CHAPTER V

DISCUSSION

Although the enzyme 1,2-dehydroreticuline reductase from *Papaver somniferum* has been characterized (De-Eknamkul and Zenk, 1992), nothing is known about its basic primary structure of amino acid. Therefore, in this research work, we aimed to obtain basic information on the amino acid sequence of this enzyme. The information is very important since it will allow us to design and synthesize oligonucleotides which will be used for gene cloning and studying the expression of the gene. However to be able to study the amino acid sequence, it is necessary to have large amounts (microgram level) of 1,2-dehydroreticuline reductase as starting material. This study, therefore, involved a large scale purification of the reductase from *P. somniferum* seedlings, preparation of blotted membrane of the purified enzyme for protein sequencing and amino acid sequence determination.

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

Since the radioactively labelled substrate 1,2-dehydroreticuline is not commercially available, it was necessary to prepare it in the laboratory. Unlike previous methods which are time-consuming (Amann and Zenk, 1987; De-Eknamkul and Zenk, 1992), we have modified the method to obtain the radiolabelled [N-C^3H_3]-1,2-dehydroreticuline with higher specific activities or yield. As described in the Experimantal, the tritium labelled 1,2-dehydroreticuline was synthesized from (S)-norreticuline using (S)-tetrahydroprotoberberine oxidase (STOX) as biocatalyst. It was clear that the complete conversion of the substrate to the product occurred within 15 hr. No side reaction or degradation product was detected (Fig. 19). It is essential to have 10 mM ascorbic acid in the incubation mixture since it effectively protected the photooxidation of both substrate and product. Partial characterization of the product showed the UV absorption bands at λ_{max} 280 and 330 nm (Fig. 21) suggesting that there is a characteristic dehydrochromophore in the molecule which is similar to that reported previously (Battersby *et al.*, 1965b). Furthermore, the APCI spectrum (Fig. 22) of the product exhibited $[M+H]^+$ at m/z 314 corresponding to the molecular formula of $C_{18}H_{19}NO_4$. Therefore, it was clear that the the reaction product was 1,2-

dehydronorreticuline. However, the APCI spectrum also exhibited another major peak at m/z 328, which might be due to an unspecific methylation of 1,2-dehydronorreticuline caused by solvent effect. Nevertheless, this step gave relatively high yield (45%) of which was sufficient for performing the second reaction step.

In the second step, the resulted 1,2-dehydronorreticuline was methylated using [C^3H_3]-SAM as cosubstrate and N-methyltransferase (NMT) as the catalysing enzyme. The NMT enzyme could be isolated from *Berberis* cell culture. However, the crude enzyme extract contained many enzymes eg. berberis bridge enzyme (BBE), STOX, berberine synthase etc. which could cause the side reactions leading to berberine synthesis (Amann *et al.*, 1988). To solve this problem, it was necessary to perform some partial purification of the enzyme before being used for catalysing the reaction of the second step of the reaction (see Experimantal). After the reaction mixture was incubated for 24 hr, it was found by radioscanning that more than 50% of [C^3H_3]-SAM was utilized and 2 peaks of radiolabelled products occurred (Fig. 23). According to preliminary study, the band with higher Rf value was [N-C^3H_3]-1,2-dehydroreticuline which we obtained [N-C^3H_3]-1,2-dehydroreticuline in 16% yield. As expected, this modified method of 1,2-dehydroreticuline synthesis is more effective than the method previously described (Amann and Zenk, 1987) which used the dehydrogenation of [N-$^3CH_3$]-(S)-reticuline using STOX as biocatalyst. Since with the rate of oxidation of (S)-reticuline by STOX is approximately only 1% of that of (S)-norreticuline (Amann *et al.*, 1988), it is obvious that our modified method is more suitable for [N-C^3H_3]-1,2-dehydroreticuline synthesis.

