CHAPTER I

INTRODUCTION

1.1 RESEARCH CONNECTION

Superoxide dismutase (SOD) and bioactive cyclotide were firstly reported from the root of *Stemona tuberosa* Lour. and the leaves of *Viola sumatrana* Miq., respectively. The two bioactive plant proteins were passed through the process of isolation, purification, characterization which described in three chapters III-V (Figure 1.1).

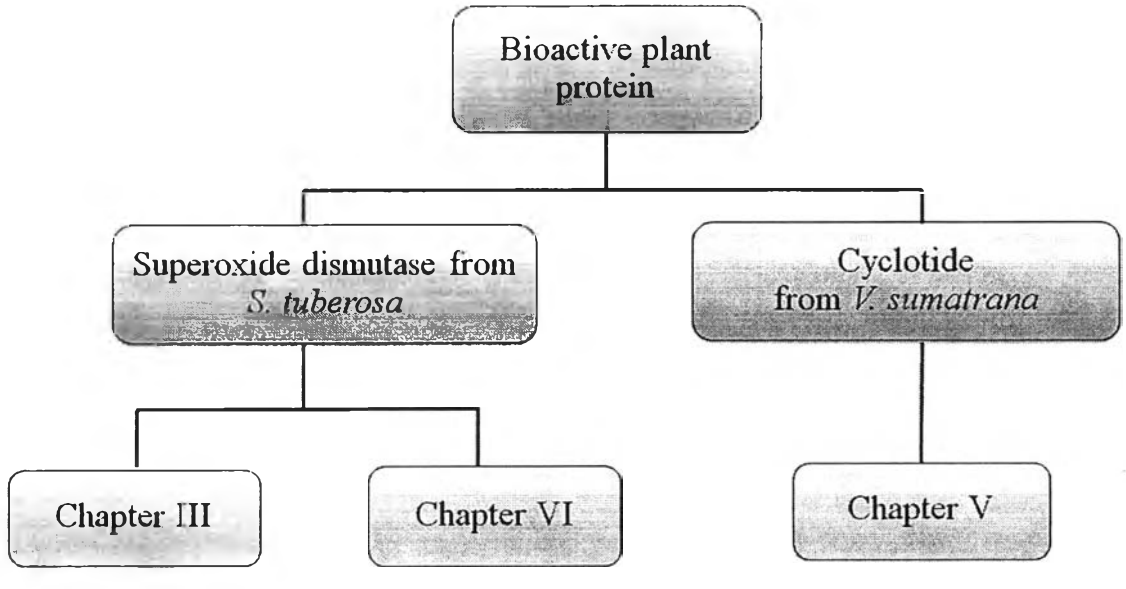

Figure 1. 1 The diagram shows the three parts (chapter III-V) of this study

Chapter III is about the developed two dimensional gel electrophoresis zymogram for detected different SOD isozymes using crude protein from *S.tuberosa* and SOD from bovine erythrocyte (a standard) to determine the efficiency of the developed method. While chapter IV is about evaluation of biochemical properties

of SOD from crude protein of *S. tuberosa*. The last is chapter VI, the chapter is about determination of amino acid sequencing and secondary structure of cyclotides from *V. sumatrana* before their activities on cell cytotoxicity were determined.

1.2 RESEARCH RATIONALE

Stemona tuberosa Lour., also called Non-Tai-Yak in Thai, is in Stemonaceae family. The botanical characteristic of *S. tuberosa*, trunk: vine, intertwined with other plants; long, round, slender, green stem, with cylindrical thumb-sized underground tubers aggregated in a cluster, leaf: single, alternate, heart-shaped, 4-6 centimeters in width, 6-10 centimeters in length, concaved base with pointy tip, smooth margins, waxy surface with indentations parallel to the veins, clearly visible parallel venation, long stem, flower: single flower, buds at leaf axils, 4 petals, outer petals with chartreuse color, deep red or white when bloomed and fruit: small capsule with pointy tip, approximately 1 centimeter in width and 2 centimeters in length, dry and open fruit. *S. tuberosa* has been found in Asia such as central China, Indochina, Taiwan, India and Thailand, tropical Australia and North America.[1, 2] The taxonomy and morphology of *S. tuberosa* is showing in figure 1.2.

