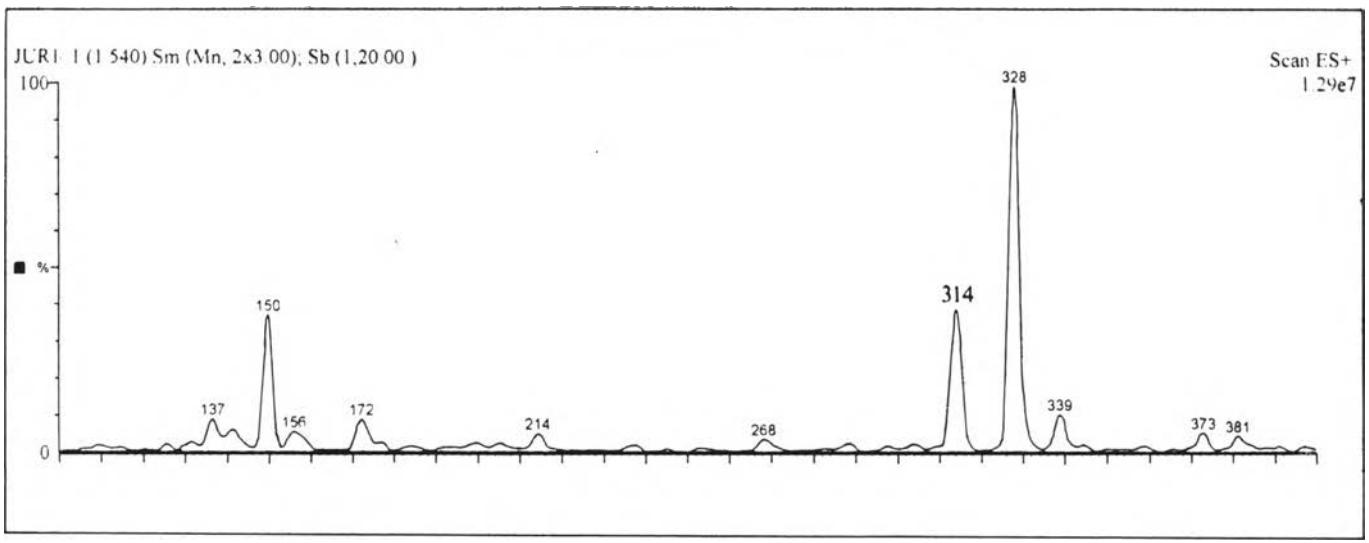


Figure 22 Mass spectrum of 1,2-dehydronorreticuline (LC-MS, APCI)

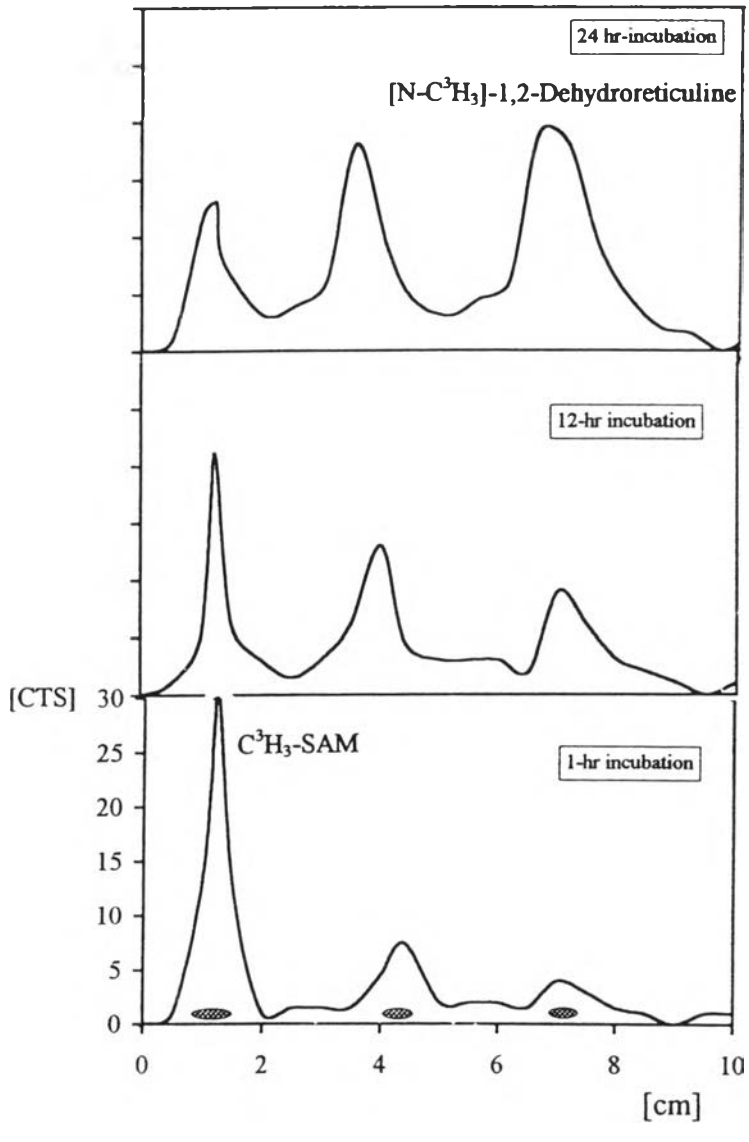


Figure 23 TLC-radiochromatogram of the conversion of 1,2-dehydronorreticuline to [N-C^3H_3]1,2-dehydroreticuline using [C^3H_3]-SAM as radioactively labelled substrate and *Berberis* N-methyltransferase as biocatalyst

2. Detection of 1,2-Dehydroreticuline Reductase Activity from *Papaver somniferum* Seedlings

It has been reported that the enzyme activity of 1,2-dehydroreticuline reductase in *P.somniferum* seedlings is highest at day 4 or 5 and then declined gradually until day 10 (De-Eknamkul and Zenk, 1992). Based on this information, we chose the five-day-old seedlings as an enzyme source for this study. Fig. 24 shows the feature of the five-day-old seedlings which were obtained by germinating the seeds in a large tray (90x46 cm).

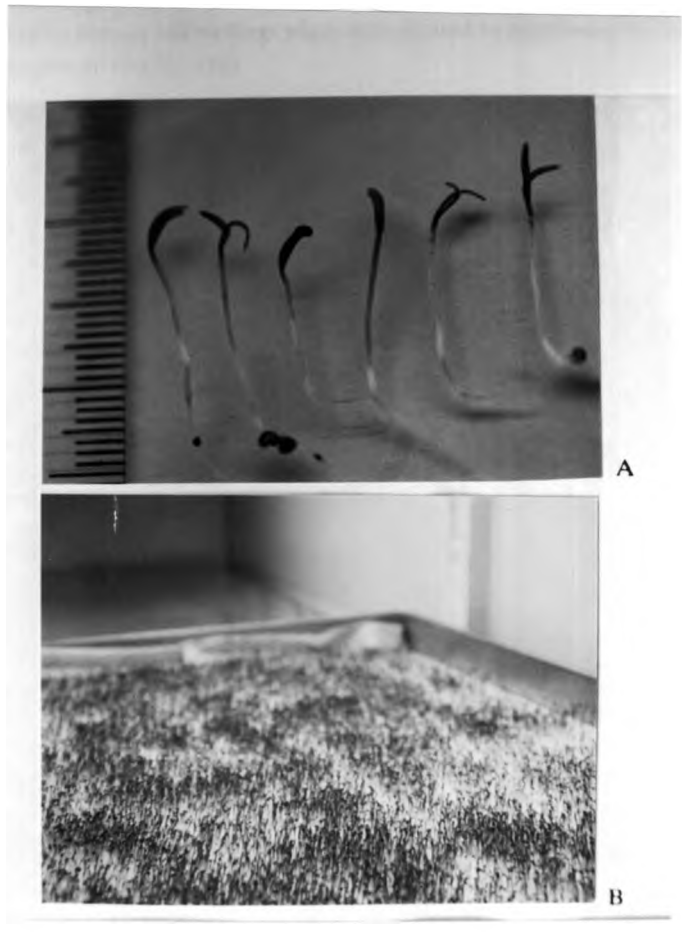


Figure 24 A. Five-day-old seedlings of *P. somniferum*
B. The germinated seedlings in a aluminium tray (90x46 cm)