2. Enzyme Detection in *Papaver somniferum* Seedlings

The enzyme 1,2-dehydroreticuline reductase has been found to occur specifically in both *P. somniferum* and, in lesser amount in *P. bracteatum* (De-Eknamkul and Zenk, 1992). These two species are known to contain morphinan alkaloids. It is, therefore, quite certain that this enzyme is involved in the biosynthesis of the morphinan alkaloids. In the past, many attempts have been made to establish tissue and cell cultures of *P. somniferum* containing morphinan alkaloids (Nessler, 1990). However, most of the cultures, either callus or suspension, produced very low amounts of the alkaloids or not at all (see Historical). In these cultures, no 1,2-dehydroreticuline reductase activity was detected (De-Eknamkul and Zenk, 1992).

Therefore, the occurrence of 1,2-dehydroreticuline reductase is highly related to the accumulation of morphinan alkaloids in higher plants.

The formation of opium alkaloids during the germination of *P. somniferum* has been studied (Wieczorek *et al.*, 1986; William and Ellis, 1989). They have shown that reticuline and thebaine are formed at the fifth day after germination whereas codeine and morphine are found in trace at sixth day. The activity of enzyme,1,2-dehydroreticuline reductase was detected at day 2 after germination. Time-course study of the enzyme activity in the *P. somniferum* seedlings showed that the measurable onset of enzyme activity was at day 2, then increased rapidly to reach a maximum value at day 4. Thereafter, the activity declined at day 10 (De-Eknamkul and Zenk, 1992). These results indicated that the biosynthetic pathway from (S)-reticuline to morphine was operated during day 2 and day 10 of the seed germination. Therefore, to obtain suitable amounts of the reductase for large purification, the four- to seven-day-old could be used as enzyme source.

For our experiment, we mostly chose the five-day-old seedlings (Fig. 24) as starting material. The harvested seedlings were used for the preparation of crude enzyme extract to monitor the reductase activity. The results showed that the reductase activity was approximately 1.65×10^{-3} pkatg^{-1} fresh weight.

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

The enzyme 1,2-dehydroreticuline reductase was first discovered and characterized from the seedlings of *P. somniferum* (De-Eknamkul and Zenk, 1992). It is highly species- and substrate- as well as stereospecific. The enzyme catalyses the provision of (R)-reticuline for the formation of morphinan alkaloids also possessing the (R)-configuration at the corresponding chiral center. The function of enzyme is to transfer the pro-S-hydride from NADPH (the cofactor) to C-1 of the 1,2-dehydroreticulinium ion to form (R)-reticuline. The enzyme has an apparent molecular weight of 30,000, a pH optimum at 8.5 and a optimum temperature at 30^{o}C. Its activity is strictly dependent on NADPH (De-Eknamkul and Zenk, 1990; 1992). Presently, at least 14 enzymes of the morphinan pathway have been discovered. Among these enzymes, 1,2-dehydroreticuline reductase is considered most important since it switches on (S)-reticuline, the central intermediate to flow through the morphinan pathway.

