## **RESEARCH ON CHARACTERISTICS AND**

# **UTILIZATION OF STORAGE PROTEIN**

IN Curcuma alismatifolia Gagnep.

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## **CHAPTER 1**

## Introduction

## **1. General Plant Background**

#### 1.1 Introduction of flower bulbs

Geophytes are plant species which have buds develop in underground and flower bulbs are geophytes which usually use for ornamental plants and has a commercial potential (Raunkiaer, 1934; Rees, 1989; Halevy, 1990; De Hertogh and Le Nard, 1993). Under adverse conditions, especially in a prolonged drought period, ornamental geophytes shed their leaves and live in the bulbs, storage organs of nutrients. When favorable conditions return, they produce leaves and flower rapidly, which gives them predominant position to other plants (Marinelli, 2004). Flower bulbs are divided into two groups, bulbous and tuberous. Most of bulbous plants belong to monocotyledonous plants, and are composed of true bulbs and corms. A bulb has a shortened stem called "the basal plate" which has one or more apical meristems and is enveloped by numerous fleshy scales. The scales are the primary storage tissue in true bulbs, i.e., Narcissus, Tulipa and Lilium. In contrast, a corm has an enlarged stem (basal plate) that has different nodes and internodes. The basal plate is surrounded by various dry scales (tunics) and is a primary storage organ, i.e., Gladiolous, Freesia and Crocus. The tuberous plant comprises tubers, tuberous roots, rhizomes and enlarged hypocotyls and mostly is dicots. A tuber consists of primary of enlarged stem tissue. It can have one or more apical buds and develops root primodia, i.e., Anemone and Gloriosa. A tuberous root is a specialized enlarged root tissue. It has crown containing one or more apical shoot meristems, i.e., Dalia and Ranunculus. A rhizome comprised primary of a specialized horizontal growing stem. Shoots and roots general emerge at right angles to the horizontal stem, i.e., Alstroemeria and Canna. Lastly, the primary storage tissues emerge from the hypocotyl, i.e., Begonia and Cyclamen (De Hertogh and Le Nard, 1993).

The perennial rhizomatous plants like a family Zingiberaceae which is one of the largest families of the plant kingdom. It is important natural resources that provide many

useful products for food, species, medicines, dyes, perfume and beauty to men. They are found in the tropical forests and are distributed mostly in tropical and subtropical areas. The greatest numerous of genera and species is in the Malaysian regions, i.e., Indonesia, Malaysia, Singapore, Brunei, the Philippines and Papua New Guinea. Although, more than 50 species of the genus *Curcuma* have been found to occur in Thailand, with only 26 of them being formally identified (Sirirugsa, 1998). However, Maknoi (2006) recently reported that *Curcuma* have been found 38 species in Thailand and distributed all areas of Thailand.

#### 1.2 Origin and distributions of Curcuma spp.

The largest genera in the Zingiberaceae family are *Curcuma* spp. which comprises about 80 species and it is generally distributed in Asia from India to South China, Southeast Asia, Papua New Guinea and Northern Australia. Larsen and Larsen (2006) reported that approximately 50 species have originated in Thailand and is commonly discovered at the altitude 500-900 masl (m above sea level). Nevertheless, Sirirugsa et al. (2007) investigated that about 38 species of this genus grows in wide range of altitudes from 100-1,300 masl such as C. alismatifolia Gagnep., C. rhabdota, C. longa L., C. parviflora, C. comosa, etc. However, Curcuma grows well in loose and sandy soils in shaded areas. C. alismatifolia is native to in the northeast Thailand and distributed to Laos and Cambodia. It is commonly found in open areas in the pine or deciduous forests at altitude 1,300 masl (Sirirugsa et al., 2007). Recently, Khumkratok et al. (2012) reported that five Curcuma species, e.g., C. angustifolia Roxb., C. alismatifolia Gagnep., C. gracillima Gagnep., C. parviflora Wall. and C. rhabdota., which were found in an altitudinal range between 290 masl and 831 masl in Northeastern Thailand. Four species (C. angustifolia, C. alismatifolia, C. gracillima and C. rhabdota.) were distributed in open gaps in dry dipterocarp forest while C. parviflora was found in the contact zone between dry dipterocarp and bamboo forest. Moreover, C. rhabdota. was found only in a habitat with high humidity and shading along the Thailand-Lao PDR border.

Due to the genus of *Curcuma* comprises many species with various sizes, flower colors and postproduction longevity that it was divided to two subgenera, Eucurcuma and Paracurcuma. Eucurcuma has remarkable feature as a lip of true flower is white or yellow colors and the inflorescence occurs from the rhizome directly or occurs from the apical meristem of pseudostem such as *C. angustifolia*, *C. roscoeana* Wall. and *C. aurantiaca*.

Paracurcuma is outstanding characteristic that a lip of true flower is white or purple colors and inflorescence occurs from the apical meristem of pseudostem such as *C. alismatifolia* Gagnep., *C. harmandii* Gagnep. and *C. parviflora* Wall. (Wannakrairoj, 1996; Sirirugsa *et al.*, 2007). *C. alismatifolia* Gagnep. has great ornamental value for exporting subordinate to orchid of Thailand and their value increased year by year (Ruamrunsri, 2015).

## 1.3 Morphology of Curcuma

Larsen and Larsen (2006) reported that *Curcuma* L. is the low herbs with welldeveloped rhizomes and often having tuberous roots. The pseudostem is most often short and the leaves radical. The inflorescence is more or less cylindric in outline, terminal on the leafy shoot or on a separate, lateral shoot. The bracts are broad, often recurved and always laterally connate in the basal part forming pouches and often surrounded by a slimy substance protecting the young flower and, later, the maturing capsule. In most species the distal part of the inflorescence consists of sterile bracts, which may have another color than the fertile ones. This terminal part of the inflorescence is called a coma. The flowers are variously colored, white, yellow, red, purple or bluish. The anther is most often versatile, sometimes with a short anther crest, and in many species with two basal spurs.

*C. alismatifolia* Gagnep. belongs to Paracurcuma subgenus which is composed of an aerial part and an underground part as describe below;

#### 1.3.1 Aerial part

**1)** Leaves and pseudostem: leaf is separated into two parts, e.g., leaf sheath and leaf blade (Figure.1.1). A pseudostem is formed by the leaf sheaths and surrounded by the bladeless sheaths at the base. Leaf blades are about 4-5 cm width and 30-35 cm length. The shape of leaf is slender which a basal leaf is wider than a tip. The tip of leaf is sharp. Midrib has green or reddish (Phubuopiend, 1992; Wannakrairoj, 1996; Sirirugsa *et al.*, 2007; Ruamrugsri, 2015).



Figure 1.1 Foliage leaves and pseudostem of *C. alismatifolia* Gagnep.

**2) Inflorescences:** Inflorescence comprises two types of bracts. First, pink coma bracts in the upper part (12-15 bracts) look like a lotus flower (Figure 1.2). Second, green coma bracts in the lower part (8-10 bracts) connect together to form the shape like a cup overlapping and has small true flowers inside the bract. Besides, the pink bracts are arranged to overlap without small true flowers (Hagiladi *et al.*, 1997; Sukhvibul and Thongtaksin, 1995; Vichailak, 2005; Ruamrungsri, 2015).



Figure 1.2 Inflorescence (left) and true flowers (right) of C. alismatifolia Gagnep.

**3) True flowers:** the color of true flower is white and the base is overlapped. One of the petal changes to the lip which has deeply purple (Figure 1.2). Calyx is tubular, unequally toothed, deeply divided along one side. Corolla-tube is more or less funnel-shaped; corolla-lobes are unequal, the dorsal slightly larger than the lateral ones, and its apex is hooded. Staminodes are petaloid, elliptic, oblong or linear. Labellum has a thickened middle part and thinner lateral lobes which overlap the staminodes. Stamen has a short and broad filament, and a constricted apex. Anther is versatile, with or without spurs, and the anther-crest is usually small. Spurs vary in several shapes and sizes, and they are important characters for infra-generic classification. Ovary is glabrous or pubescent, and 3-lobed. Stylodes can be present. Capsule is ellipsoid, and seeds are arillate. Flower forms have different arrangement of staminodes and corolla-lobes made up the 2-formed flowers, i.e., closed form: the staminodes are wrapped by the dorsal corolla-lobes or the open forms: the staminodes are free from the dorsal corolla-lobe (Sirirugsa *et al.*, 2007; Ruamrugsri, 2015).

#### **1.3.2 Underground part**

**1) Root system**: The root system is truly adventitious roots that are composed of fibrous roots and contractile roots (Figure 1.3). Fibrous roots initiate and emerge from the base of storage roots and the base of new shoot during sprouting stage under high temperature and high humidity. The contractile roots emerge from the base of rhizomes and finally develop into the new storage roots like an egg-shape root when entering the dormant stage. This organ also called 'milk tank' from the imported foreigners (Hagiladi *et al.*, 1997; Chidburee, 2008; Ruamrungsri, 2015).





**2) Storage organs**: Underground storage organs comprised of two parts, rhizome and storage roots. The rhizome is a true stem, which comprises buds, nodes and internodes (Figure 1.3). The primary rhizome has an ovoid or globose structure at the base of the aerial shoot, bearing a few to many horizontal branches and roots. It is modified from the vertically-growing stem with short internodes or stubbed rhizome. A lateral bud of rhizome germinates and develops to a pseudostem that is created from the leaf sheath, which tightly packs the inflorescence stalk and the inflorescence on aboveground parts. A new rhizome, which develops from the base of pseudostem enlarges during senescence. It stores the food and water that rhizomes play a more important role in supplying N (Sukhvibul and Thongtaksin, 1995; Vichailak, 2005; Sirirugsa *et al.*, 2007; Khuankaew, 2010a). Another storage organ is the storage root, which are morphologically changed from contractile roots after the shoots begin to grow old and they have a thick root with a spherical-ball shape at the end. The storage roots affect earlier flowering, higher inflorescence and rhizome quality (Ruamrungsri *et al.*, 2001; Ohtake *et al.*, 2006; Khuankaew, 2010a).

### 1.4 Growth cycle

Ruamrungsri (2015) separated the growth cycles of *C. alismatifolia* into five stages, i.e., sprouting, vegetative growth, flowering, new rhizome formation and dormancy stages. In general, rhizomes sprout in May in northern Thailand when theirs bud received a high temperature and enough humidity that they need. The primary roots are fibrous roots that occurred from the end of old storage roots (Figure 1.4). Totally, from planting to sprouting, it consumes for 2-6 weeks depends on dormancy levels and environment. Shoot grows and develops to the first leaf for 6-7 weeks after planting (WAP), and the second leaf for 7-8 WAP. The third fully-expanded leaf for 8-9 WAP and the fourth leaf for 9-10 WAP.



Figure 1.4 Growth cycle of C. alismatifolia Gagnep.

The leaf bud initiates and changes to a floral bud when the shoot grows as 5-10 cm height (Changjeraja, 2009) and the growth of floral part is successfully within 7 days and then the leaves growth are developed. The inflorescence appeared when plants have 3-4 leaves fully expanded and flowering stage occurs during July-August (Phubuopiend, 1992). Many new shoots sprout from the rhizome during the two weeks. Each shoot produces two to four leaf blades and a flower stalk. First shoot produces the best quality of flower and qualities are declined sequentially. A new rhizome is formed during flowering, some contractile roots elongate from the bottom of new rhizomes and some are modified to new storage roots. Then, aerial parts are withered in October, finally dormant in November-December during dry season. The new rhizomes are harvested in December-January and exported to the customer's countries during January-March (Vichailak, 2005; Chidburee, 2008; Khuankaew, 2010a). Because Curcuma plants are not truly dormant, when plants receive sufficient amount of water with continuous high humidity, it cannot be complete dormancy. Therefore, growers should use a plastic cover on the plots after senescence for stopping the irrigation and accelerates plant dormancy for harvesting (Ruamrungsri, 2015).

## 1.5 Cultivation

In Thailand, *Curcuma* plants are generally cultivated in two seasons; 1) Regular season (rainy season) and 2) Off-season (winter season). The regular season cultivation is divided into 3 periods, i.e., (1) early-season (February to March), (2) mid-season (April or May) and (3) late-season (June to July) (Vichailak, 2005). The off-season cultivation was attempted to avoid the oversupply of cut flower in the market in regular season, to shirk the risk and to raise profits of growers, satisfy customers at the time of their needs and guarantee employment throughout the year. The main area of *Curcuma* cultivation can be grouped to two patterns, a bag culture and field culture. In a bag culture, *Curcuma* plants are cultivated in a plastic bag filled with soil and separated from the ground, so the bag culture can avoid soil-borne diseases and the same field can be used for *Curcuma* cultivation repeatedly (Ruamrungsri, 2015).

## 1.5.1 Rhizome preparation

Before planting, rhizomes should be disease-free, without the nematodes and come from disease-free planting fields. The same size of rhizomes should be selected for planting in the field. The rhizome sizes are as follows; "Large", size is larger than 1.5 cm of diameter; "medium", size is between 1.0-1.5 cm of diameters and "small", size is less than 1.0 cm of diameter. Rhizomes are soaked in fresh water for three days or incubate in the mixed medium with sand, rice husk charcoal, and ground coconut husk, and the relative humidity should be about 70% for enhancing bud sprouting (Vichailak, 2005; Khuankaew, 2010a).