To confirm that the enzyme activity of 1,2-dehydroreticuline reductase was present in the five-day-old seedlings, a crude enzyme extract was prepared (see Experimental) and assayed for the activity using radioisotopic method. This radioisotopic enzyme assay involved the transformation of the radioactively labelled of [N-C^3H_3]-1,2-dehydroreticulinium ion to [N-C^3H_3]-(R)-reticuline . Since the labelled substrate and product were highly different in their solubility, they could be separated from each other by using organic solvent. In this assay, the labelled product ([N-C^3H_3]-(R)-reticuline) was extracted into toluene while the reacted substrate ([N-C^3H_3]-1,2-dehydroreticulinium ion) remained in the aqueous phase. Therefore, the radioactivity in the organic phase determined by liquid scintillation counter could be used for calculating the enzyme activity. Based on this method, the reductase activity in the seedlings was found to be $1.65x10^{-3}$ $pkatg^{-1}$ fresh weight. This comfirmed that the five-day-old seedlings could be used as an enzyme source for this study.

3. Large Scale Purification of 1,2-Dehydroreticuline Reductase

1.2-Dehydroreticuline reductase was purified from *P. somniferum* L.seedlings using a combination of various steps, including ammonium sulfate precipitation, and chromatographic separations using Phenyl-Sepharose CL-4B chromatography, DEAE-Sephacel, MonoQ HR5/5, Superose-12 HR16/50, and 2nd MonoQ HR5/5. The reductase was purified 1433-fold with an overall yield of 0.002%. A typical purification scheme is summarized in Table 8.

In the first step, a large amount of undesired proteins was removed by fractionation between 55-85% saturation of ammonium sulfate. In this saturation range , the reductase was concentrated into a small volume which was convenient for handling. In the second step, the enzyme solution (containing 0.2M $(NH_4)_2SO_4$ was initially mixed with Phenyl-Sepharose CL-4B in stead of loading the enzyme solution directly into the Phenyl-Sepharose column, in order to prevent the shrinkage and aggregation of the gel. The mixture of the gel and enzyme solution was then loaded onto a column for chromatography. This step of Phenyl-Sepharose CL-4B could separate a considerable amount of protein(60%) with only a small effect on the reductase activity. Since this was the step of hydrophobic chromatography, the enzyme was eluted with the extraction buffer containing no ammonium sulfate. Fig. 25 shows the profile of protein absorbance at 280 nm and the enzyme activity. It can be seen that the protein was eluted in a single peak which contained the reductase activity. In this

Table 8 Summary of purification procedure for 1,2-dehydroreticuline reductase from 1 kg.(fr. wt) *Papaver somniferum* seedlings

Purification step	Total volume (ml)	Total protein (mg)	Total activity $x10^{-3}$ (pkat)	Specific activity $x10^{-3}$ (pkat/mg)	Yield (%)	Purification (-fold)
55-85% $(NH_4)_2SO_4$ precipitation	165	323	337	1.0	100	1
Phenyl Sepharose CL-4B	108	130	378	2.9	40	2.9
DEAE Sephacel	44	88	116	1.3	27	1.3
1st. FPLC MonoQ HR5/5	4	7	208	29.7	2.2	30
FPLC Superose12 HR16/50	55	0.7	194	277	0.22	277
2nd. FPLC MonoQ HR5/5	6	$6x10^{-3}$	8.6	1433	0.002	1433

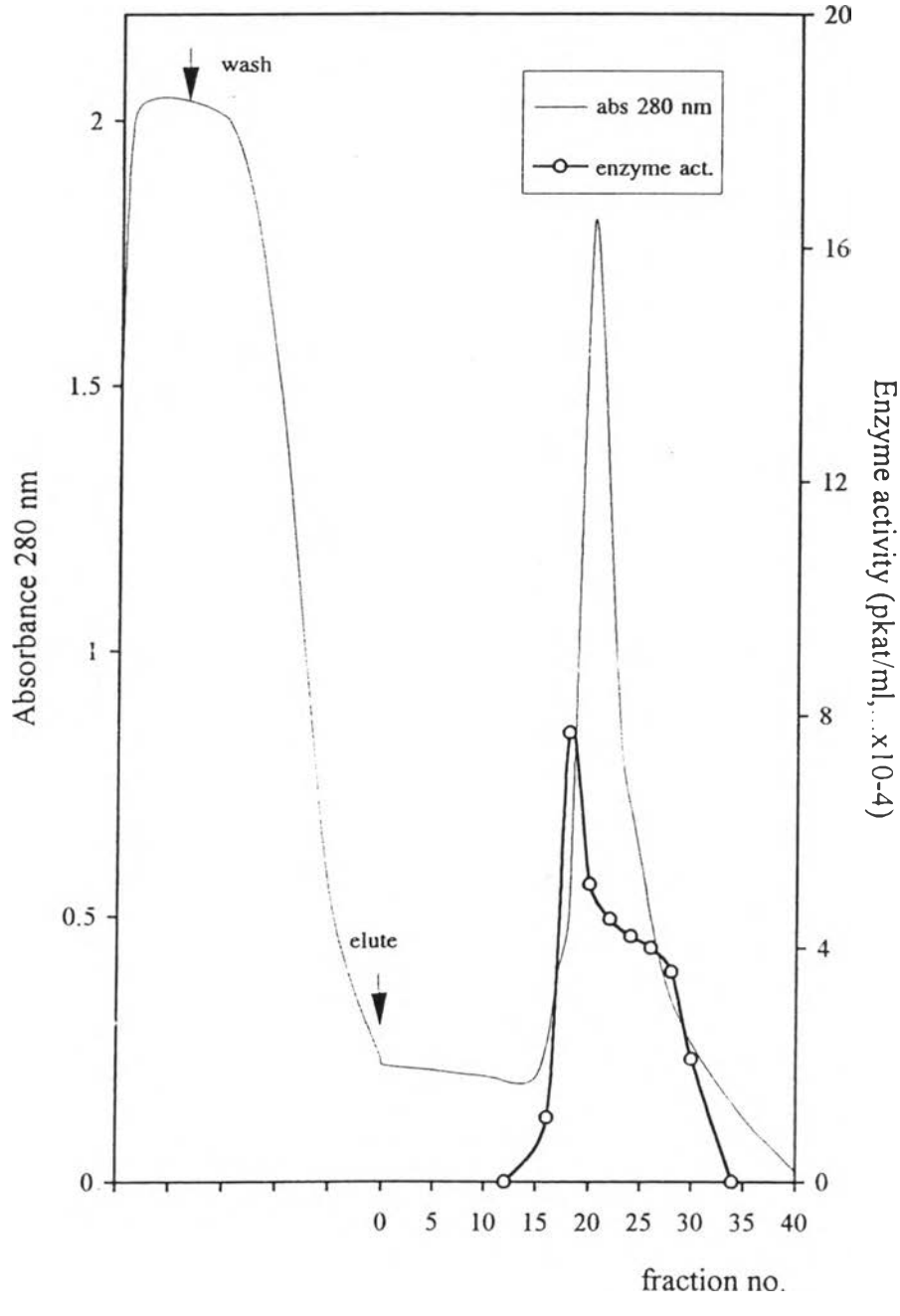


Figure 25 Phenyl-Sepharose CL-4B hydrophobic chromatography of 1,2-dehydroreticuline reductase preparation obtained after 55-85% ammonium sulphate precipitation

