CHAPTER III

EXPERIMENTAL

1. Chemicals

Optically pure (S)-norreticuline, (R)-reticuline and [C^3H_3]-S-adenosyl-L-methionine were kindly provided by Prof. M.H. Zenk, Institute of Pharmaceutical Biology, Germany.

Molecular weight standard protein for gel filtration (thyroglobulin, 670 kD; gamma globulin, 158 kD; ovalbumin, 44 kD; myoglobulin, 14 kD; vitamin B-12, 1.35 kD) and for SDS-PAGE (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) were purchased from Bio-Rad, Laboratories, Richmond, Ca., USA. Chemicals for gel electrophoresis, including acrylamide, bis-acrylamide, ammonium persulfate, N,N,N',N' tetramethylethylenediamine (TEMED), coomassie brilliant G-250(for protein checking), coomassie brilliant R-250(for coomassie blue staining), silver staining plus kit and bromophenol blue were also purchased from Bio-Rad, Laboratories, Richmond, Ca.,USA.

Materials for chromatography (Sephadex G-25 M, Phenyl-Sepharose CL-4B, DEAE-Sephacel, Superose12, MonoQ HR5/5) were purchased from Pharmacia Biotechnology, Uppsala, Sweden.

Tricine, glycine, ethylenediaminetetraacetic acid(EDTA), trisma base, bovine serum albumin, nicotinamide adenine dinucleotide phosphate (NADPH), and dextran blue were purchased from Sigma Chemical Company, St. Louis, Mo., USA. Disodium hydrogen orthophosphate($Na_2HPO_4.12H_2O$), sodium dihydrogen orthophosphate ($NaH_2PO_4.H_2O$), ammonium sulfate($(NH_4)_2SO_4$) were purchased from Farmitalia, Milan, Italy. The chemicals for culture media were all tissue culture grade. Plant growth regulators were products of Gibco Laboratories,New York, USA.,and agar was purchased from Difco Laboratories, Detroit, Michigan, USA.

Organic solvents used for TLC system were redistilled solvents of commercial grade. Organic solvents used for FPLC and for enzyme analysis were either HPLC or analytical grade. Water was triple deionized. TLC plates of silica gel 60 F_{254} on aluminium sheets and silica gel 60 F_{254S} on flatten-glass were purchased from E.Merck, Damstadt, Germany.

2. Plant Material

The seeds of *Papaver somniferum* were obtained from Prof. M.H. Zenk, Institute of Pharmaceutical Biology, Germany. They were harvested in 1991 and 1992.

For the preparation of seedlings, the opium seeds were germinated in 90x46 cm aluminium trays, each of which contained 3 layers of tissue paper and 300 ml of water. The seeds were spreaded on the soaked-tissue. The trays were then kept in the dark for two days until root germination, followed by exposing with continuous light(white fluorescent lamps) for three days. The five-day-old seedlings were harvested and kept at -20°C until being used as the source of enzyme.

3. Preliminary Study on the Activity of 1,2-Dehydroreticuline Reductase from *P. somniferum* Seedlings

Five-day-old seedlings were used as an 1,2-dehydroreticuline reductase source. Determination of its enzyme activity will be described in section 5. 55-85% Ammonium sulfate precipitation was prepared as described in section 8. The pellet was dissolved and desalted with Sephadex G-25M (0.7x10cm) column. The desalted fraction was collected and checked the activity of 1,2-dehydroreticuline reductase.

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

4.1. Preparation of partially purified S-tetrahydroprotoberberine oxidase (STOX) and N-methyltransferase (NMT)

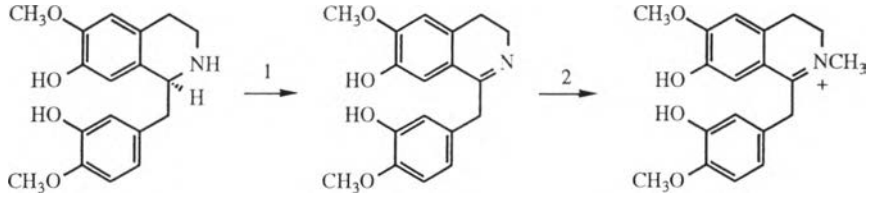
Partial purification of STOX and NMT were carried out by using the modified method from Amann *et al.* (1988) and Wat *et al.* (1985), respectively. Both enzymes were prepared from *Berberis stolonifera* cell cultures which were maintained in Linsmaier and Skoog(LS) medium containing 30 g/l of sucrose, 0.2 mg/l of 2,4-