#### 1.5.2 Planting

There are two methods for cultivating the *Curcuma* plants; 1) plot culture and 2) bag culture (Ruamrungsri, 2015)

#### 1) Plot culture

Plot should not be contaminated with rhizome rot or wilt disease and must be free of nematodes. If land has been planted with *Curcuma*, it should be rotated with other crops for at least three years before returning to *Curcuma* again. Basically, soil should be sandy loam with medium fertility, well-drained; having nutrition in medium level, and soil pH should be in the range of 6.5-7.0. Soil preparation for plot culture should plow the land once and leave it to be exposed to the sunlight for 20-30 days. As a preventive measure against rhizome rot disease, urea mixed with lime at the ratio of 1:10 is applied to the soil at the rate of 5,500 kg per hectare for 62,500 rhizomes before harrowing. The land is bedded, covered with plastic sheet and left for 15 days. The beds are sub-divided into small plots of about 400 m<sup>2</sup> and water drainage channels are provided. Within the sub-division, the beds are raised the level by 20-30 cm high and 1.0-1.2 m wide, provided a 0.5 m wide path for walking space and a 1 m wide between the sub divisions. The soil should not be plowed too deeply as the storage roots will grow down too deep, rhizome would get damaged when harvest and become undesirable for the market. Before planting, 15 g of 15-15-15 or 16-16-16 fertilizer (N: P<sub>2</sub>O<sub>5</sub>: K<sub>2</sub>O) should be used in the basal hole and placed a rhizome by 7-10 cm deep. Planting space depends on the sizes of the rhizome, i.e., large ( $30 \times 30$  cm or 62,500 rhizomes per hectare), medium ( $25 \times 25$  cm or 93,750 rhizomes per hectare) and small ( $20 \times 20$  cm or 125,000 rhizomes per hectare) (Vichailak, 2005).

## 2) Bag culture

This cultivation is planting in a plastic bag by using soilless culture for decreasing or solving the rhizome rot and wilt disease problem in the rhizome which are exported to other countries. Soilless culture comprises sand: rice husk or ground coconut husk: rice husk charcoal at ratio 1: 1: 1 by volume. Any manures or dolomites can be added together. After mixing the culture medium, then sprinkle with water, cover with clear plastic for 30 days or leave it in the sun for 1-3 months (Ruamrungsri, 2015). Then, fill into a black plastic bag size of 15×30 cm. The bags are placed on a sheet of clear plastic on a raised planting platform of 20 cm high to prevent rhizome from rot disease. The sprouted rhizome placed close to the soil surface with upright shoot, this will result in inducing flowers about two weeks earlier than usual. After that, cover it with soil thinly in order to protect the shoot from burning (Vichailak, 2005).

#### **1.5.3 Environmental factor**

**1. Light:** *Curcuma* originates in the tropical and subtropical areas, under high light intensity and consistent water supply. Low light intensity decreased photosynthesis rate in *Curcuma* that affected on long leaves, thin stem and unhealthy appearance (Ruamrungsri, 2015). In addition, Hongpakdee (2010) also found that *Curcuma* had lower photosynthesis rate in the winter season than in the rainy season. In the field, the photosynthesis rate increased when the light intensity rose up and it was constant at the

light saturation point (400-500  $\mu$ mol CO<sub>2</sub> m<sup>-2</sup>S<sup>-1</sup>). After this point, the rate was decreased (Siritrakulsak, 2010). When the plant was grown under the red light fluorescent lamp (632-660 nm) decreased plant height, number of leaves per plant, number of plants per cluster, number of new rhizomes per cluster and number of storage roots per rhizome but were not significantly different from cool day night (405-812 nm). However, the growth of plant under red light and cool day light conditions were less than under natural light. Moreover, plants were grown under the red light resulted in less the starch, total nonstructural carbohydrate (TNC), reducing sugar (RS) and total-N when compared to the other light types. Unless, total soluble sugar (TSS) of new rhizome and storage root were not different from cool day light condition (Chidburee *et al.*, 2007).

**2.** Photoperiod: *Curcuma* is a quantitative long day plant that it can be flowering all the year (Hagiladi *et al.*, 1997). However, in the winter (short day) have an effect on flower quality, e.g., flower stalk, inflorescence length and the number of pink bract were decreased. So, it should add the light about 2 hours when growing plant on November and December (Ruamrungsri and Uthaibutra, 2005). Kuehny *et al.* (2002) found that 8 hours of photoperiod declined plant height and the number of leaves of *C. alismatifolia*, *C. cordata*, *C. petiolata* and *C. thorelii*.

**3. Temperature**: The temperature should be between 25-30°C of daytime and approximately 18-25°C of nighttime. Changjeraja *et al.* (2007) reported that high temperature 36/24°C (day/night) increased vegetative growth of aboveground parts and flower but decreasing of night temperature to 24/18 °C supported dry weight accumulation of underground storage organs. Moreover, plants gave higher plant height and flower quality, i.e., stalk length, inflorescence size when grown under the 30/24 °C than at 30/18 °C. Plants supplied with NO<sub>3</sub><sup>-</sup> as the N source showed the highest N concentration (mg/plant) in the leaves. N assimilation was major in leaves at 30/24 °C, and in fibrous roots at 30/18 °C. The total amino acid concentration when fed with NH<sub>4</sub><sup>+</sup>-N was higher than supplied with NO<sub>3</sub><sup>-</sup>-N. Glutamic acid (Glu) was major N form accumulated in the old rhizome, especially in plants, which were supplied with NO<sub>3</sub><sup>-</sup>-N at 30/18 °C, while asparagine (Asn) and aspartic acid (Asp) were the main forms accumulated in fibrous roots when grown at 30/18 °C (Inkham *et al.*, 2011).

**4. Water:** it should be clean with pH in the range of 5.5-6.5, without contamination from any organic matters or toxic inorganic matters. High soil moisture is good for growth and development of *Curcuma*, thus daily or often watering during plant growth and flowering are necessary (Vichailak, 2005).

#### **1.5.4 Fertilizer application**

An optimum fertilizer application rate for *C. alismatifolia* growth should be supplied with chemical fertilizer 15: 15: 15 (N: P<sub>2</sub>O<sub>5</sub>: K<sub>2</sub>O) at 7.5 g/ pot (1/2 GAP) until flowering, then add with 13: 13: 21 (N: P<sub>2</sub>O<sub>5</sub>: K<sub>2</sub>O) at 7.5 g/ pot until senescence twice a month (Siritrakulsak, 2010). The liquid fertilizer for planting *Curcuma* in Thailand has been developed by [H.M. the King's Initiative Centre for Flower and Fruit Propagation, Chiang Mai University] named "Banrai's Centre or BC-1". The nutrient solution contains complete macro and micro elements by N 200, P 50, K 200, Mg 25, Ca 136, B 0.22, Mn 0.81, Zn 0.26, Cu 0.025, Mo 0.035 and Fe 0.41 mg 1<sup>-1</sup> (Ruamrungsri, 2005). Liquid fertilizer application supports the producing of new shoot and new rhizome quality.

#### 1.5.5 Disease and pest control

The most damaging disease in *Curcuma* is bacterial pathogens, which causes wilt and rot diseases. *Ralstonia solanacearum* is a causal bacterium, the symptom appears at the lower leaves, which will roll due to the lack of water and it is clearly prominent in the morning. The base of the plant and the new growth will appear succulent and leaf roll will spread to the upper parts, and then, the whole plant. The plant folds over, easily comes off the ground when pulled and finally, dries up or dies. The disease also causes rhizome and root parts to become succulent and transparent glassy appearance. It causes the rhizomes and roots to become dark and emits a rotting smell. The presence of nematodes will exacerbate the problem. Nematode is an important factor in spreading the wilt disease and is commonly found in sandy soil. Control measure can be made by rotating crops that are not susceptible to the disease. Addition of organic matters, such as manure, fresh manure and humus, improves the physical properties of the soil as well as increases microorganisms that are antagonistic to nematodes. Nematode-infected rhizomes and roots should collect from the planting ground and disposes by burying or burning (Vichailak, 2005).

*Curcuma* is infested by very few insects except for the leaf roller. The grasshopper damages the leaves but can be controlled easily by synthetic pyrethroid such as cypermethrin. Red spider mites cause spotting to the colorful bracts and can be controlled by dicofol with wetting agents.

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#### **1.5.6 Harvest and Post-harvest**

1. Cut flower production: Plant watering should be done before harvest. The suitable time for cut flower harvesting is in the morning when 4-6 coma bracts open and 2-3 true flowers appear. Hold the base of inflorescence stalk, then twist and pull up with one leaf attached. Soak in clean water and cover with a plastic bag. For preparation before transportation, keeping flower at low temperature 12-15°C under a high relative humidity at 85-95% about 1-2 hours before transport will prolong flower quality. Before transportation, the basal end of inflorescence stalk should be wrapped in cotton wool and dipped in disinfectant solution (50-100 mg Clorox per liter of water). Pack it in a carton box and store in a 15-18 °C room (Vichailak, 2005). C. alismatifolia is an attractive cut flower with a great length of vase life (usually more than 2 weeks in freshly-harvested stems). The flowers are chilling-sensitive, and cannot be stored dry but they can be stored in water at 7 °C for about 6 days. Since vase life is rather long, it is also possible to store the flowers in water for a few days at ambient temperatures (25°C). The stems have a very short vase life after 3 days of dry storage. When stored in water, the optimum temperature for storage is 7 °C. After 3 or 6 days of storage at 7 °C, the vase life is not different from that of unstored controls (about 18 days). However, if flowers are held at 5 °C for 3 days, they have a vase life of only 2 days (Bunya-atichart et al., 2004).

2. Rhizome production: After the aboveground part of plant becomes senescent, the underground parts become dormant. The aboveground organs wilt and dry and rhizomes will be harvested. In case of planting *Curcuma* in a field, the bed should be watered before harvesting rhizomes. Dig up rhizomes and wash out the soil, then separate rhizomes from a cluster. Cut the unhealthy rhizomes or storage roots by using the clean pruning shear (clean with 70% alcohol) and grade rhizomes according to rhizome size. Rhizomes will be immerged in the insecticide and fungicide solution and dried in the air under the shaded place for 14 days, then store rhizomes in well-ventilated place (shelf) (Vichailak, 2005).

#### 2. Proteins Background

### 2.1 General properties and classification of proteins

Proteins are a diverse and abundant class of biomolecules, which consists of more than 50% of the dry weight of cells. Their diversity and abundance reflect the central role of protein in virtually all aspects of cell structure and function. Proteins are unbranched polymers of amino acids linked head to tail, from carboxyl group to amino group, through formation of covalent peptide bonds, a type of amide linkage. Their molecules composed of one or more polypeptide chains (Garrett and Grisham, 2005). Proteins are polypeptides that normally range in length around 100-2000 amino acids or can reach sizes of more than a thousand amino acids in length. The information stored in the sequence of nucleotides in DNA is transcribed and translated into amino acids sequence that will ultimately bring about the genetically coded phenotype (Buckingham, 2012). Boulter and Derbyshire (1976) have classified proteins by involves chemical structure, mechanism of action, biological function, location and the separator procedures employed in protein purification as described bellows;

1) Chemical structure: proteins can be classified as conjugated or unconjugated ones. Unconjugated proteins are composed entirely of amino acids while conjugated proteins have other chemical groups bound to the polypeptide chain in stoichiometric proportions. A variety of chemical groups may be involved, including carbohydrates, lipids, metals, porphyrins, open-chain tetrapyrroles and flavins.

2) Function: proteins are classified on the basis of their function into enzymic, enzyme inhibiting, recognition, regulatory, transporting, storage and structural proteins as follow;

**2.1) Enzymes:** Enzymes are the largest groups in the classification of protein, which are classified concerning to the nature of their reaction towards specific substrates.

**2.2) Enzyme inhibitors:** Enzyme inhibitors are identified in plants including trypsin, chymotrypsin, and other mammalian proteinase, insect and bacterial proteinase, amylase, invertase and lipase (Liener, 1969).

**2.3) Recognition proteins**: Recognition proteins may be loosely defined as those which determine the compatibility or otherwise of incoming materials by specific interactions such as lectins.

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**2.4) Regulatory proteins:** Regulatory proteins are classified such as histone which is probably regulated the transcription and replication of DNA, either by inhibition of DNA-dependent RNA polymerases or by interaction with the DNA template. Other regulatory plant proteins include the photosynthetic receptors, chlorophyll *a* and *b*, phytochome ( $P_r$  and  $P_{fr}$ ), and other morphogenic proteins are associated with microtubules (tubulin), etc.

**2.5)** Transporters: Transporters are membrane bound proteins, which mediate the transport of ions such as  $H^+$ ,  $K^+$ ,  $NO_3^-$ , amino acids, sugars etc. through cell membrane.

**2.6)** Storage proteins: These proteins are laid down in one phase of development but function subsequently at a later metabolic phase to supply intermediary nitrogen compounds for biosynthesis are called storage proteins (Newcomb, 1967). The amino acids formed by nitrate assimilation can be stored in terms of storage proteins, which have neither metabolic nor structural roles (Heldt and Piechulla, 2011) and often have a relatively high proportion of N-rich amino acids, especially arginine and the amides.

Normally, plants accumulate reserve foods, e.g., proteins, lipids and starch in their seeds, which are hydrolyzed after germination and utilized for the heterotrophic growth of the seedling. Plants also store reserve foods in their vegetative parts such as stems, root, leaves and floral axes (Srivastava, 2002).