Condition: flow rate 3 ml/min, fraction size 10 ml, chart speed 0.5 mm/min, detection, 280 nm at 2.0AUFS

step the yield of 1,2-dehydroreticuline reductase was 40% with 2.9-fold purification. Those fractions containing the enzyme activity were pooled and passed through the anion exchange column of DEAE-Sephacel (Fig. 26). In this step, proteins could not bound to the column was washed out of the column but the 1,2-dehydroreticuline reductase was bound to the column. The enzyme was later eluted with the extraction buffer containing 0.2 M ammonium sulfate. The enzyme activity was detected in the fractions of a single major protein peak (fractions 10 to 25). This step gave on 1.3-fold of purification with 27% yield of the protein. The next step of enzyme purification was MonoQ anion exchange chromatography. The resulted active enzyme fractions obtained from DEAE-Sephacel column was first dialyzed against the sugar free extraction buffer. No significant amount of protein loss occurred in this step. The enzyme solution was then loaded onto MonoQ HR5/5 (0.5x5cm) which was preequilibrated with the same buffer used bound to column. When the column was eluted with linear gradient of 0-350 mM KCl in buffer, the protein profile shows two major peaks and the reductase enzyme activity was found in the first peak (70-140 mM KCl)(Fig. 27). The fractions containing the reductase activity (fractions 6 to 10) were combined and passed through the next column. This step of FPLC MonoQ chromatography appeared to be very efficient in separating the reductase from other proteins resulting in a 30-fold purification with 2.2% yield of the protein. For the step of FPLC Superose-12 gel filtration (Fig.28), the reductase activity was found within a trailing shoulder of a major protein peak suggesting that the reductase was a minor protein. This Superose-12 HR16/50 column (1.6x50cm) was also effective in separating the higher and lower molecular weight proteins of of the reductase enzyme (Fig. 29). This step produced 277-fold of purification with 0.22% yield of protein. The proteins were eluted out of column depending on their molecular weight. In the last step, we chose a Mono Q HR5/5 for separating the protein from each other by salting gradient. The Mono Q HR5/5 was preequilibrated with 10 mM phosphate buffer pH 7.5, then the protein fractions (pooled from gel filtration step) was loaded into the MonoQ HR5/5. The proteins which could not boumd the column were washed out of the column. The 1,2-dehydroreticuline reductase was eluted by gradient of 50-150 mM KCl in 10 mM phosphate buffer pH 7.5 . The result of gradient is shown in Fig. 30. Finally, the second MonoQ HR5/5 gave 1,2-dehydroreticuline reductase in 0.002% yield with 1433-fold of purification. The 1,2-dehydroreticuline reductase was eluted at the gradient between 100-110 mM KCl. The purity of the reductase enzyme in each fraction was checked by SDS-PAGE (Fig. 31). The purified 1,2-dehydroreticuline reductase was kept at -20 ^{o}C for further determining of amino acid sequence.

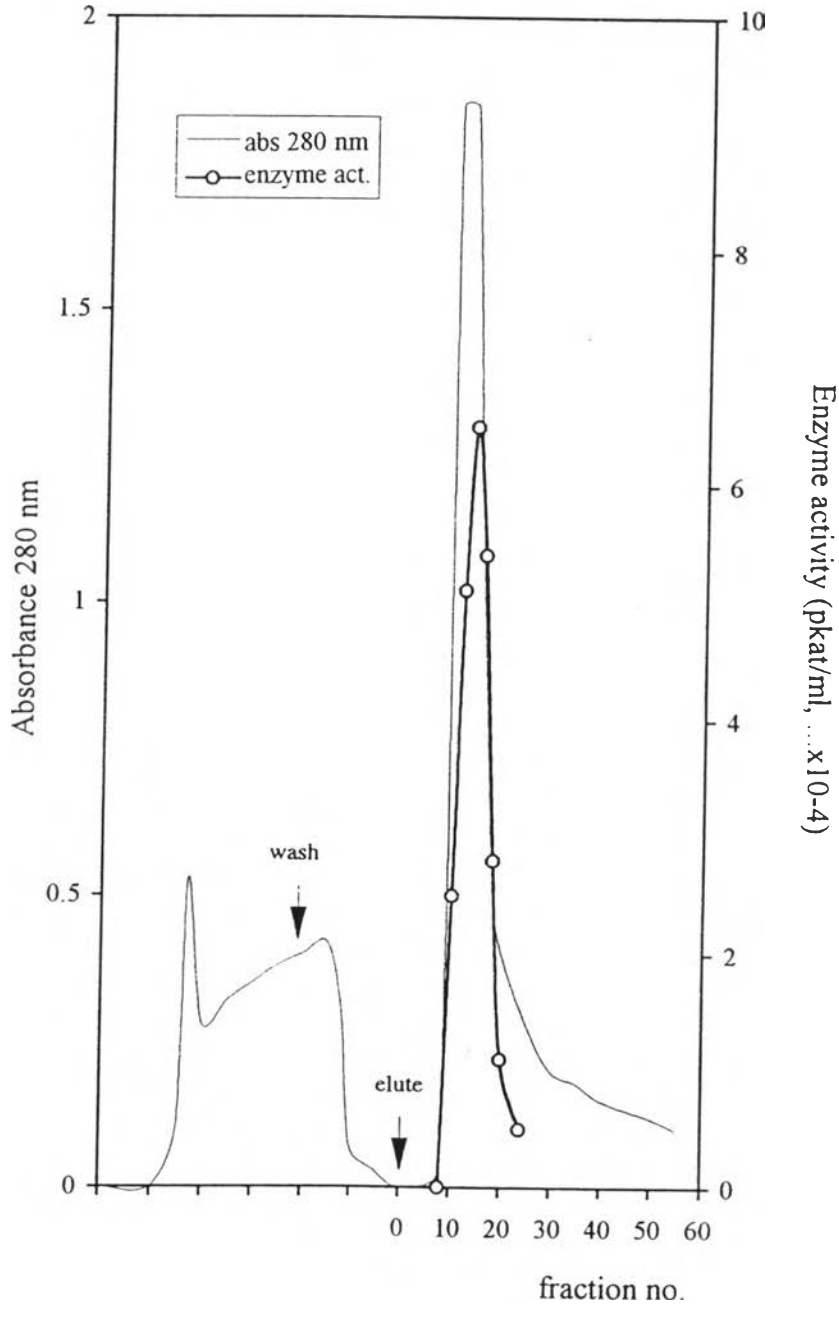


Figure 26 DEAE-Sephacel anion exchange chromatography of the pooled fractions from Phenyl-Sepharose CL-4B column