dichlorophenoxyacetic acid (2,4 D), and 0.2 mg/l α-naphthaleneacetic acid (NAA). Harvested-cells(30 g) of *B. stolonifera* (14 days old) were ground in mortar and stirred with 60 ml of 50 mM phosphate buffer pH 7.5 for 10 min. The homogenate was filtered through four layers of cheesecloth and clarified by centrifugation at 13,000*g* for 10 min in Hitachi High-Speed Refrigerated Centrifuge Model CR20B3. The pellet was discarded and the supernatant was made up to 70% saturation with $(NH_4)_2SO_4$. The protein precipitate was separated by centrifugation at 13,000*g* for 10 min. The protein pellet was resuspended in 50 mM phosphate buffer pH 7.5 (minimum volume,20 ml). This protein solution was desalted on Sephadex G-25M column (2.5x10 cm) which was preequilibrated and eluted with 50 mM phosphate buffer pH 7.5. The resulted protein fraction was divided into two parts. The first part was used directly as an the oxidase enzyme(STOX). the other part was further run through DEAE-Sephacel (1x10 cm, flow rate1 ml/min). The adsorbed enzyme was eluted with 0-400 mM KCl in linear gradient in the same buffer as that used in the desalting step. NMT activity was monitored by the method of Amann *et al.* (1988) and the active NMT enzyme fractions were pooled and used as the NMT enzyme.

Figure 17 *Berberis stolonifera* cell suspension culture in LS medium

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

[N-C^3H_3]-1,2-Dehydroreticuline used as the substrate of the enzyme 1,2-dehydroreticuline reductase was synthesized from (S)-norreticuline in two steps as followed:



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

Step 1 (S)-norreticuline(1mg) was first converted to 1,2-dehydronorreticuline by STOX enzyme. The reaction mixture contained: 150 mM borate buffer pH 8.9, 10 mM ascorbate and 500 µl of the partial purified STOX enzyme(from 4.1). The total volume of the reaction mixture was 800 µl. Fifty tubes of the reaction mixture were prepared and incubated at 30°C. Time course of the substrate-product conversion was monitored by TLC-Scanner Model CS-930. After 15 hr, the reaction tubes were frozen in a refrigerator and dried by a lyophyllizer. The dried-reaction tubes were extracted with methanol and concentrated by a rotary evaporator. The reaction product 1,2-dehydronorreticuline was then purified by preparative TLC(dichloromethane: methanol: ammonium hydroxide, 90:9:1, Rf 0.52). The intense yellow band of 1,2-dehydronorreticuline was excised and eluted with methanol. The eluate was evaporated to obtain the dried, yellow product.

Step 2 The purified 1,2-dehydronorreticuline was then used for the synthesis of [N-C^3H_3]-1,2-dehydroreticuline by using the NMT enzyme. In this reaction, S-adenosyl-L-[C^3H_3]-methionine ([C^3H_3]-SAM) was used as a methyl group donor to 1,2-dehydronorreticuline(see the reaction above). The reaction was catalysed by using *Berberis* NMT enzyme. The enzymic reaction contained, in a total volume of 1 ml, 200mM tricine buffer pH 7.5, 10 mM sodium ascorbate, [C^3H_3]-SAM(0.7 µCi) and 800 µl of NMT enzyme (from 4.1).To start the reaction, 1,2-dehydronorreticuline (from step1) dissolved in water with gentle heat was added to the mixture. The reaction mixture was then incubated for 24 hr at 30°C. The product [N-C^3H_3]-1,2-dehydroreticuline was purified from the mixture by preparative TLC (acetone: water: diethylamine, 8:1:1; Rf 0.70). Before purification, potassium chloride was added to the

reaction in a final concentration of 0.2 M. The rate of [N-C^3H_3]-1,2-dehydroreticuline forming was monitored by Automatic TLC-Linear Analyzer. The radioactive band of product exhibiting high count of radiolabelled of 1,2-dehydroreticuline was excised and eluted with methanol. The eluate was then evaporated to obtain the dried product and dissolved in 50 mM borate buffer pH 8.9 supplement with KCl, to the final concentration of 0.1 M. Specific activity of [N-C^3H_3]-1,2-dehydroreticuline was similared value to that of S-adenosyl-L-methionine (194 µCi/µmol).