1) Vegetative storage proteins (VSPs): VSPs have been identified in a large number of plant species and can constitute up to 50% of the total soluble proteins in various vegetative storage organs (Staswick, 1994; Ourry *et al.*, 2001). VSPs are heterogeneous group of proteins that are accumulated in vegetative tissues, usually in vacuoles of parenchyma cells associated with vascular tissues. They are accumulated under high N or sugar conditions and are hydrolyzed to provide building blocks for synthetic activities elsewhere. Many VSPs are enzymes, but their major function seems to be to serve as a nitrogen source to sink areas, including developing seeds and new growth in the spring (Srivastava, 2002). VSPs accumulate temporary and are degraded within the life cycle of the plant. It can be indirectly affected by changes in sources-sink relationships in relation to N within the plant or directly by exogenous hormone (methyl-jasmonate) or modifications of soil N availability (Staswick, 1994; Ourry *et al.*, 2001; Meuriot *et al.*, 2003).

2) Seed storage proteins (SSPs): About 50-85% of SSPs are storage proteins which are synthesized during seed development and serve as principal source of amino acids for germination and seedling growth (Shewry, 2007). SSPs have been studied based

on their solubility properties by Osborne (1924), it was classified into; 1) albumins (soluble in pure water), 2) globulins (soluble in dilute salt solution), 3) glutelins (soluble in diluted alkali or acidic solution) and 4) prolamins (soluble in aqueous ethanol). The predominant storage proteins of cereals are prolamins while globulins are major protein in legumes and rice (Holding and Larkins, 2008). The increase in grain protein with high N fertilization is due to greater synthesis and accumulation of storage proteins (Kindred *et al.*, 2008; Godfrey *et al.*, 2010).

**2.7) Structural proteins**: Structural proteins have no enzymic activity and a purely structural function although some of the enzymes associated with membranes and ribosomes, etc. may also play an important structural role. An important structural protein of plants is the cell wall glycoprotein, extension, it occurs closely bound to the a-cellulose fraction of the wall (Lamport, 1970).

**3)** Location: subcellular organelles have multi-enzyme complexes associated with them in which the component proteins occur in a fixed spatial arrangement upon which the proper functioning of the system depends. Some of these proteins are only found in a particular organelle, e.g. cytochrome-oxidase in mitochondria and sometimes they are classified following their subcellular location, e.g. chloroplast, mitochondria and nuclear, etc. Moreover, some of proteins occur in particular organ such as leaf protein and seed protein, etc.

4) Methodological: proteins are classified base on solubility criteria, Osborne (1924) grouped proteins into albumins, soluble in water; globulins which soluble in salt; prolamins, soluble in 70-80% ethanol and glutelins soluble in dilute acids and alkalis. In addition, some classifications have been on these reparatory techniques, i.e. chromatographic, electrophoretic, molecular sieving and centrifugal techniques following protein size, shape or charge.

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## 3. Protein Techniques

Proteomics is the study of the protein complement of a genome using the tools of protein biochemistry on a proteome-wide scale. It is devoted to monitoring changes in expression levels or post-translational modifications of all the proteins in an organism, organ, cell, or organelle as a function of time or biological state. Ideally, it should also address protein structure-function in terms of interactions with substrates, drug, inhibitors, lipids, DNA, or other protein (Sem, 2007). Due to cells contain thousands of different proteins; a major problem for protein chemists is to purify a chosen protein so that they can study its specific properties in the absence of other proteins. Proteins can be separated and purified on the basis of their two prominent physical properties, size and electrical charge. A more direct approach is to use affinity purification strategies that take advantage of the biological function or similar specific recognition properties of a protein (Garrett and Grisham, 2005).

#### 3.1 Protein concentrations methods

Cell extracts are complex mixtures that typically contain protein molecules of many different molecular weights, so the results of protein estimations cannot be expressed on a molar basis. Also, aside from the rather unreactive repeating peptide backbone, little common chemical identity is seen among the many proteins found in cells that might be readily utilized for exact chemical analysis. Most of their chemical properties vary with their amino acid composition (Garrett and Grisham, 2005).

1) Lowry method: uses  $Cu^{2+}$  ions along with Folin-Ciocalteau reagent, a combination of phosphomolybdic and phosphotungstic acid complexes that react with  $Cu^+$ .  $Cu^+$  is generated from  $Cu^{2+}$  by readily oxidizable protein components, such as cysteine or the phenols and indoles of tyrosine and tryptophan. The  $Cu^+$  reaction with the Folin reagent gives intensely colored products measurable spectrophotometrically.

2) BCA method: Bicinchoninic acid (BCA) forms a purple complex with  $Cu^+$  in alkaline solution. This reagent reacts more efficiently with  $Cu^+$  than Folin-Ciocalteau reagent.

**3) Bradford assay**: The Bradford assay is a rapid and reliable technique that uses a dye called Coomassie Brilliant Blue (CBB) G-250, which undergoes a change in its color upon non-covalent binding to proteins. The binding is quantitative and less sensitive to

variations in the protein's amino acid composition. The color change is easily measured with a spectrophotometer. A similar, very sensitive method capable of quantifying nanogram amounts of protein is based on the shit in color of colloidal gold upon binding to proteins.

#### 3.2 Protein separation methods

Separation methods were based on the size exclusion chromatography, ultrafiltration, and ultracentrifugation. The ionic properties of peptides and proteins are determined principally by their complement of amino acid side chains. Furthermore, the ionization of these groups is pH-dependent. A variety of procedures have been designed to exploit the electrical charges on a protein as a means to separate proteins in a mixture. These procedures include ion exchange chromatography, electrophoresis, and solubility (Garrett and Grisham, 2005).

**3.2.1. Electrophoresis:** is the movement of molecules by an electric current to analysis of proteins and nucleic acids (Buckingham, 2012). Electrophoresis is general carried out not in free solution but in a porous support matrix such as polyacrylamide or agarose gel, which retards the movement of molecules according to their dimensions relative to the size of the pores in the matrix (Garrett and Grisham, 2005).

1) Polyacrylamide gel electrophoresis with a sodium dodecyl sulfatecontaining buffer (SDS-PAGE): SDS-PAGE is a technique for separating proteins in a polyacrylamide gel medium by the application of an electrical field (Spangler, 2002). The hydrophobic tail of dodecyl sulfate interacts strongly with polypeptide chains. The number of SDS molecules bound by a polypeptide is proportional to the length of the polypeptide. SDS is also a detergent that disrupts protein folding (protein 3<sup>rd</sup> structure). SDS-PAGE is usually run in the presence of sulfhydryl-reducing agents such as  $\beta$ -mercaptoethanol so that any disulfide links between polypeptide chains are broken. SDS-PAGE is often used to determine the molecular weight of protein (Garrett and Grisham, 2005).

2) A non-denaturing PAGE (Native-PAGE): Native-PAGE gel separates proteins as a function of both charge and size using simple buffers and applied voltage. The more highly charged a molecule is, the faster it will be pulled through the gel by a constant voltage applied. However, the larger the molecules will be retarded in the gel matrix based on molecular size and shape (Spangler, 2002).

**3) Isoelectric focusing:** Isoelectric focusing is an electrophoretic technique for separating proteins according to their isoelectric points (pIs). A solution of ampholytes is first electrophoresed through a gel, usually contained in a small tube. The migration of these substances in an electric filed establishes a pH gradient in the tube. Then a protein mixture is applied to the gel and electrophoresis is resumed. As the protein molecules move down the gel, they experience the pH gradient and migrate to a position corresponding to their respective pIs. At its pIs, a protein has no net charge and thus moves no farther (Garrett and Grisham, 2005).

4) Two-dimensional gel electrophoresis (2D-PAGE): this separation technique uses isoelectric focusing in one dimension (1D) and SD-PAGE in the second dimension (2D) to resolve protein mixtures. The proteins in a mixture are first separated according to pI by isoelectric focusing in a polyacrylamide gel in the tube. The gel is then removed and laid along the top of an SD-PAGE slab, and the proteins are electrophoresed into the SDS polyacrylamide gel, where they are separated according to size. The gel slab can be stained to reveal the locations of the individual proteins (Garrett and Grisham, 2005).

**3.2.2 Gel staining**: the separated protein bands are visualized by staining after electrophoresis, particularly with Coomassie Brilliant Blue (CBB); ( $\mu$ g sensitivity) or silver stain (ng sensitivity). The stain is applied in a solvent that serves as a fixative (precipitant) to immobilize the protein bands in the gel.

1) CBB is believed to interact electrostatically with lysine residues in a protein but it does not interact effectively with polyacrylamide so the gel is de-stained using dilute acetic acid, leaving the background clear and the blue dye associated with the protein bands (Spangler, 2002). Usually this staining can be detected 0.2-0.5  $\mu$ g of any protein in a sharp band and at least 15-20  $\mu$ g of protein quantity (Hames and Rickwood, 1990).

2) Silver stain is originally developed for protein visualization after electrophoresis. The sample is fixed with methanol and acetic acid. Then, the gel is saturated with ammoniacal silver (silver diamine) solution or silver nitrate in a weakly acid solution (Rabilloud, 1992). Interaction of silver ions with acidic or nucleophilic groups on the target results in crystallization or deposition of metabolic silver under optimal pH condition. The insoluble black silver salt precipitates upon introduction of formaldehyde in a weak acid solution, or alkaline solution for silver nitrate. Although, silver nitrate is considered to be more stable, silver diamine is the best for thick gels (Merrill, 1990).

3) Periodic acid-Schiff (PAS): PAS staining is the most usual technique for detecting the glycoproteins that readily located in polyacrylamide gels and distinguished

from other non-glycosylated proteins (Zacharius *et al.*, 1969). Periodic acid oxidizes the 1,2-glycol groups to aldehyde in polysaccharides, which react with fuchsin sulfide to form magenta or pink colored bands (Deepak *et al*, 2003).

## 3.3 Protein identification methods

Proteins and peptides are linear polymers made up of combinations of the 20 most common amino acids linked with each other by peptide bonds. Moreover, the protein produced by ribosome may undergo covalent modifications, called post-translational modifications, after its incorporation of amino acids. Over 200 such modifications have been detected already, the most important being glycosylation, the formation of disulfide bridges, phosphorylation, sulfation, hydroxylation, carboxylation and acetylation of the N-terminal acid (Hoffmann and Stroobant, 2007). Presently, 2D gel electrophoresis, mass spectrometry and bioinformatics have become important tools in correlating the proteome with the genome (Larsen and Roepstorff, 2000). It has two methods, Edman degradation and mass spectrometry is described below;

1) Edman degradation: Edman degradation method is sequencing amino acids in a peptide by labeled the amino-terminal residue and cleaved from the peptide without disrupting the peptide bonds between other amino acid residues (Edman et al., 1950). Firstly, the reaction occurs between an uncharged N-terminal amino acid group and phenylisothiocyanate under mildly alkaline conditions to form a cyclical phenylthiocarbamoyl derivative. Then, this derivative is cleaved as a thiazolinone derivative under acidic condition. Finally, the thiazolinone derivative is then selectively extracted into a form of phenylthiohydantoin (PTH)-amino acid derivative in an organic solvent and treated with acid. PTH can be identified by using chromatography or electrophoresis. The next amino acid can be repeated again as above reaction to identify. An advantage of the Edman degradation is that it only uses 10 - 100 pico-moles of peptide for the sequencing process. The Edman degradation reaction was automated in 1967 by Edman and Beggs to speed up the process and 100 automated devices were in use worldwide by 1973 (Edman and Begg, 1967; Niall, 1973; Mortz et al., 2013).

Edman degradation has a few significant limitations; it does not work well for very hydrophobic proteins or peptides (a potential problem for membrane proteins), and it cannot work when the N-terminus of the protein or peptide is chemically blocked (e.g., with an acetyl group). In the latter case, however, it may be possible to cleave the protein

(chemically or enzymatically) into peptides, most of which (except for the one derived from the original protein N-terminus) will have free amino-termini and can therefore be sequenced. Moreover, this technique requires a long analysis time and a large amount of sample (Gevaert and Vandekerckhove, 2000; Hoffmann and Stroobant, 2007).

2) Mass spectrometry (MS); MS is a very sensitive and accurate method to determine the precise molecular weight of a protein. Moreover, MS can be used to determine protein sequences and thus to determine the identities of proteins in a manner conceptually similar to that described above based on Edman degradation. It can be applied to large numbers of samples more rapidly is a more powerful method. MS is perfectly adapted to provide such information about the products obtained upon cleaving an isolated (unknown) protein. MS is uniquely well-suited for such analyses because it can yield very accurate determinations of molecular weights from very even very small amounts of fragments resulting from the digestion of a particular protein. Techniques have now been developed by which proteins separated in 2D gels can be digested within the gels and then injected directly into a mass spectrometer for analysis of the resulting fragments.

MS is not only allows the precise determination of the molecular mass of peptides and proteins but also the determination of their sequences, especially when used with tandem mass techniques. Indeed, fragmentation of peptides and proteins gives sequence information that can be used for protein identification, *de novo* sequencing, and identification and localization of post-translational or other covalent modifications (Aebersold and Mann, 2003; Standing, 2003; Sechi and Oda, 2003; Jensen, 2004; Meng *et al.*, 2005; Hernandez *et al.*, 2006).