Condition: flow rate 2 ml/min, fraction size 5 ml, chart speed 0.5 mm/min, detection, 280 nm, 2.0AUFS

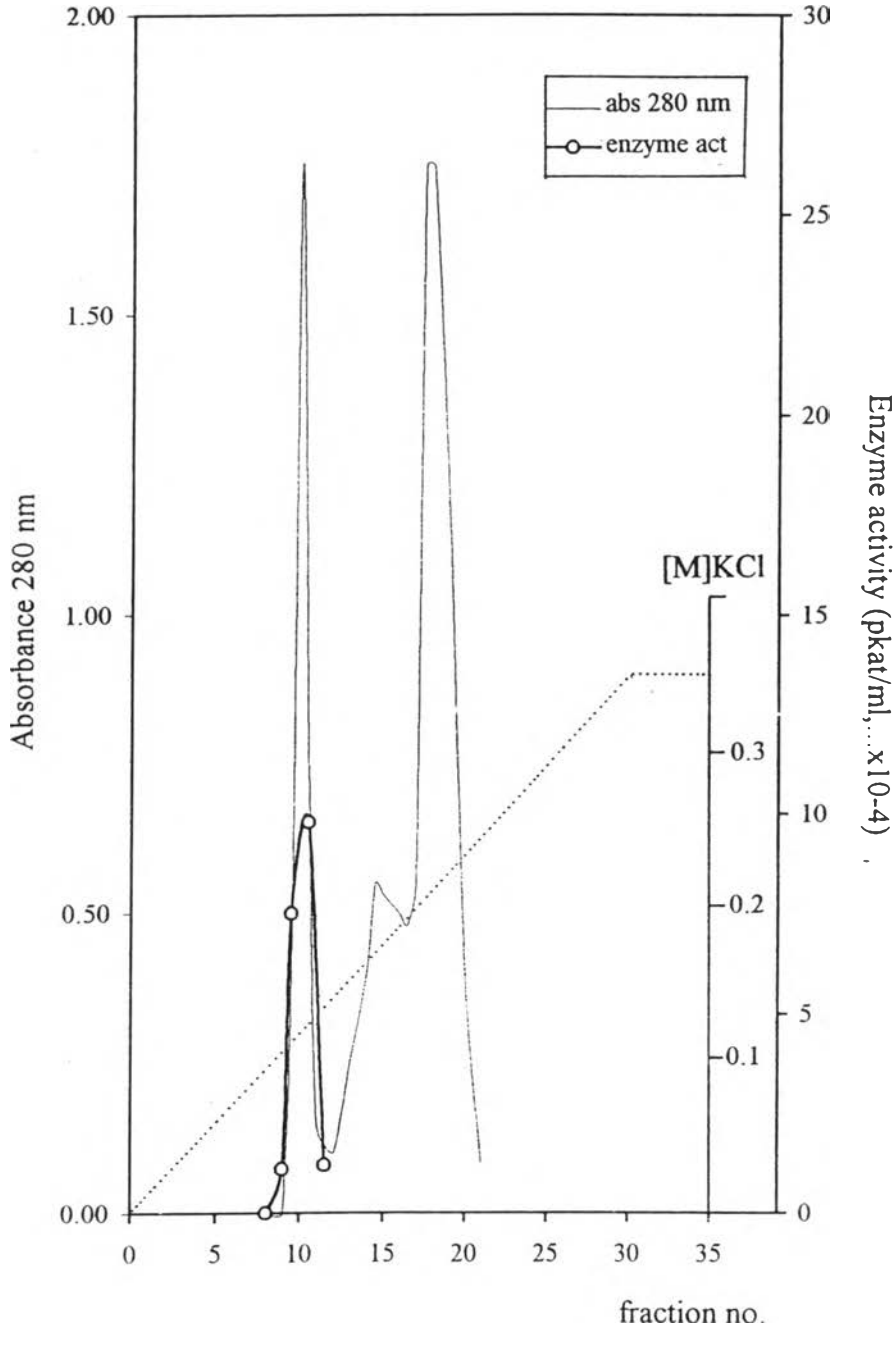


Figure 27 The first MonoQ HR 5/5 anion exchange chromatography of the enzyme from DEAE-Sephacel column

Condition: flow rate 1 ml/min, fraction size 1 ml, chart speed 2 mm/min, detection, 280 nm, 2.0AUFS, buffer A, 100 mM tricine-NaOH pH 7.5, 1 mM EDTA, 10 mM β-mercaptoethanol, buffer B, 1.0 M KCl in buffer A, linear gradient 0-35% B(·····) in 30 min.