5. Determination of 1,2-Dehydroreticuline Reductase Activity

The enzyme activity of 1,2-dehydroreticuline reductase was measured in the protein fractions which were eluted from various chromatographic columns. The specific activity of the enzyme was expressed in the unit of pkat per mg of protein. The enzyme assay was modified from the method of De-Eknamkul and Zenk (1992). The reaction mixture (final volume 200 µl) contained: 200 mM tricine-NaOH buffer pH 7.5, 0.67 mM NADPH, 0.1 µM[N-C^3H_3]-1,2-dehydroreticuline (8,000 dpm, 194 µCi/ µmol)(from 4.2) and protein fraction. The reaction mixture was incubated at 30°C in Multi-Block Heater, Lab-Line for 1 hr. A blank was also run concurrently in which the cofactor (NADPH) was omitted. The reaction was terminated by the addition of 1 ml of toluene. Tubes were vortexed for 30 seconds to extract radioproduct ([N-C^3H_3]-R-reticuline) into organic layer. Phase separation was achieved by centrifugation in Fisher Microcentrifuge Model 235C. The toluene layer (600 µl) was pipetted into Eppendorf tube and scintillation fluid (600µl) was added into the tube. The mixture was mixed well and measured in Wallac 1409 Liquid Scintillation Counter. The activity of 1,2-dehydroreticuline reductase was expressed as pmole of [N-C^3H_3]-reticuline per 1 second.

The activity of 1,2-dehydroreticuline reductase was determined by the following formula .

$$\text{Total count (cpm/assay) (test-blank)} \times \frac{1}{\text{sp.act. of [N-}C^3H_3\text{]-R-reticuline (cpm/µmol)}} \times \frac{1}{\text{incubation time (sec)}}$$

And the specfic activity was determined by the formula:

$$\text{Total count (test-blank) (cpm/assay)} \times \frac{1}{\text{sp.act. of [N-C}^3\text{H}_3\text{]-R-reticuline (cpm/}\mu\text{mol)}} \times \frac{1}{\text{incubation time (sec)}} \times \frac{1}{\text{total protein (mg)}}$$

6. Protein Determination

During chromatographic separation, The protein profile was monitored by UV detector which was set up at 280 nm. For each step of protein purification, the total protein of active enzyme fraction was determined by the method of Bradford (1976). Bovine serum albumin was used as protein standard. The linear range of microtiter plate assay was from 5.0 µg/ml to 30.0 µg/ml. The sample solution (160 µl) was pipetted into separated 96-well microtiter plate. Then, dye reagent concentrate (Bio-Rad protein assay)(40 µl) was added to the sample in each well. The solution was mixed thoroughly using a micropipet, and incubated at room temperature for 5 min. The absorbance was measured rapidly at 595 nm using Bio-Rad Model 450 Microtiter Plate Reader.

7. Preparation of Crude Enzyme Extract from *P. somniferum* Seedlings

All the procedures were carried out at 4°C to minimize proteolysis. Frozen five-day-old seedlings(1 kg) of *P. somniferum* were ground to fine powder in a precooled mortar. The frozen powder was thawed and stirred in 2 litres of extraction buffer (100 mM tricine-NaOH buffer pH 7.5 containing 250 mM sucrose, 1 mM EDTA, 10 mM β-mercaptoethanol) for 20 min. Then, the mixture was filtered through four layers of cheesecloth. Brei cell passing through was not filtered by the cheesecloth was separated by centrifugation at 8,000 rpm, for 20 min in Hitachi High-Speed Refrigerated Centrifuge Model CR20B3. The supernatant was used as a crude enzyme extract.

8. Preparation of 55-85% Saturated Ammonium Sulfate Fractionation

Solution of the crude enzyme extract (from 7) was fractionated with 55-85% saturation of $(NH_4)_2SO_4$. By this method, the proteins were cut off to desire protein region. Finely ground solid $(NH_4)_2SO_4$(55%saturation) was added slowly into the stirred solution and equilibrated protein precipitation for 30 min,at 4°C. The precipitate

was separated by centrifugation at 8,000 rpm for 20 min. The 55%$(NH_4)_2SO_4$ protein precipitation was discarded. Then, finely ground solid $(NH_4)_2SO_4$ (made up to 85% saturation) was added to the supernatant and stirred for 30 min. The precipitate was separated by centrifugation at 8,000 rpm for 40 min. In the latter step, pellet of the centrifugated between 55-85% saturation of $(NH_4)_2SO_4$ fractionation was collected, so supernatant was discarded. The pellet was dissolved in a minimal volume of extraction buffer supplemented with $(NH_4)_2SO_4$ to 0.2 M. So it was 55-85% saturation of $(NH_4)_2SO_4$ protein fractionation, was subjected to further purification.