#### 4. Research Background

#### 4.1 Nitrogen effect on growth and development in C. alismatifolia and other plants

In C. alismatifolia plants, N supply plays an important role on growth, flower and rhizome qualities. The increase of N supply typically enhances the number of flowers and rhizomes. However, it depressed the new storage roots formation (Ruamrungsri and Apavatjrut, 2003; Ohtake et al., 2006). When the Curcuma plants were cultivated in the lack of N solution, plants showed the deficiency of N, i.e., stunted growth, the leaf area were decreased; leaf changed to yellow and reduced flower quality. Moreover, it decreased the number of new shoot and leaves per plant when compared with the plant in complete solution. However, a continuous supply of N was important for flower and rhizome production (Ruamrungsri et al., 2006). A high N concentration applied to Curcuma plants can increase free amino acid content in rhizomes and storage roots while plants grown in lack of N culture solution decreased total N concentration, total amino acids and protein concentrations in both rhizomes and storage roots (Ohtake et al., 2006). From the commercial Curcuma fields in Chiang Mai, Thailand, the better plant height, number of new shoots, and stalk lengths were found in the field that used N fertilizer 6.9 g N/plant in soil than other site. However, N supplied at 15 g N/plant that gave the highest N concentration in rhizome and leaves at flowering stage and in storage roots at harvesting stage, but in terms of rhizome and storage roots quality (fresh weight, dry weight and diameter) was lower than other site (at 4.1- 6.9 g N/plant). So, the N ranges between 4.1-6.9 g N/plant was required for maximum rhizome yields, and the highest (15 g N/plant) or lowest (1.95 g N/plant) of N supply decreased rhizome yields (Inkham, 2012).

Anyway, N fertilization applied to soil in carrot caused a significant increase in total-N concentration in storage root and N uptake by storage roots while foliar spray (2% urea) affected a rise on the NO<sub>3</sub><sup>-</sup> concentrations and total-N in storage roots and N uptake by carrot storage roots (Smolèn and Sady, 2009), similar to broccoli, it was found that the optimum urea concentrations was about 0.61% and 0.96%, foliar applications could be successfully used to obtain better growth and yield for broccoli cultivars AG 3317 and AG 3324, respectively (Yildirim *et al.*, 2007). Moreover, foliar fertilizer application (NPK 24:24:18) increased the quality of the tested tea, which had significantly higher levels of total polyphenols content as compared with soil fertilizer (NPK 20:5:5) (Njogu *et al.*,

2014). Although, in apple trees, it indicated that soil N application significantly increased shoot length compared to free-N (control) or foliar N application, and leaf color, leaf N and N concentration of one-year-old wood and roots were similarly increased compared to control by both soil and foliar N application. There was not significantly difference in yield and fruit quality between soil and foliar N application (Dong *et al.*, 2005) whereas the foliar application of urea in wheat significantly increased plant height, spike length, number of grains spike<sup>-1</sup>, hundred grain weights, biological yield, grain yield and N uptake by the crop (Khan *et al.*, 2009). Similary, Amanullah *et al.* (2013) revealed that plant height, leaf area, biomass and grain yield of maize was increased when foliar-N was applied late (45 and 60 days after emergence; DAE) than early (15 and 30 DAE) application.

#### 4.2 Nitrogen in storage organs

Nitrogen (N) is an essential major element for all organisms, and is required for the synthesis of amino acids, proteins, nucleic acids, and other important cellular compounds. Plants usually absorb N from soil via their roots. However, before the root system develops and starts to absorb N, plants use the N reserves contained in storage organs including seeds, bulbs, rhizomes, tubers, and storage roots. N is stored mainly in the form of proteins in these organs (Ohyama and Sueyoshi, 2010). In flower bulbs, food reserves in storage organs for survival mechanism such as shoot and root emergence, floral initiation and development. The storage nitrogen in bulbs is mainly protein-N that assimilated from inorganic forms both NH<sub>4</sub>-N and NO<sub>3</sub>-N. Protein-N fraction commonly amounts to 80-85% of the total N in a higher plant while, the others are the nucleic acids-N (10%) and the soluble amino-N about 5% (Mengel and Kirkby, 1987).

Generally, researches often studied the N fraction in the storage organs of flower bulbs. In *Narcissus*, found that about 90-93% of N in bulb scales was in the 80% ethanol-insoluble fraction (protein-N) and only 1-2% was in the 80% ethanol-soluble fraction (non-protein N; the lower molecular weight nitrogenous compounds). Moreover, 30% of N was in PBS-insoluble fraction suggested that N was assimilated into conjugated and structural proteins which were the components of cell wall, membrane and organelles. However, after harvesting stage, *Narcissus* bulb contained 30% of insoluble-N and 33% of soluble-N fractions (Ruamrungsri *et al.*, 2010). From the previous research in *Curcuma* has been reported that rhizomes store higher amounts of N than the storage roots, while the

storage roots store larger amounts of carbon (C) especially, in the starch form. Rhizome illustrated that about 4-5% of N on dry weight (DW) and 97% of N was in insoluble form while, storage roots stored approximately 1-2% of N and 88% in insoluble form at beginning of dormancy (Ruamrungsri *et al.*, 2001). After that, the N concentration in mother rhizome and storage roots changed during growth and development. Tapun and Ruamrungsri (2006) exposed that N in mother rhizome was about 3.96% and 1.36% in storage roots at planting stage. After shoot and leaves growth, N concentration in mother rhizome and storage roots was about 4.6% and 1.2%, respectively. Then, the N in both organs was decreased at flowering.

## 4.3 Changes of protein characteristic during plant growth and development

Proteins have been studied in more genera than amino acids. The general research have focused on protein identifications and content (De Hertogh and Le Nard, 1993). Nevertheless, there have been several studies about protein metabolism on physiological responses during plant growth and development. For example, Paulin (1973) illustrated drought stress decreased the protein content on cut Iris germanica flowers and it can be synthesis in a few hours after the flowers were rehydrated. Moreover, several researches have been found in tulip, Higuchi and Sisa (1967) also studied protein changes in tulip scales during cooling. Barber and Steward (1968) and Steward et al. (1971) have studied protein changes and metabolism in tulips during morphogenesis. Sopanen and Carfantan (1976) studied peptidase activity in the petals during senescence. Recently, Koksal et al. (2011) was studied the change of total soluble protein (TSP) contents and protein pattern of tulip and freesia by comparing the different storage conditions and durations on the sprouting and the proportion of plant formation. The TSP contents decreased sharply after 40 and 60 days in all storage conditions, which it showed the storage proteins are breakdown. A 18 kDa is a major polypeptide in tulip bulbs which was detected with different band intensity throughout the end of storage periods. However, 58 and 66 kDa proteins might be related to dormancy or sprouting in freesia. In Hyacinthus orientalis cv. Carnegie indicated that a 29 kDa was presented most strongly polypeptide in bulb scales at beginning culture, and after 4 weeks of culture its intensity began to decrease and finally disappeared while the 53-55 kDa appeared after 4 weeks and the 32-34 kDa disappeared after 8 weeks of culture (Yi et al., 2002). Similar to Chung et al. (2006) reported that the total protein contents gradually decreased during culture after coldpretreated at 4 °C for 4 months, and found that the 45 kDa polypeptide are frequently accumulated during the bulblet development and belong to a family of major storage proteins in *H. orientalis* L. cv. Anna Marie. In the rhizomes of *C. longa* L., an 18 kDa and a 23 kDa storage proteins disappeared after 45 days in storage, indicating that protein degradation and synthesis preceded shoot sprouting in this plant (Jayakumar *et al.*, 2001). In oilseed rape (*Brassica napus* L.), a 23 kDa polypeptide act as a vegetative storage protein (VSP) as it accumulated in the taproots during flowering and is hydrolyzed completely when N filling of the grain occurred (Rossato *et al.*, 2001).

Typically, in dicotyledonous plants, the major seed reserves are storage proteins like globulins, legumins and vicilins (Sheoran *et al.*, 2005). Huang *et al.* (2012) revealed that a number of proteins with molecular weight of 20.1-30.0 and 45.0-66.0 kDa were identified as vicilin-like embryo storage protein and globulin. These proteins accumulated gradually during development and then disappeared during germination and increased in desiccation tolerance of maize embryo during desiccation condition. Lecona-Guzmán *et al.* (2012) observed the protein profiles in somatic embryos of Habanero pepper (*Capsicum chinense J.*) during different developmental stages (globular, heart-shaped, torpedo and cotyledonary stages), which found that nine exclusive bands but only five of these bands were reported for storage proteins in other plant species. Protein content tendency decreased throughout the somatic embryos development.

## 4.4 Protein characteristic investigation in plants

#### 4.4.1) Zingiberaceae

Zingiberaceae families are widely used in the traditional medicine, along with a food flavoring and spice agents. Many studies have focused on the bioactive small organic compounds from these plants and have supported the traditional medicinal use of plant extracts, such as curcumin, sesquiterpene, and various essential oils, flavonoids and phenolic compounds (Chopra *et al.*, 1954; Kojima *et al.*, 1998; Lai *et al.*, 2004; Makabe *et al.*, 2006; Kundu *et al.*, 2009; Kim *et al.*, 2009; Sun *et al.*, 2009; Ghasemzadeh *et al.*, 2010; Shehzad *et al.*, 2010). Moreover, the biologically active proteins reported in terms of antifungal, antioxidant and anti-inflammatory activities (Wang and Ng, 2005; Krishnaraj and Mathivanan, 2008; Petnual *et al.*, 2010). For instance, Angel *et al.* (2013) found that a 12 and 14 kDa proteins in aqueous extracts from the rhizomes of eight *Curcuma* species (*C. aeruginosa, C. amada, C. aromatic, C. brog, C. caesia, C. malabarica, C. rakthakanta* 

and C. sylvatica) showed significant antioxidant activity and also exhibited high antiinflammatory activity. Similar to C. longa rhizome has been isolated turmerin (34 kDa) and a turmeric antioxidant protein (28 kDa). These proteins were powerful antioxidants, which could inhibit lipid peroxidation and scavenge free radicals (Srinivas et al., 1992; Cohly et al., 2003; Selvam et al., 1995; Smitha et al., 2009; Ramadas and Srinivas, 2011). The protein profiles of C. comosa Roxb. rhizomes was improved by separation by microscale solution-phase isoelectrofocusing (IEF), and identified by using high throughput two-dimentional IEF-SDS-PAGE together with tandem mass spectrometry, reported that a high abundance of protein spots in the acidic ranges. Several interesting proteins were identified as lectins and antioxidant proteins, which appear to be related with their activity and cysteine protease that are found in other Zingiberaceae plant species (Boonmee et al., 2011). Recently, a cystatin (CaCPI) gene was isolated from cDNA library of Curcuma alismatifolia cv. Chiang Mai Pink from bracts that the deduced amino acid sequence consisted of a putative N-terminal secretory signal peptide of 22 amino acids and an estimated molecular mass for the mature protein of 11.239 kDa. The CaCPI protein contains all of the highly conserved blocks, including Gly<sup>31</sup>-Gly<sup>32</sup>, the reactive site motif QXVXG and the LGRFAVDQHN block that are common among plant cystains (Porruana et al., 2013).

#### 4.4.2 Other bulbous plants

There have been informs on lectins in *Galanthus*, *Hippeastrum* and *Narcissus* (Van Damme *et al.*, 1987, 1988; Van Damme and Peumans, 1990). Structure of storage protein in *Lilium tigrinum* was investigated by Thaler and Amelunxen (1975). The localization, enzymatic, and anti-genetic activity of proteins in *Gladiolus* pollen have been reported by Knox (1971). In garlic (*Allium sativum*), can identify the immunomodulatory proteins (13 kDa) from the raw bulbs (Clement *et al.*, 2010). Other proteins was found in garlic such as allivin (13 kDa); the antifungal protein from round-cloved garlic (Wang and Ng, 2001), alliumin (13 kDa); the anti-microbial protein (Xia and Ng, 2005). An antifungal protein from *Urginea indica* bulbs (29-kDa) was identified to be a glycoprotein by periodic acid-Schiff (PAS) staining and N-terminal amino acid sequence was determined as SQLKAXIXDF. Although, it had not similar to any antifungal proteins, this protein was completely inhibits the germination of spores and hyphal growth of *Fusarium oxysporum* (Deepak *et al.*, 2003).

## **CHAPTER 2**

## Localization of protein in storage organs of C. alismatifolia

### 2.1 Introduction

The products of CO<sub>2</sub> assimilation are accumulated in plants in the form of oligoand polysaccharides. Amino acids, the products of nitrate assimilation, are finally stored as proteins. These are special storage proteins, which have no enzymatic activity, and often deposited in the cell within protein bodies. Protein bodies are surrounded by a single membrane and are derived from the endomembrane system of the endoplasmic reticulum and the Golgi apparatus or the vacuoles. Storage proteins can be accumulated in various plant organs, e.g., leaves, stems, and roots. They are stored in seeds and tubers and also in the cambium of tree trunks during winter, to enable rapid formation of leaves during seed germination and sprouting. Moreover, storage proteins are located in the endosperm in cereal and the cotyledons in legume seeds (Heldt, 1997). Storage proteins are both soluble and insoluble which store N and C compounds in seeds or bulbs to support plant growth especially, when they are harvested and it is consumed after planting (Ohyama, 2010). *Curcuma* plants have both rhizomes and storage roots as underground storage organs. The rhizome is important organ for nitrogen storage, while the storage roots are the major organ for carbohydrate storage such as starch and soluble sugar (Ruamrungsri et al., 2001). However, the predominant storage compound in underground storage organs is generally starch. For instances, Ranunculus asiaticus tuberous roots were found that the starch grains were present in the cortical cells of roots during their development which stained positively of stem tubers cellulose/ hemicelluloses (Kamenesky et al., 2005) and similar demonstrated in bulbs of Hyacinth and Nerine bowdenii (Addai and Scott, 2011; Theron and Jacobs, 1996).