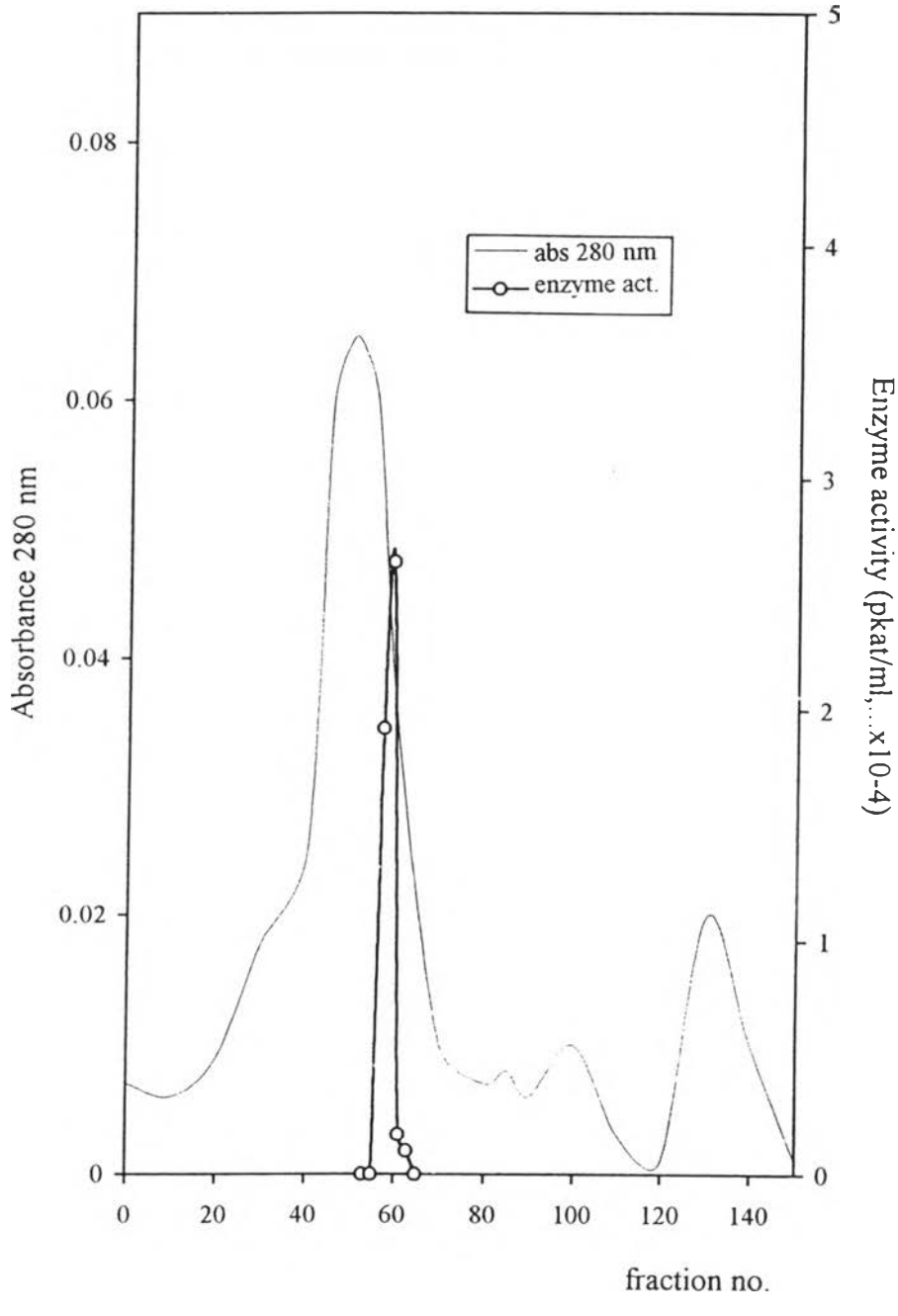


Figure 28 Gel filtration chromatography Superose12 HR 16/50 of the pooled fractions after purified by MonoQ column on

Condition: flow rate 0.5 ml/min, fraction size 1 ml, chart speed 1 ml/min, detection, 280 nm at 0.1AUFS

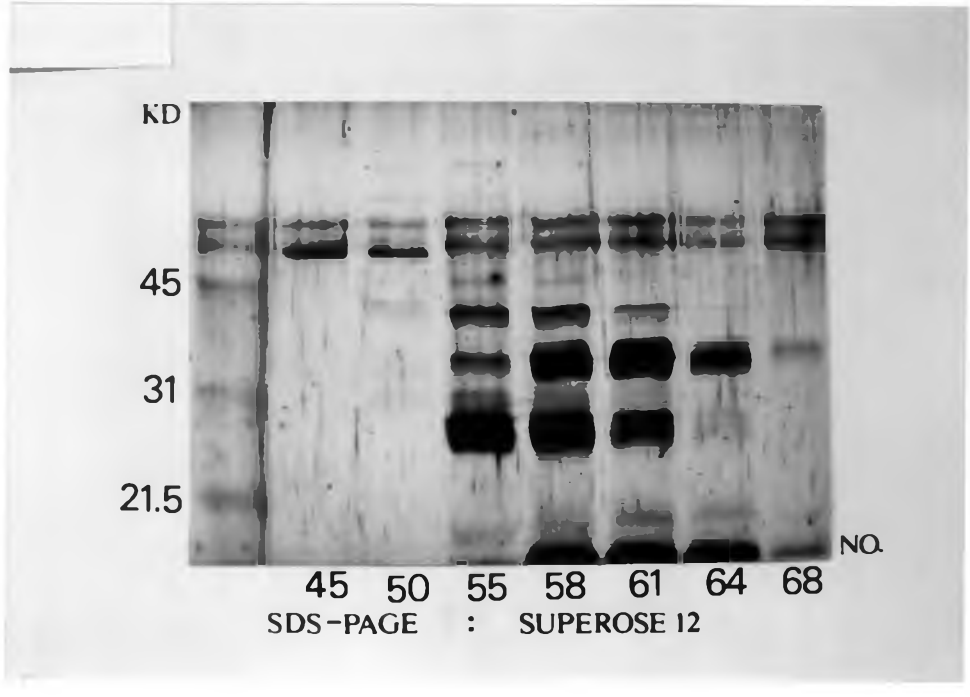


Figure 29 SDS-PAGE of each fraction from Superose12 column

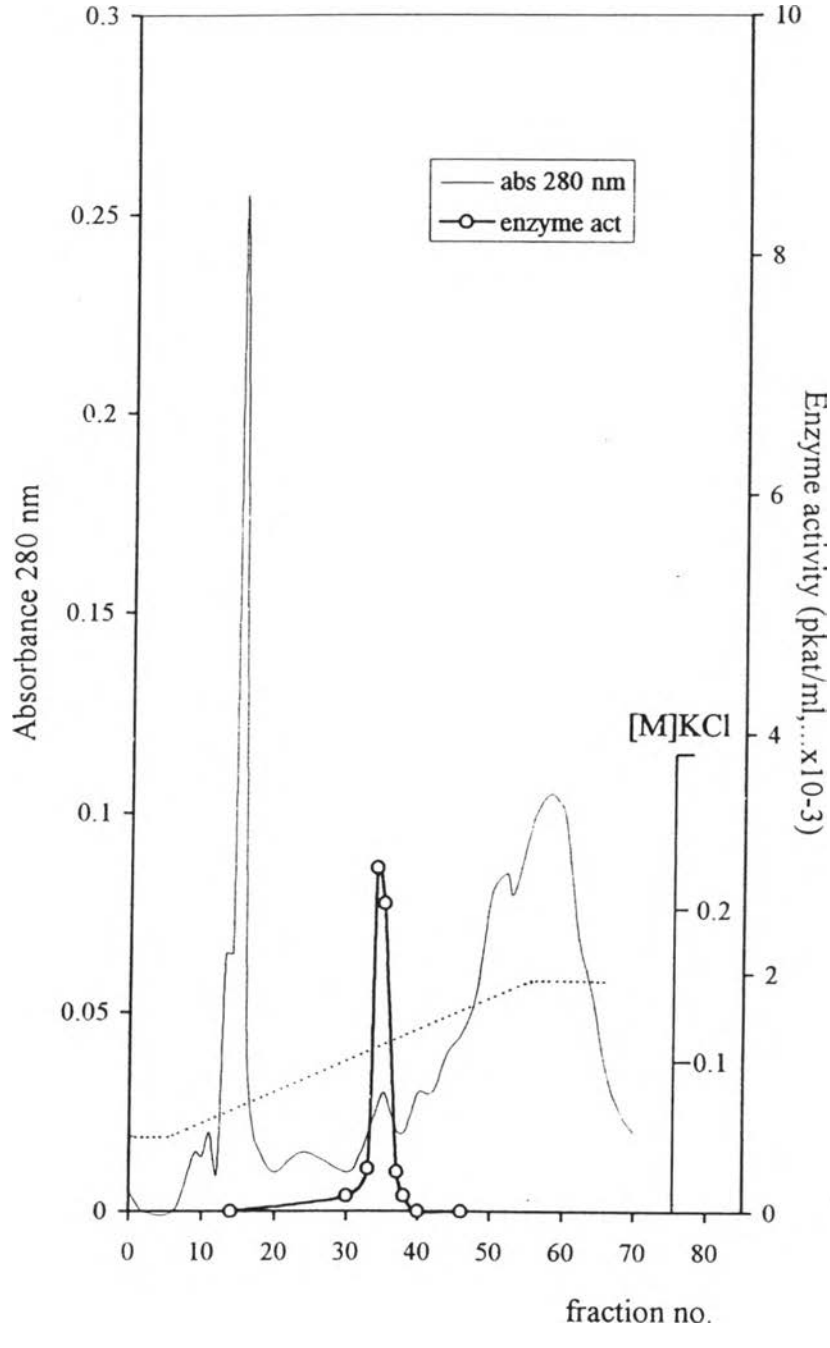


Figure 30 The second MonoQ HR5/5 anion exchange chromatography of the pooled fractions from Superose12 column

Condition: flow rate 1 ml/min, fraction size 1 ml, chart speed 2 mm/min, detection, 280 nm at 0.05AUFS, buffer A, 10 mM phosphate buffer pH 7.5, buffer B, 1 M KCl in buffer A, linear gradient (·····), 5-15% buffer B in 50 min.