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

The 55-85% saturation of $(NH_4)_2SO_4$ crude enzyme extract (from 8) was mixed with Phenyl-Sepharose CL-4B preequilibrated with the extraction buffer containing $(NH_4)_2SO_4$ to 0.2 M. The mixture was swirled for 20 min by a shaker. The slurry was packed into glass column (2.5x120 cm). The column was washed with the same buffer (maximum flow rate, 3 ml/min) to remove unbound substances. After the absorbance at 280 nm went down to the baseline, the column was eluted with extraction buffer(without $(NH_4)_2SO_4$). Fractions of 10.0 ml were collected. Every 2 fractions were analyzed for the enzyme activity(as described in section 5). Fractions showing activity of 1,2-dehydroreticuline reductase were pooled and the pooled fraction was applied onto DEAE-Sephacel column (2.5x10 cm), preequilibrated in extraction buffer. A flow rate of 2 ml/min was maintained. The column was first washed with 200 ml of the extraction buffer and then eluted with extraction buffer containing 0.2 M $(NH_4)_2SO_4$. Fractions of 5.0 ml were collected and assayed for 1,2-dehydroreticuline reductase activity, as well as for protein content at absorbance of 280 nm (A_{280}). Fractions possessed the enzyme activity were pooled. The pooled fraction from DEAE-Sephacel column was dialysed overnight against 2x2000 ml of 100 mM tricine-NaOH buffer pH 7.5 contained 1 mM EDTA, 10 mM β-mercaptoethanol. The dialyzed sample was further purified by chromatography on a column of FPLC MonoQ HR5/5 (0.5x5 cm), preequilibrated with the same buffer (in dialysis step) at a constant flow rate of 1 ml/min. The dialysed sample was applied onto FPLC MonoQ column. The column was washed with the same buffer, and followed by a linear gradient of 0-350 mM KCl in the same buffer. The increase ionic strength of the buffer caused the elution proteins out of the column and each fraction was collected for 1.0 ml per fraction. The 1,2-dehydroreticuline reductase activity and A_{280} were determined. Fractions contained the enzyme activity were pooled and further purified on FPLC-Superose12 HR16/50

column (1.6x50 cm). The pooled fraction was then applied onto the column by superloop, which injected the sample of 1.0 ml per injection. Flow rate of 0.5 ml/min was maintained. The column was preequilibrated and eluted with 10 mM phosphate buffer pH 7.5. The fractions of 1.0 ml was collected and assayed for 1,2-dehydroreticuline reductase activity. Those fractions with the enzyme activity were pooled and once again loaded onto the column of FPLC-MonoQ HR5/5 again which preequilibrated with the same buffer as in the gel filtration step. After washing with 10 ml of the buffer at a constant flow rate of 1 ml/min, the adsorbed enzyme was eluted with linear gradient of 50-150 mM KCl in 10 mM phosphate buffer pH 7.5 within 50 min. Fractions of 1.0 ml were collected and analysed for the enzyme activity. The fractions contained the enzyme activity and were pooled and stored at -20°C until use.

10. Molecular Weight Determination

10.1 Determination of molecular weight of 1,2-dehydroreticuline reductase using denaturing gel electrophoresis (SDS-PAGE)

Denaturing gel electrophoresis was performed according to the method of Laemmli (Laemmli, 1970). This experiment used the Bio-Rad Mini Protean II Apparatus, casting discontinuous polyacrylamide gel. Mini gel(70x80x0.75 mm) was prepared as a slab gel. The separating gel(50x80x0.75 mm) contained 12% polyacrylamide(prepared from a stock containing 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 1% of 10%w/v sodium dodecyl sulfate (SDS). The gel was polymerized chemically by addition of 0.5% of 10% w/v ammonium persulfate(APS) and 0.05% N,N,N',N'-tetramethylethylenediamine (TEMED),polymerizing agent(Table 7, Appendix II). The mixture was mixed well by swirling and poured into the assembled gel sandwich, using a pipet. Then, the top of separating gel was gently overlayed with a small volume of distilled water to keep the gel surface flat. The gel was allowed to polymerize for 45 min to 1 hr, and rinsed off with distilled water. Then, the stacking gel (20x80x0.75 mm) was prepared. The stacking gel contained 4% polyacrylamide (prepared from same stock as mentioned in Table 7) in 0.125 M tris-HCl pH 6.8, 1.0% of 10%w/v SDS polymerized by addition of 0.5% of 10%w/v APS and 0.1% TEMED. Comb was placed in the gel sandwich. Mixture of monomer solutions was poured to the upper part of separating gel, until all the teeth had been covered by solution. The stacking gel was allowed to polymerize for at least 30 min, and rinsed the well with distilled water. The