Kingdom: Plantae

Order: Pandanales

Family: Stemonaceae

Genus: *Stemona*

Species: *Stemona tuberosa* Lour

***Stemola tuberosa* Lour.**

Figure 1. 2 The taxonomy of *S. tuberosa*

Up to now the root tuber of *S. tuberosa* has been used in Chinese, Japanese and Korean traditional medicines to treat respiratory disorders, e.g., bronchitis, pertussis and tuberculosis, and prevent human and cattle parasites, agricultural pests and insects[3]. At present, the publications have reported on the various biological activities from the organic compounds from root tuber of *S. tuberosa* such as antitussive[4, 5] and antibacterial activities.[6] Moreover, antifungal and anticancer activities against enteric helminthes and ectoparasites have also been reported[7]. Compounds from the root also affected on medullary and thyroid carcinoma cells[8]. Recently, the potential antioxidant agents such as dehydrotocopherols and phenolic compounds have been found from the root of *S. tuberosa*. The antioxidant agents from previously have been also found in other plants from *Stemona* genus such as *Stmona. curtisii*, *Stemona. collinsae*, *Stemona. burkillii*, *Stemona. cochinchinensis* and *Stemona. kerrii*[9] but there are no report on antioxidant protein from *S. tuberosa* so far.

Superoxide dismutase (SOD) is one of the important antioxidant enzymes which plays a role in catalyzing superoxide radical to hydrogen peroxide and oxygen molecule. Nowadays, many publications have been reported on the purification and characterization of plants SODs such as FeSOD purified from *Citrus limonum*. In addition there are two publications reported on CuZnSOD and MnSOD purified from *Radix lethospermi* seed and *Camellia sinensis*(L.) O. Kuntze respectively. Moreover, several researches have also reported on the purified SODs from plants such as garlic, wheat seedling, pea, water melon, pine, tobacco, rice and melon. There are various publications have been also reported on biological activities of SOD such antioxidant, anti-inflammatory, anti-aging activities[10, 11] and applied to use in medicine, cosmetic, chemical and food industries. In addition some different SOD isozyme in plants can be used as an indicator to evaluate the growth stage or stress/infection circumstances of that plant.[11] Interestingly none of publications have currently reported on superoxide dismutase discovery from *S.tuberosa* so far.

Viola sumatrana Miq., called as Hong-Ron in Thai, is normally found in South-East Asia such as Indonesia, Malaysia, Myanmar, Vietnam, and Thailand. The botanical characteristic of *V. sumatrana,* for instance, leaves are deep green or dark green, triangular-ovate or oblong-ovate, 2-5 × 1.5-3 cm, petioles are narrowly winged in upper part unequal in length, 2-9 cm, flowers violet to light purple or white, rhizome is 1-20 cm, 1.5-2.5 mm in diam, with brown remains of stipules at nodes and stolon is slender, glabrous, producing adventitious roots 15-20 cm, longest to 40 cm.

The taxonomy and morphology of *V. sumatrana* is represented in figure 1.3.

V. sumatarna is in Violaceae family (violets flowering plant family) and Viola genus. Interestingly, every *Viola* sp plants contain an important bioactive peptide named as cyclotide. Cyclotides are a cyclic peptide binding membrane of which on several biological activities such as uterotonic activity,[12] anti-HIV activity,[13, 14] haemolytic activity,[15, 16] antimicrobial activity,[17] antifouling activity,[18] neurotensin antagonism[19] and cytotoxic activity have been reported.[20-22] Recently, there are some studies on the membrane binding properties of cyclotide giving more understanding about their mode of action on those biological activities. From previous publications, it has been also reported on cyclotide discovery in various *Viola* sp such *Viola abyssinica*,[20] *Viola arvensis*,[22-24] *Viola biflora*,[25] *Viola hederacea*,[26] *Viola odorata*,[15, 27] *Viola tricolor*,[16] *Viola yedoensis*[28] and *Viola philippica*[20] but none of have been reported from *V. sumatrana* so far.

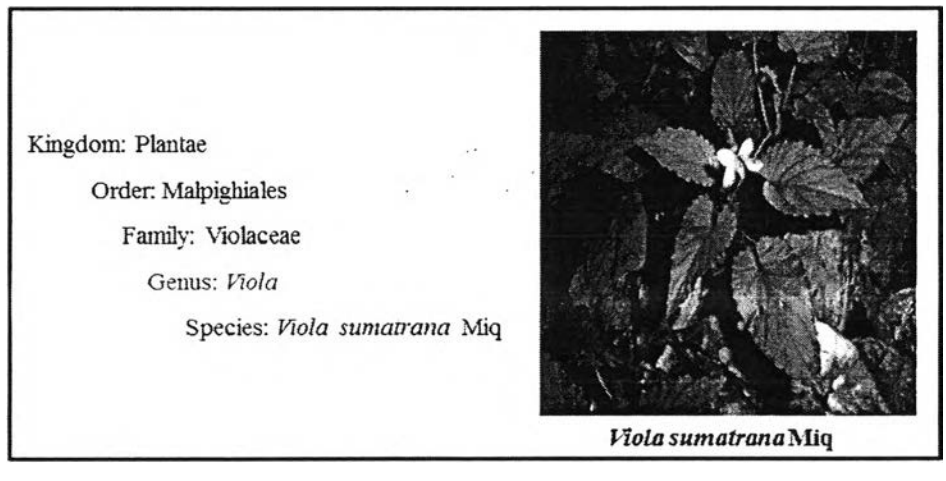

Figure 1. 3 The taxonomy of *V. sumatrana*

Consequently it is interesting to purify the two bioactive proteins from both plants and study on their biochemical characterizations because both bioactive