As all proteins are composed of amino acids, direct histochemical staining of proteins is only possible via the properties of their amino acid residues. Thus, protein staining means amino acid staining and this clearly gives the limits of the field. However, to characterize and localize individual protein types in a much more precise way than via staining of amino acid side-group (James and Tas, 1984). There are several types of staining media; each can be used for a different purpose. For example, many conjugated

proteins can also be stained more or less selectively on the basis of their non-protein moiety, such as the periodic acid-Schiff (PAS) staining which can be used to demonstrate glycoproteins, Sakaguchi method for arginine, Millon reaction for tyrosine, DMAB nitrate reaction for tryptophan, DDD reaction for the thiol group (-SH) of cysteine and the disulfide bridge (-SS) of cysteine, Naphthol Yellow S are generally suitable for lysine, arginine and histidine, Coomassie blue commonly stains proteins a brilliant blue, and is often used in gel electrophoresis (Pearse, 1975). The iodine staining in seed plants has been useful for determining starch such as rice, wheat, maize, barley and sorghum (Nakamura *et al.*, 1998). Nevertheless, it was used to study the characteristics of waxy protein and amylose-free mutants in wheat and potato (Hovenkamp-Hermelink *et al.*, 1987; Nakamura *et al.*, 1995).

#### 2.2 Objectives

There are few reports about the location of storage proteins in the storage organ in *Curcuma* plants by using staining and light microscopy. Therefore, we aim to investigate the localization of storage proteins to understand the accumulation of protein in different parts.

#### 2.3 Materials and methods

#### **2.3.1 Rhizome and storage roots preparation**

The dormant rhizomes of *C. alismatifolia* 'Chiang Mai Pink' were obtained from H.M. the King's Initiative Centre for Flower and Fruit Propagation, Thailand, and it was divided into rhizome and storage roots part. Both organs were peeled and cut the tissue approximate 1.0 cm x 1.0 cm of size for cross section generation.

## 2.3.2 Freezing microtome (Cryostat) and Free-hand section

Frequently, the researcher requires a quick microscopic investigation of a certain tissue or organ and does not want to spend the time to fully prepare the tissue for paraffin or plastic embedding and sectioning (Ruzin, 1951). In this study alternative is to cross-sections of dormant rhizomes by frozen and encased in a support matrix using a

cryostat (Leica CM 1850; Leica Microsystems Nussloch GmbH, Nussloch, Germany) and sections of storage roots were prepared by hand using a razor blade.

## 2.3.2.1 Freezing microtome (Cryostat)

1. Cool the mounting stage to -35 °C by pressing the "Quick Freeze" bottom and wait for 30 min for the stage to reach temperature.

2. Pour a small amount of tissue-freezing medium (TFM) by Jung tissue freezing medium (Leica Microsystems, Wetzlar, Germany) on the mounting stub so that it will freeze and create a pad.

3. When the pad is frozen, add more TFM to the stub and place the tissue in the TFM. Wait until the TFM freezes; carefully manipulate the tissue to the correct orientation (upright for cross sections). Continue to surround the tissue until the sample is completely encased in solid TFM.

4. With a cold razor blade, face off the block to a broad square or trapezoid.

5. Transfer the block to the microtome and allow about 10 minute for the block to equilibrate to the temperature of chamber. With most plant material start sectioning at - 20 °C.

6. Adjust the antiroll plate by pushing it directly against the face of the knife and slightly below the knife edge.

7. Section at 40  $\mu$ m thickness. Ribbons are picked up with a cold forceps and put on the glass slides. Place the glass slide in the room temperature and then it will melt on slide. Specimens are already for staining further.

## 2.3.2.2 Free-hand section

Free-hand sectioning refers to the process of making sections or slices of tissues by hand using only a razor blade. Storage roots of *Curcuma* were sliced by a razor blade approximate 0.5 mm thickness that it can be seen through under microscopy. Cut tissues were put onto a wetted slide before staining.

#### 2.3.3 Staining specimens

## 2.3.3.1 Protein staining by Coomassie Brilliant Blue (CBB)

1. Prepare 0.25% (w/v) CBB solution; weigh 0.5 g of CBB R-250 dissolved in 200 ml of 50% (v/v) methanol and 10% (v/v) acetic acid.

2. Drop CBB solution into the specimens and leave it about 10 minutes.

3. Wash specimens by drop  $dH_2O$  in three times.

4. Observed under an Olympus BX51 light microscope. Images were taken using a DP 12 digital camera (Olympus, Tokyo, Japan.)

## 2.3.3.2 Starch staining by iodine solution (Nakamura et al., 1998)

1. Prepare KI-I<sub>2</sub> solution; weigh 0.74 g of I<sub>2</sub> and 1.48 g of KI dissolved in 400 ml of distilled water ( $dH_2O$ ).

2. Drop the KI-I<sub>2</sub> solution into the specimens. After 10 minute, each section was washed three-times in  $dH_2O$ .

3. Wash specimens by drop dH<sub>2</sub>O until clear specimen without stain.

4. Observed under an Olympus BX51 light microscope. Images were taken using a DP 12 digital camera (Olympus, Tokyo, Japan).

## 2.4 Results and discussions

## Localization of proteins in rhizomes and storage roots of C. alismatifolia Gagnep

Figure 2.1 showed the location of proteins and starch granules in the rhizome. Proteins in the rhizomes were stained with CBB as shown in Figure 2.1b and e.



**Figure 2.1** Transverse sections of dormant *C. alismatifolia* Gagnep. rhizomes in different staining and viewed with 10X (a, b and c) and 20X (d, e and f) of light microscopy.

A blue color was present in the cytosol and cell walls in rhizomes, and small particles, which may have been protein bodies, were heavily stained in the cytosol and also found that in the storage roots in Figure 2.2b and e. However, the intensity of CBB staining was stronger in rhizomes than in storage roots. Starch granules were stained with the KI-I<sub>2</sub> solution in the rhizome as shown in Figure 2.1c and f, and storage roots in Figure 2.2c and f. Storage roots contained a higher quantity of larger starch granules than rhizomes.



Figure 2.2 Transverse sections of dormant *C. alismatifolia* Gagnep. storage roots in different staining and viewed with 10X (a, b and c) and 20X (d, e and f) of light microscopy.

This result similar to Ruamrungsri *et al.* (2001) observed that nitrogen (N) and carbohydrates (C) in both the rhizomes and storage roots of *C. alismatifolia*, with rhizomes being the principal organ for N storage and storage roots being the major organ for C storage. Starch grains were present in cortical cells during the development of tuberous roots in *Ranunculus asiaticus* (Kamenetsky *et al.*, 2005), whereas a cross-section of stem tubers of *Plectranthus esculentus* showed that starch granules were abundant in the inner layer of the storage parenchyma (Allemann *et al.*, 2003).
## 2.5 Conclusion

It was confirmed that the rhizome of *C. alismatifolia* is a greater storage organ of N than storage roots, while the storage roots mainly store starch by using freezing microtome and free-hand section techniques together with CBB and KI-I<sub>2</sub> staining. The intensity of CBB staining of tissues was stronger in rhizomes than in storage roots. Protein staining was present in the cytosol and in the cell walls in rhizomes, and was intense in small particle-like protein bodies in the cytosol. This result is a basic data for study protein profile in both organs further.

## **CHAPTER 3**

## Changes of nitrogenous compound in storage organs of C. alismatifolia

### 3.1 Introduction

The underground storage organs play an important role for survival of some plant species by reserving foods, nutrients and moisture during seasonal growth and development (De Hertogh and Le Nard, 1993). Nitrogen (N) is one of the storage compounds that stores in the form of both soluble and insoluble protein in seeds or bulbs when plant are harvested and it is consumed after planting the seeds or bulbs (Ohyama, 2010).

*Curcuma alismatifolia* Gagnep. is a tropical geophytes, which has storage organs comprises of a stubbed stem or rhizome and storage roots (Apavatjrut *et al.*, 1999). It is a valuable crop for the cut flower industry and for potted plant production (Ruamrungsri, 2015). A rhizome is a major organ of nitrogen storage which nitrogen is a major element stored in bulbs and it is assimilated into free amino acids, proteins and other nitrogenous compounds related to the growth and developmental stages (Ruamrungsri, 2010). Most of N in new storage organ of *Curcuma* during dormancy was in the 80% ethanol insoluble fraction of high-molecular weight constituents such as protein, starch, cellulose, etc., and arginine was dominant free amino acid in new rhizomes and glutamic acid in new storage roots in soluble fraction (Ruamrungsri *et al.*, 2001). In *Tulipa*, storage N in both roots and mother bulb scales during the winter was used for leaf and stem growth after shooting in spring season (Ohyama *et al.*, 1988). Moreover, N absorption and accumulation was actively in roots of *Narcissus* after sprouting (Ruamrungsri *et al.*, 1996).

Although, Khuankaew (2010b) has been reported about assimilation and translocation of nitrogen during *Curcuma* growth by using <sup>15</sup>N-isotope in each organ, the changes of the nitrogenous compound in storage organs during different growth stages of *Curcuma* have been less discussed, which is considered to be essential for understanding N accumulation and utilization during growth cycle of this plant and to know the accumulated N form in storage organs for the further finding protein profiles.

## 3.2 Objectives

The aim of this experiment was to extract and separate the soluble N and insoluble N fractions in each stage of *Curcuma* growth for understanding N accumulation and utilization and for knowledge the basic information to investigate change of storage protein.

## 3.3 Materials and methods

## 3.3.1 Plant materials and cultivation

*C. alismatifolia* Gagnep cv. Chiang Mai Pink rhizomes were obtained from H.M. the King's Initiative Centre for Flower and Fruit Propagation, Thailand. Size of rhizome was approximately 1.5-2.0 cm of diameter and four storage roots. Rhizomes were soaked in fresh water for 3 days and changed the water every day to accelerate sprouting before planting. Each rhizome was planted in a 15 cm-diameter pot containing a 1: 1: 1 (v/v/v) mixture of perlite: vermiculite: sand. It was put on the bench and grown under the plastic house at Chiang Mai University, Thailand (Figure 3.1).



Figure 3.1 *C. alismatifolia* Gagnep. were planted in soilless medium pots in the plastic house at Chiang Mai University, Thailand

## **3.3.2** Watering and fertilizer supply

During cultivation, de-ionized water was supplied twice a week, as required, and 30 ml of a complete nutrient solution, consisting of N, P, K, Mg, Ca, Fe, Mn, Zn, Cu, Mo, B at the concentrations of 100, 50, 100, 25, 25, 5, 0.5, 0.025, 0.015, 0.015 and 1.0 mg/l respectively, was applied once a week from the time of shoot sprouting at 6 weeks after planting (WAP) until the above-ground plant parts senesced at 23 WAP.

### 3.3.3 Sampling

After that, plants were sampled at each of five different growth stages (Figure 3.2 A–E) where stage 1 (ST1) was at 0 WAP; Stage 2 (ST2) was 1 cm-long shoots sprouting at 6 WAP; Stage 3 (ST3) was the second fully-expanded leaf stage at 11 WAP; Stage 4 (ST4) was when the first floret opened at 17 WAP; and Stage 5 (ST5) was senescence of the above-ground plant parts and rhizome dormancy at 23 WAP; with four replications at each growth stage and five plants per replication. Old rhizomes and storage roots were separated at each stage and were washed several times by de-ionized water, and freeze-dried using a freeze dryer (Dura-Top/Dura Stop MP; Dura Dry MP; FTS Systems<sup>TM</sup>, Stone Ridge, NY, USA) for 3 days. Then, their dry weights (DWs) were measured.



Figure 3.2 C. alismatifolia Gagnep. in each growth stage for sampling; A, stage 1: before planting at 0 week after planting (WAP); B, stage 2: 1cm shoot sprouting at 6 WAP; C, stage 3: second fully-expanded leaf at 11 WAP; D, stage 4: the first floret open at 17 WAP; E, stage 5: dormancy at 23 WAP.

#### **3.3.4** N fraction of storage organs analysis

**3.3.4.1 Total N concentration;** Total N concentration was measured using Kjeldahl digestion and Indophenol method (Ohyama *et al.*, 1991) as follows;

1) Digestion method; to convert organic N in the sample to  $NH_4^+$ -N by digestion with concentrated  $H_2SO_4$  containing substances that promote oxidation of organic matter and conversion of organic N to  $NH_4^+$ -N.

## Procedure

1) Put 50 mg of a fine sample powder into a test tube ( $21 \times 200$  mm), add 1 ml of mixture (30 ml concentrated H<sub>2</sub>SO<sub>4</sub> and 1 g Salicylic acid) to it. Seal the tube by parafilm and mix well thoroughly by use of vortex mixer.

2) Leave the mixture at room temperature overnight.

3) Then, take off the parafilm add about 50 mg of Sodium thiosulfate and vortex thoroughly. Hat the tube at 100 °C for 1 hour, at 150 °C for 30 minutes and at 200 °C for about 1 hr. serially. After cooling at room temperature, put 0.3 ml of 30%  $H_2O_2$  into each tube for catalyst reaction, increase temperature to 230 °C (This process is repeated every 30 min until the digest solution is clear and colorless). This digestion is carried out under a hood with good circulation.

4) After cooling at room temperature, add a little deionized water, agitating gently and leave it overnight.

5) Transfer a digested solution to a 25 ml of volumetric flask fill up with deionized water to the final volume of 25 ml. This serves as sample for analysis.

### 2) Analysis method

The indophenol method to determine total nitrogen in the Kjeldahl digested solution. This method consists basically of reaction a sample containing  $NH_4^+$  with phenol (actually phenate) and hypochlorite at pH to form a blue color, the intensity of which is proportional to the  $NH_4^+$  concentration in the sample.