comb was removed. Then, ready gel was attached to electrode assembly and inserted into electrode tank. Sample was diluted with sample buffer 1:4 in ratio, and heated for 5 min at 95°C. Samples were pipetted into each of well, and run approximately 1.5 hr at 25 mA constant current. Gel was removed from the electrophoresis cell and stained. The procedure of staining was described in Appendix II. The gel was calibrated 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). A standard curve was plotted in the semilog scale of known molecular weight versus relative mobility(Rf), calculated as followed:

$$Rf = \frac{\text{distance of the protein migration}}{\text{distance of tracking dye migration}}$$

The molecular weight of interested protein could be extrapolated from this curve, based on its Rf value.

Table 7 Gel composition

Components	Separating gel 0.375M Tris, pH 8.8 (12%AA)	Stacking gel 0.125M Tris,pH 6.8 (4%AA)
Distilled water	3.35 ml	6.1 ml
1.5M Tris-HCl,pH 8.8	2.5 ml	-
0.5M Tris-HCl,pH 6.8	-	2.5 ml
10%w/v SDS stock	100 µl	100 µl
Acrylamide/Bis (30%stock)[a]	4.0 ml	1.3 ml
10% Ammonium persulfate[b]	50 µl	50 µl
TEMED	5 µl	10 µl
Total monomer	10 ml	10 ml

a.Degas at least 15 minutes at room temperature

b.Freshly prepared daily

The stained gel was preserved by drying according to the following procedure. Took two sheets of cellophane, and were wet in water. One sheet was laid onto a glass plate, and stained gel was placed on it. The bubbles were completely smooth out. Then, the second sheet was laid over the gel. A frame was made around

the edge using thin strips of glasses and held in place with fold-back paper clips. This frame prevented the cellophane from moving while being at room temperature for 24-48 hr. The frame was then removed, and cellophane was trimmed from edges The dried gel should be stored in a book to prevent curling.

10.2 Determination of molecular weight of 1,2-dehydroreticuline reductase using gel filtration

To determine the native molecular weight of 1,2-dehydroreticuline reductase, 55-85% ammonium sulfate precipitation was prepared as described in section 8. Precipitated protein was redissolved in 10 mM phosphate buffer pH 7.5. The sample of 500 µl was injected to FPLC-Superose12 HR16/50(1.6x50 cm) preequilibrated with 10 mM phosphate buffer pH 7.5 and eluted with the same buffer at a constant flow rate of 0.5 ml/min. Fractions of 1.0 ml were collected and protein content was monitored by measuring the absorbance at 280 nm. And 1,2-dehydroreticuline reductase activity was also determined as described in section 5.

For protein determination, the column was calibrated using the following standard protein for gel filtration standard protein: thyroglobulin (670 kD), gamma globulin (158 kD), ovalbumin (44 kD), myoglobulin (17 kD), vitamin B-12 (1.35 kD). Blue dextran was used to determine the position of the void volume (V_o). The relative molecular weight of the enzyme was determined from a calibration curve between molecular weight and V_o/V_e (V_e=elution volume) of standard protein plotted on semilogarithmic axis.

11. Amino Acid Sequence Determination

11.1 Protein blotting

The SDS-PAGE of purified protein was prepared as described in section 10.1. The protein was transferred to polyvinylidene difluoride (PVDF) membrane (Immobilon-P) by electroblotting. The Immobilon-P membrane was wet with methanol for a few seconds and equilibrated it in blotting buffer. The SDS-PAGE of purified protein was soaked in blotting buffer for 20 min. Then, the transblotting sandwich was assembled and electroblotted at 25 mA current constant, room temperature for 2 hr. After electroblotting, the Immobilon-P membrane was dried on a clean Whatman paper

No. 3MM and excised the desired band with a clean razor blade. The excised membrane was kept in clean Eppendorf tube at 4°C until sequencing.

11.2 Determination of amino acid sequence

Before protein sequencing, the 1,2-dehydroreticuline reductase was digested into smaller peptides using the enzyme, TPCK-trypsin. The peptide mixture was separated by reversed-phase HPLC [columns: Baker Bakerbond RP8 and RP4 (4.1x250 mm): solvent system, A. 0.1% TFA, B. 0.1% TFA, 60% acetonitrile: gradient 1%/min; flow rate 1 ml/min]. The amino acid sequence of the peptide was determined the amino acid sequencing by Applied Biosystems Model 470A Gas Phase Sequencer.