proteins from new plant sources could be applied using in agricultural and pharmaceutical applications in the future. In addition it's also interesting to study on the developed 2D-GE method coupled with SOD staining activity for different SOD isozymes separation which none of data have been reported. Furthermore, the developed 2D-GE method generally has more resolution for SOD separation than the classical method (1D-Native-PAGE couple with SOD staining activity). The developed method could be applied to be used in various areas related to SOD enzyme detection and develop for using in other enzymes detection on 2D-GE in the future as well.

1.3 OBJECTIVES

1. To characterize superoxide dismutase and cyclotide from *S. tuberosa* and *V. sumatrana* respectively.
2. To develop 2D-GE method coupled with SOD staining activity.

1.4 SCOPE OF DISSERTATION

Crude protein from *S. tuberosa* was obtained by using buffer extraction and ammonium sulfate precipitation. Crude protein was separated to be used for evaluation of the efficiency of the developed 2D-GE method coupled with SOD staining activity and for further purification and characterization. Crude protein was

then purified by using anion exchange chromatography and characterized biochemical properties by using various techniques such as SDS-PAGE, Native-PAGE coupled with SOD staining activity and mass spectrometry.

Crude protein from *V. sumatrana* was extracted by using 50% (v/v) acetonitrile in 1% formic acid then purified by using solid phase extraction (SPE) and HPLC, giving purified cyclotides. The purified cyclotides were subjected to determine amino acid sequences by using mass spectrometry. Cyclotide called kalata S was submitted to evaluate the secondary structure due to the absence of NMR study. Finally, all cyclotides were tested for cytotoxicity against different human cancer cell lines.

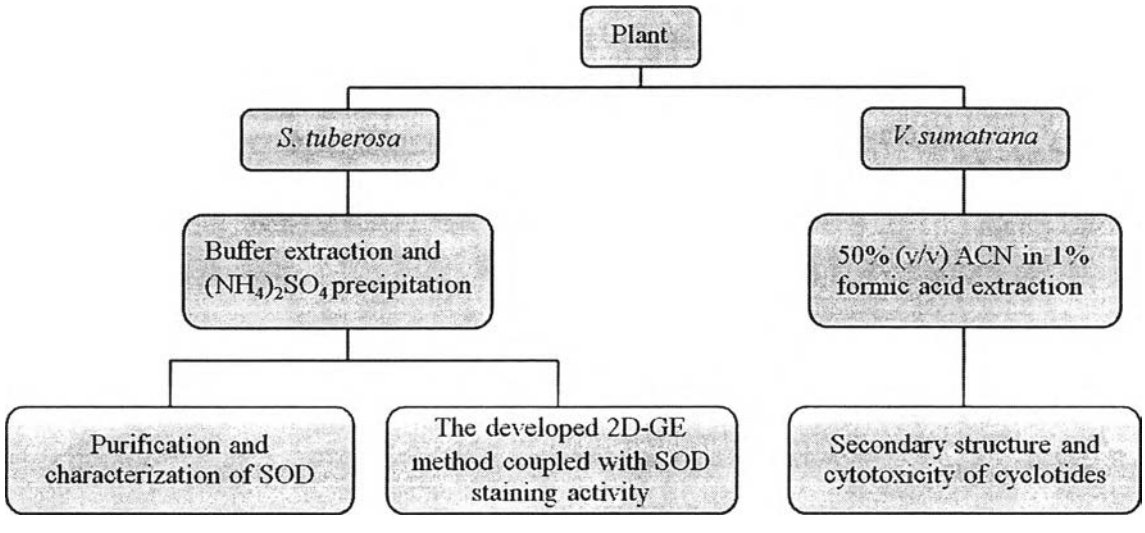

Figure 1. 4 Diagram of the scope of this study

1.5 EXPECTED RESULTS

The developed 2D-GE method coupled with SOD staining activity was expected to be obtained. Furthermore, the biochemical characterizations of purified SOD from *S. tuberosa* and cyclotides from *V. sumatrana* were expected to be obtained and the activity of cyclotide on cytotoxicity assay was also expected to be gained in this study.