#### **Reagents prepare**

- **Reagent A** (EDTA reagent): Dissolve EDTA (ethylenediaminetetra acetic acid disodium salt) 6 g in 80 ml of deionized water, adjust pH 7, mix well and dilute to a final volume of 100 ml.

- **Reagent B** (1 M of KH<sub>2</sub>P0<sub>4</sub>): Dissolve KH<sub>2</sub>P0<sub>4</sub> 136.09 g and benzoic acid 2.75 g in 1 l of deionized water.

- Reagent C (Phenol-nitroprusside reagent): Dissolve sodium nitroprusside 100 mg in

phenol 10.25 ml, dilute to a final volume of 1 l with deionized water (use the sodium nitroprusside as a catalyst)

- **Reagent D** (Buffer hypochlorite reagent): .Put 10 g of NaOH (adjust pH 10 by 10N of NaOH), Na<sub>2</sub>HPO<sub>4</sub>.7H<sub>2</sub>O 7.07 g, Na<sub>3</sub>PO<sub>4</sub>.12H<sub>2</sub>O 31.8 g into a 500 ml of beaker, dissolve in deionized water and transfer to 1 l of volumetric flask, add 10 ml of sodium hyperchlorite, dilute to 1 l of flask with deionized water.

- **Standard** ammonium solution: Dissolve  $(NH_4)_2SO_4$  471.7 mg in 0.5 N of  $H_2SO_4$  1 l for 100 mg N l<sup>-1</sup> of a stock solution. Make standard concentration 0-0.7 mg N l<sup>-1</sup>

#### Procedure

1) Pipette sample solution of the Kjeldahl digested solution 0.1-2 ml into a 25 ml of volumetric flask; add 0.5 ml of reagent A, 0.5 ml of reagent B.

2) Add a small amount of 2N NaOH, for pH adjusts, until color changed.

3) Add 2.5 ml of reagent C; follow by 2.5 ml of reagent D.

4) Immediately dilute the flask to volume with deionized water and mix well. Maintain the flask at  $30^{\circ}$ C for 3 hr. and determine the absorbance of the colored complex at a wavelength of 625 nm against a reagent blank solution. Determine the NH<sub>4</sub><sup>+</sup>-N concentration of the sample by reference to a calibration curve plotted from the results obtained with a standard curve.

# **3.3.4.2** N fraction in PBS and TCA precipitation methods Reagents

1. Phosphate buffer solution (PBS); dissolved potassium dihydrogenphosphate ( $KH_2PO_4$ ) 1.75 g and dipotassium hydrogenphosphate ( $K_2HPO_4$ ) 1.36 g with de-ionized water, then adjusted pH to 6.8 by mixed these solutions and filled volume up to 1000 ml.

2. 20% (w/v) of trichloroacetic acid (TCA) cool at 4 °C in the ice box.

#### Procedure

1. Each dry powder (100 mg) was extracted three-times with 1.0 ml of PBS and separated into the PBS-soluble and PBS-insoluble fractions by centrifugation at 10,000 x g for 15 min.

2. Then 0.5 ml of 20% (w/v) trichloroacetic acid (TCA) was added to each PBS-soluble fraction to precipitate the proteins.

3. The TCA-insoluble and TCA-soluble sub-fractions were then separated by centrifugation at 10,000 g for 15 min.

4. The N concentration in these sub-fractions was determined by the Kjeldahl method (Ohyama *et al.*, 1991).

#### 3.4 Results and discussion

# 3.4.1 Changes of rhizome and storage roots dry weight at different stages of growth

The mean dry weights (DWs) of each old rhizome decreased slightly from 2.45  $\pm$  0.28 g at planting (ST1), to 1.02  $\pm$  0.21 g at dormancy (ST5; Table 3.1). The mean DW of the storage roots decreased similarly from 3.53  $\pm$  0.56 g at ST1, to 1.01  $\pm$  0.24 g at ST5.

Storage organ	<b>Growth stages</b> <sup>1/</sup>	Dry weight <sup>2/</sup>	
		(g)	
Rhizomes	ST1	$2.45\pm0.28$	
	ST2	$1.91 \pm 0.26$	
	ST3	$1.36 \pm 0.13$	
	ST4	$1.41 \pm 0.41$	
	ST5	$1.02 \pm 0.21$	
Storage roots	ST1	$3.53 \pm 0.56$	
	ST2	$3.46 \pm 0.58$	
	ST3	$1.46 \pm 0.12$	
	ST4	$1.40 \pm 0.29$	
	ST5	$1.01 \pm 0.24$	

 Table 3.1 Dry weights variations in the rhizome and storage roots of C. alismatifolia

 Gagnep. at different growth stages.

<sup>1/</sup> ST1: at planting, ST2: at 1 cm of shoot sprouting, ST3: at the second fully-expanded leaf,

ST4: at the first floret opened, ST5: at dormancy.

 $^{2/}$  Value are mean  $\pm$  standard derivation (SD).

In general, more than 90% of the dry matter (DM) in a plant consists of organic compounds such as starch, cellulose, lipids, and proteins (Marschner, 1986). Significant reductions in the DWs of rhizomes and storage roots from ST1 to ST3 indicated that the reserves of organic compounds were used during the early stages of growth, until 11 WAP. After ST3, the DWs of rhizomes and storage roots did not decrease significantly, as shown in Table 3.1. The re-mobilization of storage compounds to other organs is essential for

*Curcuma* growth. Khuankaew et al. (2010b) found that the translocation of newlyassimilated N and C to new rhizomes started 12 WAP, when new leaf formation terminated.

# 3.4.2 Total nitrogen concentration and total nitrogen content in rhizome and storage roots at different stages of growth

Table 3.2 showed the N concentrations (in mg N/ g DW) and N contents (in mg N/ organ) in the rhizomes and storage roots of *C. alismatifolia* from ST1 to ST5. Total N concentration in old rhizomes increased gradually from ST1 ( $23.87 \pm 2.89 \text{ mg N/ g DW}$ ) to ST3 ( $29.13 \pm 2.02 \ 29 \text{ mg N/ g DW}$ ) and it decreased rapidly at ST4 ( $14.89 \pm 3.25 \text{ mg N/ g}$  DW) and ST5 ( $15.52 \pm 2.08 \text{ mg N/ g DW}$ ). Total N concentration in the old storage roots at ST3 ( $6.53 \pm 1.00 \text{ mg N/ g DW}$ ) and ST5 ( $6.87 \pm 0.33 \text{ mg N/ g DW}$ ) was significantly higher than ST2 ( $4.51 \pm 0.42 \text{ mg N/ g DW}$ ). However, total N contents in old rhizomes decreased continuously from 58.62  $\pm 10.70 \text{ mg N/ organ}$  (at ST1) to  $16.04 \pm 4.94 \text{ mg N/ organ}$  (at ST5). A similar trend was observed in storage roots, from  $18.26 \pm 6.80 \text{ mg N/ organ}$  to  $6.99 \pm 1.88 \text{ mg N/ organ}$  during growth from ST1 – ST5.

Organ	Growth	N concentration <sup>2/</sup>	N content <sup>2/</sup>
	stages <sup>1/</sup>	( mg N/ g DW)	(mg N/ organ)
Rhizome			
	ST1	$23.87\pm2.89^{b}$	$58.62 \pm 10.70^{a}$
	ST2	$27.68\pm2.41^{ab}$	$52.92 \pm 8.13^{a}$
	ST3	$29.13 \pm 2.02$ <sup>a</sup>	$39.57\pm4.23^b$
	ST4	$14.89 \pm 3.25^{\circ}$	$20.20 \pm 3.53^{\circ}$
	ST5	$15.52 \pm 2.08^{\circ}$	$16.04 \pm 4.94^{\circ}$
Storage roots			
	ST1	$5.06 \pm 1.23^{ab}$	$18.26 \pm 6.80^{a}$
	ST2	$4.51\pm0.42^{b}$	$15.43 \pm 1.52^{ab}$
	ST3	$6.53 \pm 1.00^{a}$	$9.58\pm2.27^{bc}$
	ST4	$5.53\pm2.21^{ab}$	$7.97 \pm 4.45^{c}$
	ST5	$6.87\pm0.33^a$	$6.99 \pm 1.88^{\circ}$

 Table 3.2 Total N concentration and N content in the rhizome and storage roots of C.

 alismatifolia Gagnep. at different growth stages.

<sup>1/</sup> ST1: at planting, ST2: at 1 cm of shoot sprouting, ST3: at the second fully-expanded leaf, ST4: at the first floret opened, ST5: at dormancy.

<sup>2/</sup> Value are mean  $\pm$  standard derivation (SD) in the same column followed by different uppercase letters indicate significant difference at P  $\leq$  0.05 by the LSD test.

# 3.4.3 PBS soluble N and insoluble N in rhizome and storage roots during growth periods

The N content of the PBS-soluble fraction of rhizomes increased significantly from  $12.50 \pm 3.62 \text{ mg N}/\text{ organ}$  (at ST1) to  $19.74 \pm 4.00 \text{ mg N}/\text{ organ}$  (at ST2; Table 3.3). After ST2, N contents decreased markedly to  $3.69 \pm 1.32 \text{ mg N}/\text{ organ}$  (ST5). This indicated that some new proteins were synthesized at the shoot sprouting stage (ST2) and that storage proteins in these organs were used during leaf expansion and flowering thereafter. From the soluble properties of proteins, this storage protein might be albumins and globulins group which theirs can be extracted by PBS. They are predominant in legumes and oats (Bewley and Greenwood, 1990).

Organ	Growth	PBS-soluble <sup>2/</sup>	PBS-insoluble <sup>2/</sup>
	stages <sup>1/</sup>	( mg N/ organ)	(mg N/ organ)
Rhizome			
	ST1	$12.50 \pm 3.62^{b}$	$46.12 \pm 10.01^{a}$
	ST2	$19.74\pm4.00^{a}$	$33.18\pm7.02^{b}$
	ST3	$9.62\pm3.01^{bc}$	$29.95\pm5.59^{b}$
	ST4	$5.70\pm2.56^{cd}$	$14.50 \pm 2.31^{\circ}$
	ST5	$3.69 \pm 1.32^{d}$	$12.35 \pm 3.62^{\circ}$
Storage roots			
	ST1	$4.85\pm2.35^{a}$	$13.42 \pm 6.91^{a}$
	ST2	$6.35\pm1.01^{a}$	$9.08 \pm 1.10^{ab}$
	ST3	$2.02\pm0.69^{b}$	$7.56 \pm 1.68^{b}$
	ST4	$1.32\pm0.15^{b}$	$6.65\pm4.32^b$
	ST5	$1.50\pm0.51^b$	$5.49 \pm 1.53^{b}$

 C. alismatifolia
 Gagnep. at different growth stages.

<sup>1/</sup> ST1: at planting, ST2: at 1 cm of shoot sprouting, ST3: at the second fully-expanded leaf, ST4: at the first floret opened, ST5: at dormancy.

<sup>2/</sup> Value are mean  $\pm$  standard derivation (SD) in the same column followed by different uppercase letters indicate significant difference at P  $\leq$  0.05 by the LSD test.

Rapid protein synthesis was also observed in *C. longa* rhizomes at sprouting (Jayakumar *et al.*, 2001). In *Tulipa gesneriana*, N stored in both roots and old bulb scales was used rapidly for leaf and stem growth after sprouting in the spring (Ohyama *et al.*, 1988). In contrast, the N content of the PBS-insoluble fractions of rhizomes decreased continuously from  $46.12 \pm 10.01$  mg N/ organ (at ST1) to  $12.35 \pm 3.62$  mg N/ organ (at ST5). Moreover, the percentage of N in each fraction showed that N in the PBS-insoluble fraction accounted for 63-79% of the total N, and was higher than in the PBS-soluble fraction at every stage (Figure 3.3). This storage protein might be like glutelins in cereal seeds that can be soluble in alkali and acid (Bewley and Greenwood, 1990), but they are not readily extractable by dilute salt water (PBS). However, some N remained in PBS-insoluble fraction in rhizome at ST5, they should be the structural proteins.



**Figure 3.3** Percentage of total N in each PBS fraction at five different growth stages of *C*. *alismatifolia* Gagnep. rhizomes: ST1: at planting, ST2: at 1 cm of shoot sprouting, ST3: at the second fully-expanded leaf, ST4: at the first floret opened, ST5: at dormancy.

The N contents of the PBS-soluble and PBS-insoluble fractions of storage roots decreased significantly throughout the growth period (Table 3.3), and a higher percentage of N was observed in the PBS-insoluble fraction than in the PBS-soluble fraction (Figure 3.4). This result indicated that the majority of the N in storage roots was assimilated into conjugated or structural proteins as components of cell walls, membranes, and organelles, or into saline-insoluble storage proteins such as prolamin and glutelin, storage proteins in wheat, maize and rice, theirs are not readily extractable by dilute salt water (Bewley and Greenwood, 1990), rather than soluble proteins. The PBS-insoluble fraction decreased continuously from planting to dormancy, which may have been caused by the senescence and disintegration of cell components in the storage roots. In *Narcissus*, the percentage of N in the PBS-insoluble fraction was 67% in old bulb scales and increased in new scales at harvest (Ruamrungsri et al., 1997).



Figure 3.4 Percentage of total N in each PBS fraction at five different growth stages of *C*. *alismatifolia* Gagnep. storage roots: ST1: at planting, ST2: at 1 cm of shoot sprouting, ST3: at the second fully-expanded leaf, ST4: at the first floret opened, ST5: at dormancy.