For biotechnological point of view, it is interesting to understand the control at the genetic level of the flux of (S)-reticuline to the morphinan pathway. To be able to get to the genetic level, it is necessary to have the purified enzyme in a quantity that is sufficient for doing protein sequencing. Once the amino acid sequence of the enzyme is known, it is possible to synthesize the oligonucleotide for doing DNA cloning. However large scale enzyme purification is more complicated than the small scale. There are number of problems of scaling up of the purification process such as difficulty in handling large volume, aggregation and denaturation of high protein concentration, selection of chromatographic method in protein purification etc.. In these research work, we modified the previous method of De-Eknamkul and Zenk (1992) for scaling up the purification of 1,2-dehydroreticuline reductase from *P. somniferum* seedlings. As a summerized in Table 8, this modified method gave the final enzyme preparation with 1433-fold purification and with 0.002% of total recovery of enzyme activity. The purified enzyme had the specific activity of 1.4 pkat mg^{-1} protein. The low percentage recovery of the enzyme was due to the loss of the enzyme activity during purification. To obtain sufficient enzyme, we purified the reductase enzyme more one time starting with 1 kg of fresh seedlings. The efficiency of the enzyme purification in each step was evaluated by SDS-polyacrylamide gel electrophoresis (Fig. 32). The results showed that Phenyl Sepharose CL-4B and DEAE-Sephacel could get rid of some other proteins. The most effective step of the purification appeared to be MonoQ HR5/5 anion exchange chromatography (Fig. 27). For Superose12 HR16/50 gel filtration, it could get rid of higher and lower molecululer weight proteins. In the last step. the second MonoQ HR5/5 could separate the remaining protein impurities and yield a homogeneous enzyme. The molecular weight of the reductase was determined by both SDS-polyacrylamide gel and gel filtration (Fig. 34, Fig. 37). The results indicate that 1,2-dehydroreticuline reductase has molecular weight of 34 kD which is a monomeric protein as reported previously (De-Eknamkul and Zenk, 1992).

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

The synthesis of compounds in plants is regulated at the genetic level. Each step of biosynthesis has a gene encoding the enzyme essential in catalysing each transformation step. Presently, the first alkaloid-forming enzyme encodes cDNA to be expressed in an enzymatically active form was strictosidine synthase, a key enzyme of the indole alkaloid biosynthesis (Kutchan *et al.*, 1987). An advantage of cloning the genes for the enzymes of alkaloid biosynthesis lies not only in providing a system with

which to study the regulation of the biosynthetic pathway, but also in high level heterologous expression of these enzymes to produce enough active enzyme for mechanistic studies.

In this study, we have reported the partial amino acid sequence of the enzyme 1,2-dehydroreticuline reductase. We used the specific enzyme, trypsin which cleaved the protein specifically. Of the ten 1,2-dehydroreticuline reductase tryptic peptides were purified by HPLC, one peptide was sequenced. It contained 21 amino acids. From our study, we have a partial information which is able to further studies in molecular genetics.

CONCLUSION

1,2-Dehydroreticuline reductase is the enzyme which hydrogenates 1,2-dehydroreticuline stereospecifically to (R)-reticuline. It was purified in large scale to give a sufficient amounts for amino acid sequencing. Large scale purification of the enzyme required 55-85% ammonium sulfate precipitation and five chromatographic steps including Phenyl Sepharose CL-4B, DEAE Sephacel, the first MonoQ HR5/5, Superose12 HR16/50 and the second MonoQ HR5/5, respectively. We obtained 1,2-dehydroreticuline reductase in 0.002% yield with 1433-fold purification. The apparent molecular weight was 34 kD.

In the synthesis of [N-C^3H_3]-1,2-dehydroreticuline, we was accomplished by two enzymatic reactions. The first reaction was the dehydrogenation of (S)-norreticuline to 1,2-dehydronorreticuline by STOX enzyme and the second was methylation with radioactive labelled SAM to [N-C^3H_3]-1,2-dehydroreticuline using NMT enzyme of the resulting 1,2-dehydroreticuline. The overall yield of this method was 16%.of the resulting.

The availability of the homogeneous 1,2-dehydroreticuline reductase from *Papaver somniferum* L. seedlings should facilitate basic studies on its structure. The partial amino acid of 1,2-dehydroreticuline reductase has been sequenced. It 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-.

The study of enzymes involved in alkaloid metabolism opens the new field in the study of alkaloid biosynthesis and its application to biotechnology. The basic information on, amino acid sequence of 1,2-dehydroreticuline reductase is first reported in this study. It will hopefully lead to further study their oligonucleotides, cDNA clone and expression of the protein in microorganism. Ultimately, the biotechnological production of this enzyme in microorganism will potentially leading to unrestricted quantities of the enzyme and provide to begin the regulatory mechanism of alkaloid metabolism in poppy plant or plant cell culture.