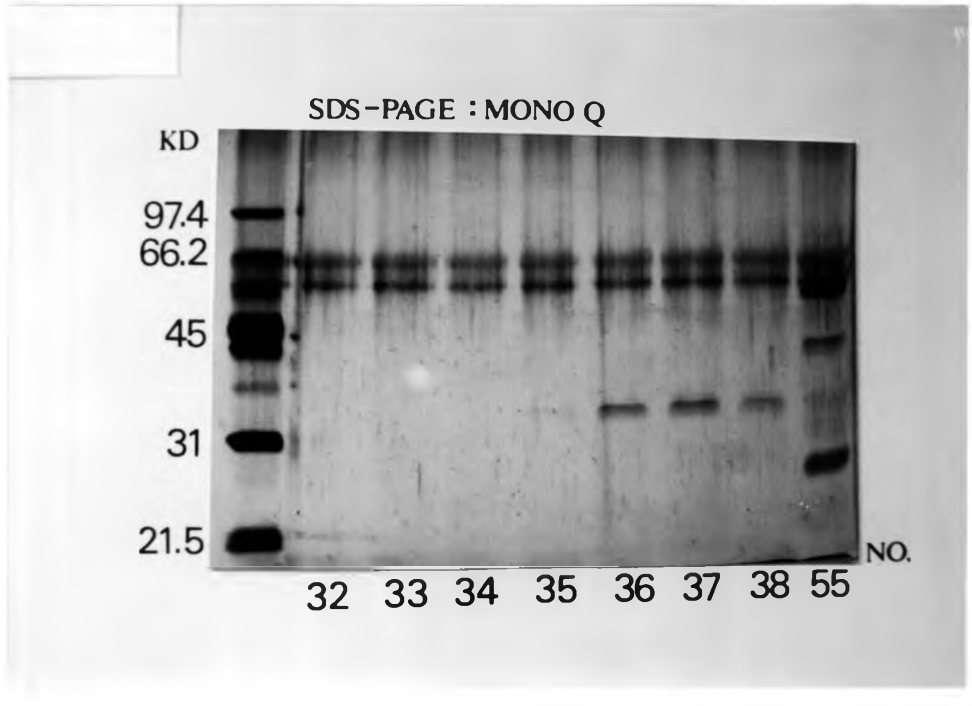


Figure 31 SDS-PAGE of each fraction from the second MonoQ column

4. Purity Check of the Purified 1,2-Dehydroreticuline Reductase

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 the enzyme solutions was loaded onto a 12% acrylamide slab gel. Electrophoresis was carried out with a constant current of 25 mA. After the operation, the gel was stained using silver staining. Fig.32 shows the SDS-PAGE pattern which indicates that 1,2 dehydroreticuline reductase was purified gradually in each step of the purification process.

5. Molecular Weight of 1,2-Dehydroreticuline Reductase

5.1 Determination by SDS-PAGE

Purified 1,2-dehydroreticuline reductase obtained from MonoQ HR5/5 II column was loaded onto electrophoresis gel. After electrophoresis, the gel was stained with Coomassie Blue staining. The molecular weight of a protein band was estimated by comparing its relative mobility (Rf) to those of known molecular weight proteins; phosphorylase b (97.4kD, Rf 0.2), bovine serum albumin (66.2, Rf 0.28), ovalbumin (45kD, Rf 0.43), carbonic anhydrase (31kD, Rf 0.66), soybean trypsin inhibitor (21.5kD, Rf 0.89) and lysozyme (14.4kD, Rf 0.92)(Fig. 33). The calibration curve of protein standard using SDS-PAGE is shown in Fig. 34. Molecular mass of 1,2-dehydroreticuline reductase is estimated as 34 kD.

5.2 Determination by gel filtration

The 55-85% ammonium sulfate precipitation was prepared as described previously (see Experimental, section 6). The pellet was dissolved in 10mM phosphate buffer pH 7.5. The solution of crude enzyme was loaded onto Superose12 HR16/50, equilibrated and eluted with the same buffer at a constant flow rate 0.5 ml/min. Fractions of 1 ml were collected and measured for 1,2-dehydroreticuline reductase activity. Fig. 35 shows the profile of 1,2-dehydroreticuline reductase activity versus the fraction number. Superose12 HR16/50 column was calibrated using the following standard proteins; thyroglobulin (670kD), gamma globulin (158kD), ovalbumin (44kD), myoglobulin (17kD), vitamin B-12 (1.35kD)(Fig. 36). When, determining the