# 3.4.4 TCA soluble N and insoluble N in rhizome and storage roots during growth periods

In the PBS-soluble fraction, the TCA-insoluble sub-fraction was composed mainly of proteins, while the TCA-soluble sub-fraction consisted of amino acids and low molecular weight compounds. Interestingly, the distribution of N in these two sub-fractions was different between rhizomes and storage roots throughout the growth period (Table 3.4).

Organ	Growth	TCA-soluble <sup>2/</sup>	TCA-insoluble <sup>2/</sup>
	stages <sup>1/</sup>	(mg N/ organ)	( mg N/ organ)
Rhizome			
	ST1	$4.10\pm3.10^{ab}$	$8.40\pm2.83^{b}$
	ST2	$7.94\pm3.40^a$	$11.79 \pm 1.77^{a}$
	ST3	$1.55 \pm 2.71^{b}$	$8.06\pm1.13^{b}$
	ST4	$2.66 \pm 1.78^{b}$	$3.04\pm0.87^{\text{c}}$
	ST5	$2.17\pm1.02^{b}$	$1.53 \pm 0.58^{\circ}$
Storage roots			
	ST1	$2.81 \pm 1.90^{ab}$	$2.03\pm0.88^{\text{a}}$
	ST2	$3.83 \pm 1.51^{a}$	$2.53 \pm 1.12^{a}$
	ST3	$1.25\pm0.67^{b}$	$0.77\pm0.28^{b}$
	ST4	$1.11\pm0.13^{b}$	$0.22\pm0.02^{b}$
	ST5	$1.27\pm0.39^{b}$	$0.24\pm0.19^{b}$

**Table 3.4** TCA sub-fraction of the PBS-soluble fraction in the rhizome and storage roots of *C. alismatifolia* Gagnep. at different growth stages.

<sup>1/</sup> ST1: at planting, ST2: at 1 cm of shoot sprouting, ST3: at the second fully-expanded leaf,

ST4: at the first floret opened, ST5: at dormancy.

<sup>2/</sup> Value are mean  $\pm$  standard derivation (SD) in the same column followed by different uppercase letters indicate significant difference at P  $\leq$  0.05 by the LSD test.

In rhizomes, N in the TCA-soluble sub-fraction increased from  $4.10 \pm 3.10 \text{ mg N/}$  organ (at ST1) to  $7.94 \pm 3.40 \text{ mg N/}$  organ (at ST2), then decreased markedly to  $1.55 \pm 2.7 \text{ mg N/}$  organ at ST3. N in the TCA-insoluble sub-fraction increased from  $8.40 \pm 2.83 \text{ mg N/}$  organ (at ST1) to  $11.79 \pm 1.77 \text{ mg N/}$  organ (at ST2), then decreased to  $1.53 \pm 0.58 \text{ mg N/}$  organ at ST5. In the storage roots, N in the TCA-soluble and TCA-insoluble sub-fractions increased from ST1 to ST2, then decreased continuously throughout the rest of the growing period. The majority of N in the PBS-soluble fraction of rhizomes occurred in the TCA-insoluble sub-fraction, especially at ST3 (84%; Figure 3.5).



Figure 3.5 Percentage of total N in each TCA sub-fraction of the PBS-soluble fraction at five different growth stages of *C. alismatifolia* Gagnep. rhizomes: ST1: at planting, ST2: at 1 cm of shoot sprouting, ST3: at the second fully-expanded leaf, ST4: at the first floret opened, ST5: at dormancy.

The major form and function of N in the rhizomes is as storage proteins. On the other hand, most N in the PBS-soluble fraction of storage roots was distributed in the TCA-soluble sub-fraction, which consisted of non-protein N compounds such as free amino acids, especially at ST4 (84%) and at ST5 (84%) as shown in Figure 3.6. The initial increase N in the TCA-insoluble and TCA-soluble sub-fractions at the sprouting stage (ST2) indicated the assimilation of N into proteins, amino acids, and other low molecular weight compounds in these organs. Moreover, there are two types of storage proteins; one is water soluble protein like albumin, and other is insoluble protein like glutelin. Ruamrungsri *et al.* (1997) reported that approximate 70% of the N in *Narcissus* roots was assimilated into the TCA-insoluble fraction, with 8% being TCA-soluble N at the sprouting stage. Increases in protein and RNA contents in *C. longa* rhizomes were also found at the initial stages of growth, and marked increases were observed at shoot sprouting, while some storage proteins were synthesized then disappeared at later stages (Jayakumar *et al.*, 2001).



**Growth stages** 

Figure 3.6 Percentage of total N in each TCA sub-fraction of the PBS-soluble fraction at five different growth stages of *C. alismatifolia* Gagnep. storage roots: ST1: at planting, ST2: at 1 cm of shoot sprouting, ST3: at the second fully-expanded leaf, ST4: at the first floret opened, ST5: at dormancy.

#### 3.5 Conclusion

Every growth stage of *Curcuma* plants, N was assimilated into conjugated or structural proteins and insoluble storage protein rather than soluble protein. The PBS-insoluble fraction was higher than the PBS-soluble fraction both in rhizome and storage roots. They decreased sharply when plants become to dormancy. After sprouting stage, PBS-soluble including TCA-insoluble and TCA-soluble fraction was the highest and it decreased thereafter, the fluctuation of the fraction associated the storage protein or amino acids and low molecular weight compound were utilized during leaf unfolding and flowering stage. Moreover, it might be synthesized a new proteins at sprouting stage.

## **CHAPTER 4**

### Protein characteristics in storage organs of C. alismatifolia

#### 4.1 Introduction

Proteins are extremely important for all organisms. There are several functions in protein such as enzymes, transporters, redox protein, structural proteins, storage proteins and proteins for moving (muscle) and etc. (Haschemeyer, 1973). The enzymes, which catalyze chemical reactions in cells (metabolism), are usually water-soluble protein. Transporters and redox protein such as cytochrome, and structural proteins are usually insoluble protein binding in membrane or cell wall. Storage proteins are both soluble and insoluble which store N and C compounds in seeds or bulbs when they are harvested, and it is consumed after planting the seeds or bulbs (Garrett and Grisham, 2005; Ohyama, 2010).

Foods reserves in storage organs are essential for flower bulb as a survival mechanism, they are utilized for shoot and roots emergence, floral initiation and development (Ree, 1972). Major storage nitrogen in bulbs is protein-N that assimilated from inorganic forms both NH<sub>4</sub>-N and NO<sub>3</sub>-N. Protein-N fraction in higher plant generally has amounts 80-85% of the total-N, by the others are nucleic acids-N (10%) and the soluble amino-N about 5% (Mengel and Kirkby, 1987). Curcuma has been reported by Ruamrungsri et al. (2001) that highly amount of N are stored in the rhizome more than in storage roots similar to Ohtake et al. (2006) found that Curcuma plant mainly stores N as protein in the rhizomes, while the storage roots accumulated N differently from amino acids or protein compounds and this corresponds to the N fertilization level. Moreover, the predominant low-molecular-weight peptides of approximately 12.0 kD and 10.6 kD, with minor bands of 29.1, 23.8 and 16.8 kD, was detected in the rhizome when separated by SDS-PAGE, while in storage roots could be detected many types of peptide bands. In C. longa rhizomes indicated an 18 kDa and a 23 kDa disappeared after 45 days of storage that occurred the degradation of storage protein during shoot sprouting (Javakumar et al., 2001). However, the protein profiles of the rhizomes and storage roots of this plant related to their growth stages have not yet been reported.

## 4.2 Objective

Therefore, we examined the characteristics and profiles of storage proteins in the rhizomes and storage roots of *C. alismatifolia* to understand the characteristics of N storage and N use throughout the plant growth cycle.

#### 4.3 Material and methods

#### 4.3.1 Plant materials and cultivation

*C. alismatifolia* Gagnep cv. Chiang Mai Pink rhizomes were obtained from H.M. the King's Initiative Centre for Flower and Fruit Propagation, Thailand. Each rhizome was planted as follows in Chapter 3 and plants also were sampled at each of five different growth stages e.g. stage 1 (ST1) was at 0 WAP; Stage 2 (ST2) was 1 cm-long shoots sprouting at 6 WAP; Stage 3 (ST3) was the second fully-expanded leaf stage at 11 WAP; Stage 4 (ST4) was when the first floret opened at 17 WAP; and Stage 5 (ST5) was senescence of the above-ground plant parts and rhizome dormancy at 23 WAP; with four replications at each growth stage and five plants per replication.

Old rhizomes and storage roots were separated at each stage and were washed several times by de-ionized water, and freeze-dried using a freeze dryer (Dura-Top/Dura Stop MP; Dura Dry MP; FTS Systems<sup>™</sup>, Stone Ridge, NY, USA) for 3 days. Then, their dry weights (DWs) were measured.

# 4.3.2 Protein concentration analysis by CBB method (The Bradford assay)4.3.2.1 Plant extraction

- 1. Weigh the freeze dry sample powder about 0.1 g in the mortar.
- 2. Add 1 ml of PBS and Quartz sand 0.01 g in the mortar.
- 3. Grind the sample to be fine (put the mortar on ice box).
- 4. Pour the solution in centrifuge tube and rinse mortar twice times by adding 500  $\mu$ l of PBS.
- 5. Balance weight each tube by add PBS, and then centrifuge at 14,000 rpm for 10 minutes at 4 °C.
- 6. Transfer only supernatant in 10 ml volumetric flask and fill up by PBS.

7. Transfer the extract to a 1.5 ml microcentrifuge tube and keep in refrigerator.

## 4.3.2.2 Analysis

#### **Standard reagents**

- 1. Weigh 10 mg of Albumin, from Bovine Serum (BSA) and put to 10 ml volumetric flask.
- 2. Adjust the volume by PBS and transfer to 1.5 ml microcentrifuge tube.

#### Procedure

- 1. Take the BSA standard solution 0, 20, 40, 60, 80 and 100 μl into the test tube and adjust to 100 μl by water each test tube.
- 2. Take the extract sample 10  $\mu$ l to a test tube and fill up to 100  $\mu$ l by water each test tube.
- 3. Add 1.9 ml CBB solution into standard and sample test tube and vortex to mix solution.
- 4. Measure absorbance of standard and sample solution by spectrophotometer at 595 nm.
- 5. Protein concentration determine by reference of calibration curve plotted from the results obtained with a standard curve.

## 4.3.3 Protein Content by SDS-PAGE gel

### 4.3.3.1 Plant extraction

- 1. Use the samples are extracted from protein concentration analysis (2.2.2) and calculate amount of samples and SDS-lysis buffer to add in each tube
- 2. Then, mix by vortex and heat the microcentrifuge tube at 120 °C for 2 minutes, then put in the ice box or keep in the refrigerator
- 3. Add 10  $\mu$ l of bromophenol blue (BPB) in each tube and mix by vortex.

### 4.3.3.2 SDS-PAGE gel (12.5% gel) preparation and analysis

- 1. Prepare the glasses for making a gel.
- 2. Prepare separation gel solution by 12.5% gel by the way:
- 2.1) Water 6.7 ml
- 2.2) Separation gel buffer 5 ml
- 2.3) 30% Acrylamide stock solution 8 ml

- 2.4) 10% Ammonium peroxodisulfate (APS) 0.2 ml
- 2.5) 10% Sodium Dodecyl Sulfate (SDS) 0.1 ml
- 2.6) N,N,N', N'-Tetramethyl-1,2-ethanediamine(TEMED) 20 µl
- 3. Pour the separation gel solution into the glasses about 80% of glasses.
- 4. Cover above part with water and wait for 20 minutes for gel is hardened.
- 5. Prepare staking gel solution by the way:
  - 5.1) Water 6.0 ml
  - 5.2) Staking gel buffer 2.5 ml
  - 5.3) 30% Acrylamide stock solution 1.4 ml
  - 5.4) 10% APS 0.1 ml
  - 5.5) 10% SDS 0.1 ml
  - 5.6) TEMED 20 µl
- 6. Pour the staking gel solution into the glasses and put the comb.
- 7. Wait the gel is hardened for 3 hours.
- 8. Prepare SDS-PAGE (10X) Running Buffer 400 ml.
- 9. Set the glasses into the box and pour the running buffer both inside and outside of glasses.
- 10. Load 5 and 10  $\mu$ l of samples and Marker into the gel.
- 11. Run gel under 120 voltages and 400 mA about 2 hours.
- 12. Cut the edge of gel and put into CBB solution for staining overnight.
- 13. Soak the gel with De-staining solution about 1 hour and rinse with water two or three times.
- 14. Dry gel about 3 hour and scanning gel by imaging scanner (Canon MG8100, Tokyo, Japan).
- 15. Use Image J program for calculated the Protein content (ng).

### 4.3.4 Screening protein profiles

### 4.3.4.1 Native PAGE (12.5% gel)

- 1. Prepare the glasses for making gels.
- 2. Prepare separation gel solution by 12.5% gel by the way:
  - 2.1) Acrylamide for separation 6 ml
  - 2.2) Buffer for Separation 4.5 ml
  - 2.3) Water 7.5 ml
  - 2.4) 10% APS 160 µl

2.5) TEMED 10 µl

- 3. Pour the separation gel solution into the glasses about 80% of glasses.
- 4. Cover above part with water and wait for 20 minutes for gel be harden
- 5. Prepare staking gel solution by the way:
  - 5.1) Acrylamide for stacking 1.5 ml
  - 5.2) Buffer for stacking 0.75 ml
  - 5.3) Water 3 ml
  - 5.4) Riboflavin (4 mg in 100 mL of water) 0.75 µl
  - 5.5) TEMED 5 µl
- 6. Pour the staking gel solution into the glasses and put the comb.
- 7. Wait the gel become hardens for 3 hours.
- Prepare Native-PAGE running buffer by Tris 7.57 g and Glycine
   36.05 g in the water fill up to 250 ml.
- Prepare the sample by take 20 μl of sample, 5 μl of BPB and 20 μl of Sucrose, then mixed by vortex.
- Set the glasses into the box and pour the running buffer both inside and outside of glasses.
- 11. Load 10 µl of samples and Marker into the gel.
- 12. Running gel at 120 voltages and 400 mA about 2 hours.
- 13. Cut the edge of gel and put into CBB solution or Silver staining overnight.
- De-staining solution and dry gel about 3 hours, and then scanning gel by imaging scanner (Canon MG8100, Tokyo, Japan).

