CHAPTER I

INTRODUCTION

"Secondary metabolites" are substances which are derived from primary metabolites. Many biosynthetic pathways of secondary metabolites lead to diverse groups of complex chemical structures (Luckner, 1990). For a long time, these compounds were regarded as waste products which had interesting structures and, in many cases, exploitable biological properties. Presently, however, most secondary metabolites are believed to be important for the overall survival fitness of the producing plants in the environment. Recent studies in the area of biochemical ecology have suggested that these compounds are involved in the defense mechanism of plants against herbivores (insects, molluscs, vertebrates) and microorganisms(viruses, bacteria, fungi)(Levin, 1976; Swain, 1977; Rosenthal and Janzen, 1979; Harborne, 1982; Wink, 1988). Secondary metabolites are also very important for mankind since they have long been used by men for medicines, flavoring, poisons, and model compounds for pharmacological studies. It is these compounds which have fascinated natural product chemists, pharmacognosists, biochemists for their structures, functions and their commercial application.

Since 1960s, a picture of the biosynthesis of secondary metabolites began to emerge, largely speculative, based on isotopically labelled precursor feeding experiments to differentiated plant (Spenser, 1968). Today, many researchers are concentrating on the detection of intermediates and on the elucidation of mechanistic detail of biosynthetic pathways and regulatory aspects of secondary metabolism (Zenk *et al.*, 1985; Rees, 1991; Zenk, 1991). The most basic part is the sequence of various steps leading from primary metabolites to the final products. However, the studies using feeding experiments or tracer works only suggest probable biosynthetic sequence. Powerful support for any biosynthetic pathway as well as detailed information on the reactions involved has to be gained by isolation, purification, and characterization of the involved enzymes which catalyse individual steps of the biosynthesis (Herbert, 1981). In addition, more information has to be sought and gained on the compartmentation of enzymes involved in the biosynthetic chain leading to secondary plant products and on the mechanism by which these products and their

precursors are transported and stored (Zenk *et al.*, 1985). This is the way to solve the question of how these metabolites are really formed. In addition, these studies will lead not only to understand the control of metabolism, but also to discover the precise role of the individual enzymes, and to integrate the study of metabolism with that of the physiology and ecology of the whole plant (Rees, 1991). For the aspect of biotechnological potential, the key enzymes should be analyzed at the molecular genetic level in order to clarify the complex regulation of the biosynthesis of compounds in the plant. The expression of plant enzymes (which are often present in minute quantities in plants) in heterologous systems such as bacteria, yeast, or other plants would allow for detailed biochemical analysis of mechanisms of reaction. Likewise, heterologous expression would exploit the biotechnological potential of these enzymes for improved productivity of the desired compounds in intact plant or cell cultures and production of known and new compounds for use by mankind (Heinstein, 1985; Zenk *et al.*, 1985; Kutchan *et al.*,1991; Robins *et al.*, 1991; Zenk, 1991; Verpoorte *et al.*, 1993).

Among various secondary pathways in higher plants, the biosynthesis of morphinan alkaloids is of particular interest. Morphinans have become a great success in pharmaceutical applications. Morphine itself is still the most frequently used as analgesic in surgery, and codeine is widely used as an antitussive drug in different medicines (Szantay, *et al.*,1982; Lindner, 1985). Morphine and codeine are found only in *P.somniferum* and closely related species. Although the alkaloids of this plant are pharmaceutically valuable, *P.somniferum* can only be cultivated in some countries under restrictive control of the governments of these producing countries and supervision of the International Narcotic Control Board of the United Nation. This is because morphine can be converted to its diacetyl-derivative, heroin, which causes of many legitimate crimes and destroys of mankind. At the present, the alternative method of opium alkaloid production by means of plant cell cultures has been extensively studied. So far, however, no success has been achieved. Most studies have shown very low level of morphine and codeine production in the various types of the cultures: callus (Hsu and Pack, 1989), suspension (Khanna and Khanna, 1976; Heinstein, 1985) and shoot (Kamo *et al.*, 1982). Some production of these alkaloids occurred has been observed only the cases of cytodifferentiation leading to laticifer types of cell (Kamo *et al.*, 1982; Schuchmann and Wellman, 1983; Yoshikawa and Furuya, 1985; Siah and Doran, 1991; Yoshimatsu and Shimomura, 1992). For these reasons, the study of morphine biosynthesis is still very interesting, particularly the study on how to regulate the biosynthesis of these compounds by using biotechnology.

It has been proposed that the enzymes catalyzing the biosynthetic pathway from L-tyrosine to morphine consist of 16 enzymes, 14 of which have been discovered already (see Historical, section 7). Among these the most important enzyme is the one which reacts on (S)-reticuline, a central intermediate of benzylisoquinoline alkaloid, to reach the compound to the morphinan pathway leading to morphinan pathway. Since morphinan alkaloids are believed to be derived from (R)-reticuline, the compound with the same absolute stereochemistry as the configuration of morphine (Barton *et al.*, 1967). the inversion of configuration of (S)-reticuline to (R)-reticuline was postulated in which the compound 1,2-dehydroreticulinium ion was assumed to be an intermediate (Battersby *et al.* a 1965; Loeffler *et al.*, 1990). Support on this proposal was found when synthetic material characterized as 1,2 dehydroreticulinium chloride was efficiently incorporated into morphinan alkaloids (Battersby *et al.*, 1965b). Moreover, Borkowski *et al.*, (1978) demonstrated that 1,2 dehydroreticuline is a naturally occurring compound detecting in *P. somniferum* (Borkowski *et al.*, 1978). If the proposed biosynthetic pathway for the inversion of configuration of (S)-reticuline to (R)-reticuline via 1,2-dehydroreticuline is true, an enzyme has to be present which stereospecifically hydrogenates 1,2 dehydroreticuline to (R)-reticuline. Today, this hypothesis is clarified. The reaction is catalyzed by an oxidase enzyme which dehydrogenates (S)-reticuline to 1,2 dehydroreticuline (Amann *et al.*, 1988), and a reductase enzyme which stereospecifically hydrogenates 1,2 dehydroreticuline to (R)-reticuline, respectively (Zenk, 1985; De-Eknamkul and Zenk, 1992).

Since the 1,2-dehydroreticuline reductase enzyme has not yet been studied at the molecular level, we aimed to study indepth to the amino acid sequence of this enzyme. In this study, we choose *P. somniferum* seedlings as the enzyme source since they contained high activity of the enzyme (De-Eknamkul and Zenk, 1992). However, the amino acid sequencing needs high amount of the enzyme. We, therefore, carried out large scale purification of the reductase enzyme from *P. somniferum* seedlings with the main objective of determining its amino acid sequence.