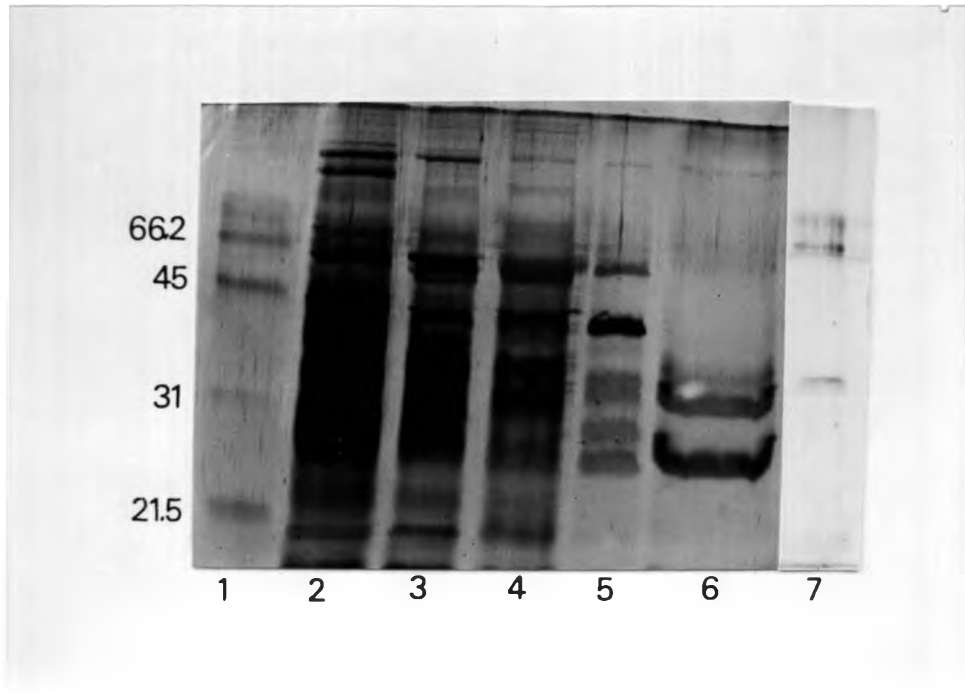


Figure 32 SDS-PAGE pattern of purified protein in each step of purification

Lane 1 : Low molecular weight markers

Lane 2 : 55-85% ammonium sulfate precipitation

Lane 3 : pooled fractions from Phenyl Sepharose CL-4B

Lane 4 : pooled fractions from DEAE Sephacel

Lane 5 : pooled fractions from 1st MonoQ column

Lane 6 : pooled fractions from Superose12 column

Lane 7 : pooled fractions from 2nd Mono Q column

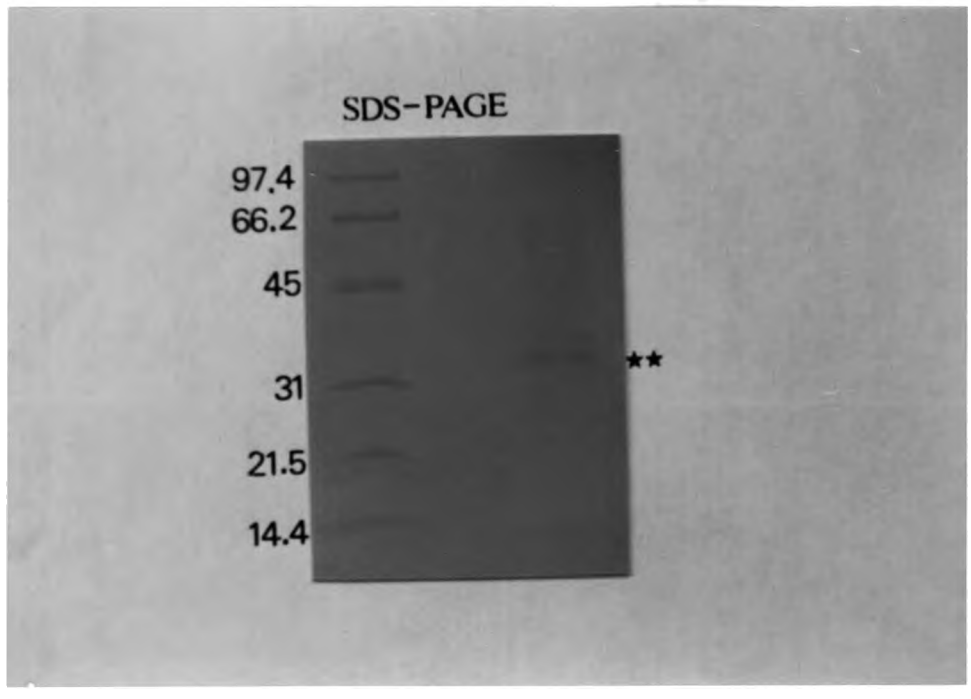


Figure 33 SDS-PAGE of purified enzyme (Coomassie blue staining)

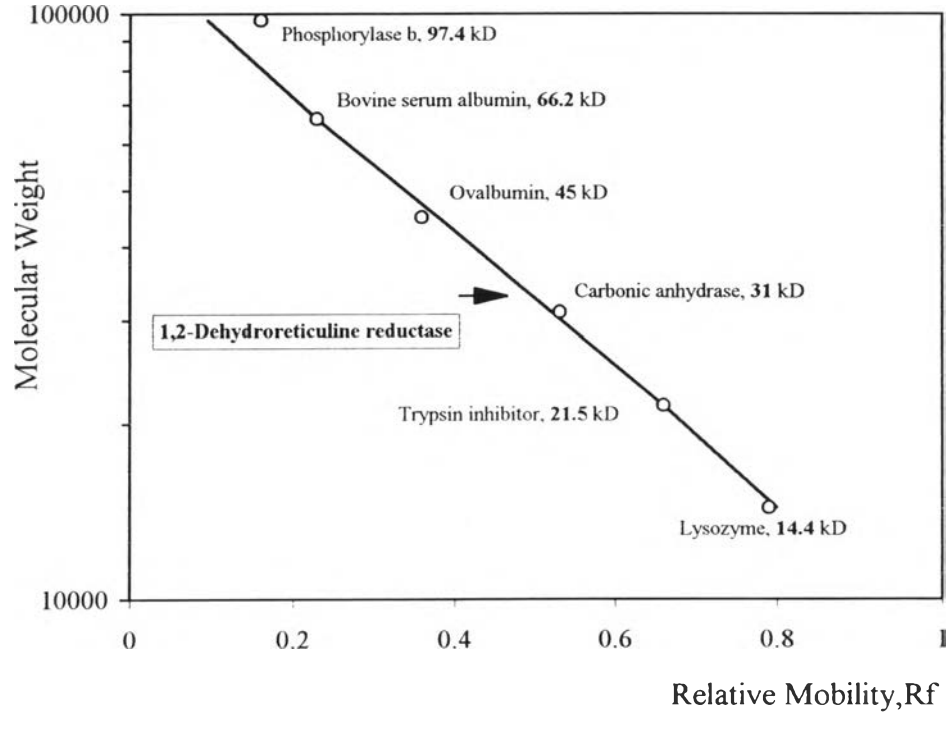


Figure 34 Standard calibration curve of molecular weight plotted against Rf

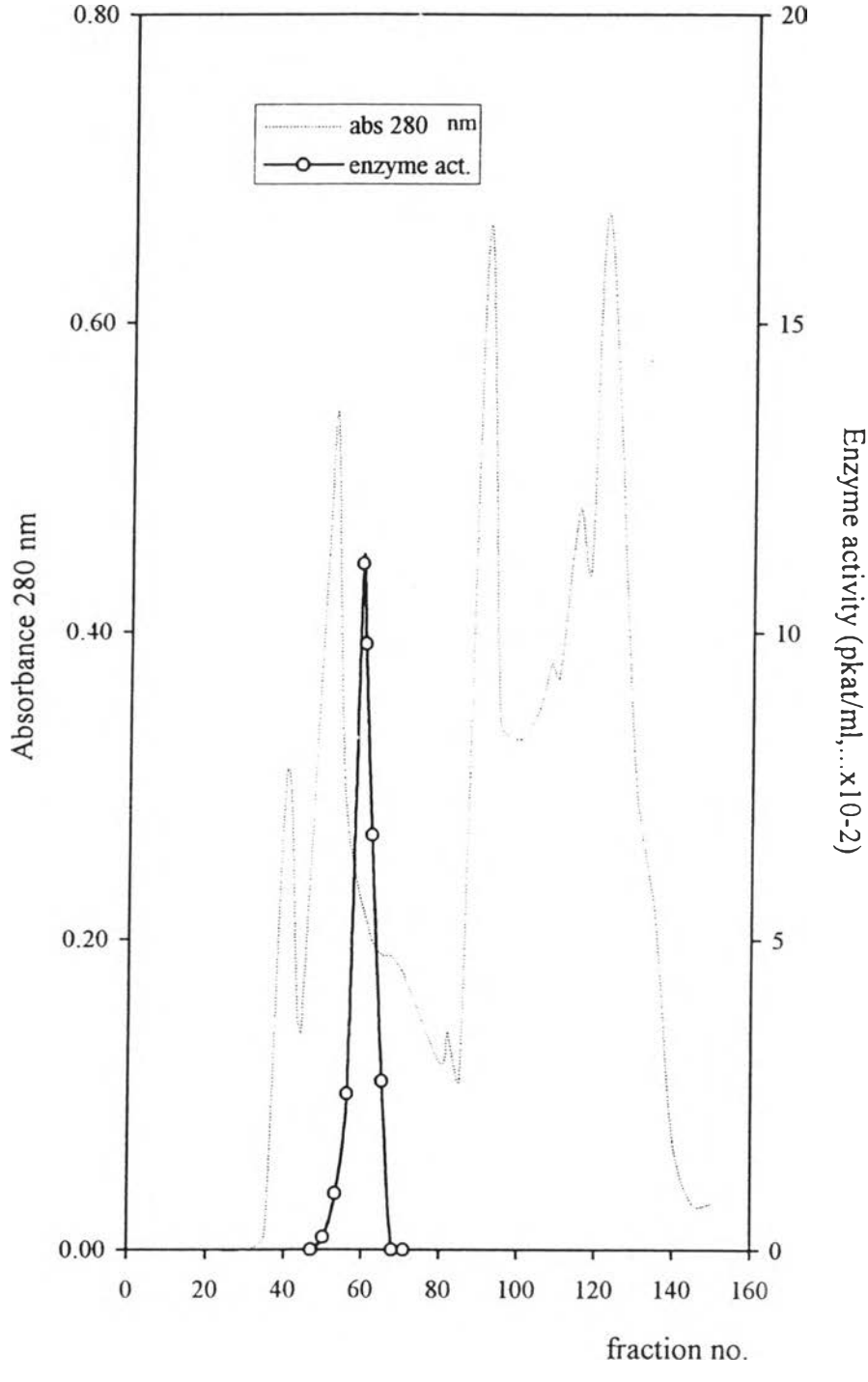


Figure 35 FPLC Superose12 HR16/50 chromatography of 55-85% ammonium sulphate precipitation

Condition: flow rate 0.5 ml/min, fraction size 1 ml, chart speed 0.5 mm/min, detection, 280 nm at 0.5AUFS

position of void volume using blue dextran. The void volumn of Superose12 HR16/50 column was determined as 38 ml. The relative molecular weight of the enzyme was determined from a calibration curve between molecular weight and V_o/V_e of standard protein (Fig.37), thyroglobulin (0.97), gamma globulin (0.80), ovalbumin (0.70), myoglobulin (0.60) and vitamin B-12(0.42) on semilog scale. 1,2-Dehydroreticuline reductase was eluted out at 58 ml, with the V_o/V_e ratio of 0.66, corresponding to a molecular mass of about 34 kD.

6. Partial Amino Acid Sequence of 1,2-Dehydroreticuline Reductase

The procedure of amino acid sequencing was performed as described in Experimental, section 11. The TPCK-trypsin was used as proteolytic enzyme to cleave the proteins specifically. The 1,2-dehydroreticuline reductase tryptic peptides were purified by reversed-phase HPLC. This experiment gave 10 fragments of small peptide, one of which was chosen and its amino acid sequence analysed using Gas Phase Sequencer. It was found that, that peptide fragment consisted of 21 amino acids as followed: -Ala-Phe-Cys-Glu-Asn-Ser-Thr-Asp-Ala-Glu-Lys-Arg-Pro-Leu-Cys-Gly-Arg-Thr-Tyr-Asp-Ile-.

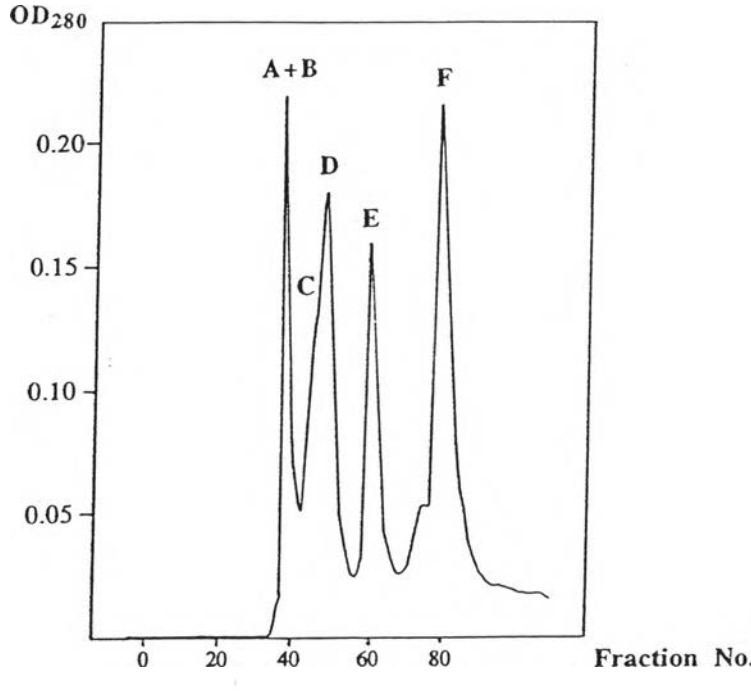


Figure 36 Elution profile of Bio-Rad molecular weight standards on Superose12 HR16/50 at a flow rate 0.5 ml/min in 10 mM phosphate buffer pH 7.5
A=void peak; B=thyroglobulin, 670kD; C=gamma globulin, 158kD;
D=ovalbumin, 44kD; E=myoglobulin, 17kD; F=vitamin B-12, 1.35kD

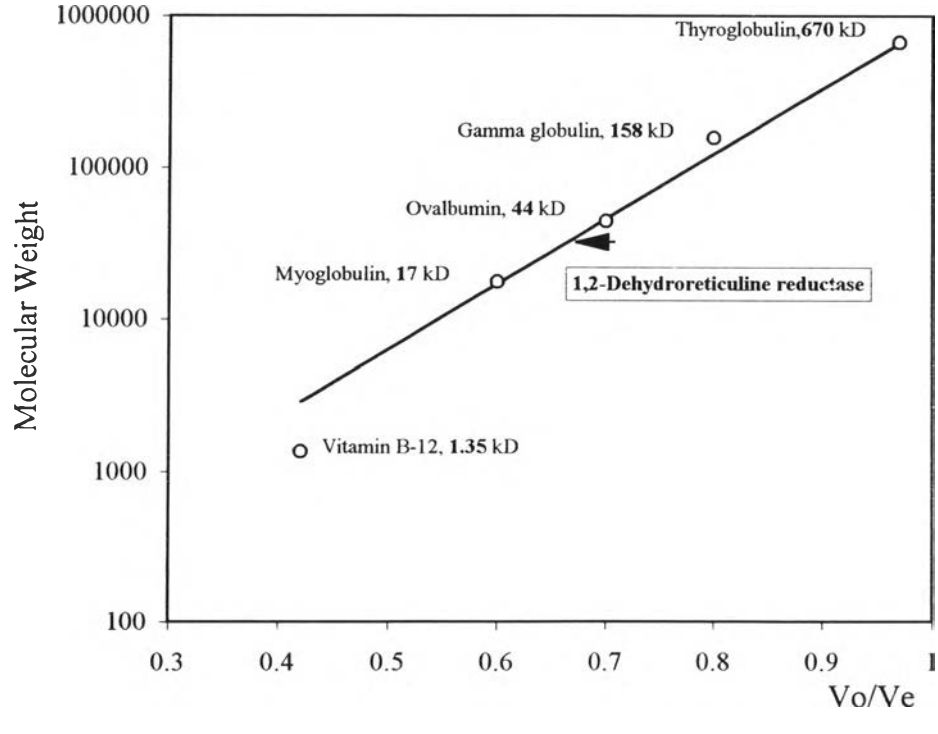


Figure 37 Standard calibration curve of molecular weight plotted against V_o/V_e