### 4.3.4.2 1D-SDS -PAGE

- 1. Prepare the glasses for making gels.
- 2. Prepare IEF Gel follows below:
  - 1) Urea 2.75 g
  - 2) Acrylamide stock solution 0.65 ml
  - 3) 10% Nonidet P-40 Stock Solution 1.0 ml
  - 4) Water 1.0 ml

#### 5) Ampholine (pH 3.5-5.0) 0.25 ml in the beaker 25 ml

- 3. Warm a few minutes in Microwave for melting.
- 4. Add 10 µl of 10% APS and 7 µl of TEMED.
- 5. Pour the solution into the glasses about 80% of glasses.
- 6. Put the comb and wait for 30 minutes.
- Prepare Cathode solution by use Phosphoric acid 3.4 ml plus water 496 ml and Anode solution by NaOH 4 g plus water 500 ml.
- When the gel is hardened put the glasses into the box and pours the Phosphate solution outside and NaOH solution inside the glasses.
- 9. Use Cylinder clear in each channel of gel.
- 10. Add the sample protection in each channel of gel.
- 11. Pre- Gel using 50 voltage and 5mA for 30 minutes.
- 12. Extract the sample by grind the 0.1 g of freeze dried sample with 1 ml of PBS and centrifuge at 14,000 rpm, 4 °C for 10 minutes, and take only supernatant in other microcentrifuge tube. Add 0.1N HCL 20  $\mu$ l for white clear sample and then centrifuge again. Use sample only participated and added 100  $\mu$ l of sample-lysis solution. Use the pipette suck solution up and down until the participated soluble.
  - 13. Loading sample 10 μl and add sample protection solution clover above sample.
  - 14. Running Gel with 50 voltages for 5 hours.
  - Cut the gel and put into the15 ml centrifuge tube and add 1 ml of SDS solution.

## 4.3.5 Purification of protein by 2D-SDS PAGE

#### 4.3.5.1 Preparation SDS-PAGE Gel (15% gel)

- 1. Prepare the glasses for making gels.
- 2. Prepare separation gel solution by 15% gel by the way:
  - 2.1) Water 4.7 ml
  - 2.2) Separation gel buffer 5 ml
  - 2.3) 30% Acrylamide stock solution 10 ml
  - 2.4) 10% APS 0.2 ml
  - 2.5) 10% SDS 0.1 ml
  - 2.6) TEMED 20 µl

- Pour the Separation gel solution into the glasses about 80% of glasses.
- 4. Cover above part with water and wait for 20 minutes for gel be hardened.
- 5. Prepare staking gel solution by the way:
  - 5.1) Water 6.0 ml
  - 5.2) Staking gel buffer 2.5 ml
  - 5.3) 30% Acrylamide stock solution 1.4 ml
  - 5.4) 10% APS 0.1 ml
  - 5.5) 10% SDS 0.1 ml
  - 5.6) TEMED 20 µl
- 6. Pour the Staking gel solution into the glasses.
- 7. Wait for 3 hour for gel is hardened.
- 8. Prepare SDS-PAGE (10X) running buffer 400 ml.
- 9. Set the glasses into the box and pour the running buffer both inside and outside of glasses.
- 10. Put the 1D-gel on the glasses and made a straight gel.
- 11. Pour Agarose solution (heated at 60-80°C) on the 1D-gel.
- 11. Running gel at 120 voltages and 400 mA about 2 hours.
- Cut the edge of gel and put into CBB solution for staining 30 minutes.
- Soak the gel with De-staining solution about 1 hour and rinse with water 2-3 times.
- Dry gel about 3 hour and scanning gel by imaging scanner (Canon MG8100, Tokyo, Japan).

### 4.3.5.2 Mini Prep Cell (MPC)

A Mini Prep Cell (Model 491 Mini-Prep Cell; Bio-Rad Laboratory,

Hercules, CA, USA) was used to isolate native protein and checking by Native-PAGE as bellows;

- 1. Prepare separation gel solution same as **4.3.4.1**
- 2. Pour the separation gel solution into the tube about 4 ml.
- 3. Cover above part about 1-2 ml with water and wait for 20 minutes for gel is hardened.
- 4. Prepare staking gel solution same as 2.2.3.1.

- 5. Pour the staking gel solution into the tube about 1 ml.
- 6. Open the lamb for help harden gel and wait for 1.5 hours for gel is harden.
- 7. Prepare Native-PAGE running buffer 500 ml.
- 8. Set the tube into the box and put the running buffer followed.
- Prepare the sample by take 0.25 μl of sample, 10 μl of BPB and 0.25 μl of Sucrose, then mix by vortex.
- 10. Load all of samples into the gel and fill the running buffer solution until full the box.
- 11. Running gel at 150 voltages and 400 mA about 2.30 hours.
- 12. The elution speed was adjusted 0.5 ml per minute and fraction was collected every10 minutes after elution of BPB dye.
- When the elution finished, selected the sample solution (blue color).
- 14. Take 1 ml of sample solution in the microcentrifuge tube.
- 15. Fill 1 spoon of Sucrose powder and 4  $\mu$ l of BPB, then mix by vortex.
- 16. Loading the sample solution into the Native-PAGE gel.
- 17. Running gel at 120 voltages and 400 mA for 2 hours.
- Staining by CBB overnight and then, De-staining 1 hour and rinse with water 2-3 times.
- Dry gel for 3 hours and scanning gel by imaging scanner (Canon MG8100, Tokyo, Japan).

## 4.3.5.3 Mini Prep Cell ----Checking by SDS PAGE

- 1. Prepare the SDS-PAGE Gel same as above 4.3.3.2
- 2. Take 400 ul of sample solution (after eluted by Mini Prep Cell) into the microcentrifuge tube
- 3. Keep in -80°C for 2 hours
- 4. Freeze dry sample for 4 hours
- 5. Fill 25  $\mu$ l of sample lysis solution and 4  $\mu$ l of BPB, then mix by vortex
- 6. Loading the sample solution into the SDS-PAGE Gel
- 7. Running gel at 120 voltages and 400 mA for 2 hours

- Staining by CBB overnight and then, De-staining 1 hour and rinse with water 2-3 times
- Dry gel for 3 hours and scanning gel by imaging scanner (Canon MG8100, Tokyo, Japan).

#### 4.3.6 Identification of proteins

2D-PAGE was performed according to above method and some gels were used for amino acid sequencing. After polypeptides were separated by SDS-PAGE and were stained by CBB, it was transferred onto a polyvinylidene fluoride (PVDF) membrane and stained with CBB. A protein spot were cut-out and was determined by using the automatic Edman degradation method and a PPSQ-21 protein sequencer (Shimadzu, Kyoto, Japan). Moreover, the sequences were reconfirmed by the Hokkaido System Science Co. Ltd, Sapporo, Japan using a PPSQ-33A protein sequencer (Shimadzu, Kyoto, Japan).

### 4.4 Results and discussion

#### 4.4.1 Protein concentration of rhizome and storage roots in different growth stages

Total protein concentrations (in mg N/ g DW) and protein contents (in mg N/ organ) in the rhizomes and storage roots of *C. alismatifolia* from ST1 to ST5 showed in Table 4.1. Protein concentrations in old rhizomes decreased extremely from ST1 (223.42  $\pm$  83.5 mg/ g DW) to ST5 (38.06  $\pm$  8.1 mg/ g DW). However, protein concentration in the old storage roots increased from ST1 (41.25  $\pm$  10.1 mg/ g DW) to ST2 (66.77  $\pm$  32.6 mg / g DW), and then it was significantly decreased at ST4 (7.24  $\pm$  2.1 mg/ g DW). Similarly, total protein contents in old rhizomes decreased continuously from 543.93  $\pm$  204.4 mg/ organ (at ST1) to 37.72  $\pm$  5.6 mg/ organ (at ST5). A similar trend was found in storage roots that increased from ST1 (147.35  $\pm$  49.5 mg/ organ) to ST2 (225.60  $\pm$  93.6 mg/ organ) and decreased sharply to 10.43  $\pm$  4.6 mg/ organ during growth from ST2 – ST4. Finally, at ST5 was increased to 18.52  $\pm$  15.7 mg/ organ.

According to this volume represented clearly and sharply bands of polypeptides such as 10.6, 12.0, 16.0 and 18.0 kDa, although, some band (10.6 kDa) was not separated clearly but the intensity of bands and protein content had higher than at 5  $\mu$ l. Thus, this volume was chose to use into loading gel.

In addition, SDS-PAGE showed that the 10.6 kDa and 12.0 kDa polypeptides were the major storage proteins in rhizomes from ST1 to ST5 (Figure 4.1), while proteins of 15 - 20 kDa disappeared during ST4 and ST5, suggesting that these polypeptides were

used during the flowering and dormancy stages. A 29 kDa band was predominant during the early stages of scale culture in *Hyacinthus orientalis* bulbs; however, this polypeptide was almost undetectable after 8 weeks in culture (Yi *et al.*, 2002). Similar results were seen in the tuberous roots of *R. asiaticus*, in which the intensities of the 14 kDa, 23 kDa, 35 kDa, and 44 kDa bands declined during growth and disappeared at the last stage (Kamenetsky *et al.*, 2005).

Organ	Growth	<b>Protein concentration</b> <sup>2/</sup>	<b>Protein content</b> <sup>2/</sup>
	stages <sup>1/</sup>	( mg N/ g DW)	(mg N/ organ)
Old rhizome			
	ST1	$223.42 \pm 83.5^{a}$	$543.93 \pm 204.4^{a}$
	ST2	$166.30 \pm 47.0^{ab}$	$326.38 \pm 138.4^{b}$
	ST3	$142.96 \pm 17.9^{b}$	$194.82 \pm 34.4^{bc}$
	ST4	$60.63 \pm 8.6^{\circ}$	$84.38 \pm 21.9^{\circ}$
	ST5	$38.06 \pm 8.1^{\circ}$	$37.72 \pm 5.6^{\circ}$
Storage roots			
	ST1	$41.25 \pm 10.1^{b}$	$147.35 \pm 49.5^{b}$
	ST2	$66.77 \pm 32.6^{a}$	$225.60 \pm 93.6^{a}$
	ST3	$29.92\pm10.7^{bc}$	$43.15 \pm 14.5^{\circ}$
	ST4	$7.24 \pm 2.1^{\circ}$	$10.43 \pm 4.6^{\circ}$
	ST5	$17.13 \pm 11.2^{bc}$	$18.52 \pm 15.7^{\circ}$

**Table 4.1** Total protein concentration and protein content in the rhizome and storage roots of *C. alismatifolia* Gagnep. at five growth stages.

<sup>1/</sup> ST1: at planting, ST2: at 1 cm of shoot sprouting, ST3: at the second fully-expanded leaf,

ST4: at the first floret opened, ST5: at dormancy.

<sup>2/</sup> Value are mean  $\pm$  standard derivation (SD) in the same column followed by different uppercase letters indicate significant difference at P  $\leq$  0.05 by the LSD test.

#### 4.4.2 Protein content in different volume of sample loading to SDS-PAGE gel

To find the optimum volume of the sample loading to SDS-PAGE gel, we were compared between 10  $\mu$ l and 5  $\mu$ l of sample solution. The result showed that 10  $\mu$ l of sample was suitable for loading to the gel than 5  $\mu$ l as shown in Figure 4.1.



Figure 4.1 Protein content (ng) comparing between 10 μl (a) and 5 μl (b) of the sample loading to SDS-PAGE gel in the rhizome of *C. alismatifolia* at five stages of growth; ST1: at planting, ST2: at 1 cm of shoot sprouting, ST3: at the second fully-expanded leaf, ST4: at the first floret opened, ST5: at dormancy.

In storage roots, the major polypeptide separated by SDS-PAGE was only 14.3 kDa (Figure 4.2) and different from the two polypeptides in rhizomes. SDS-PAGE gel showed only this predominant band in both 10 and 5  $\mu$ l of sample loading, although, the intensity of these polypeptides at 5  $\mu$ l of sample loading was higher than at 10  $\mu$ l, the predominant band was not sharply when compared to at 10  $\mu$ l. Therefore, this volume was chose to use into loading gel further.



**Figure 4.2** Protein content (ng) comparing between 10 μl (a) and 5 μl (b) of the sample loading to SDS-PAGE gel in the storage roots of *C. alismatifolia* at five stages of growth; ST1: at planting, ST2: at 1 cm of shoot sprouting, ST3: at the second fully-expanded leaf, ST4: at the first floret opened, ST5: at dormancy.

# 4.4.3 Native-PAGE profiles comparing between CBB and silver staining in the rhizomes and storage roots of *C. alismatifolia* at each stage of growth

The protein profile of rhizomes separated by ND-PAGE showed four strong bands corresponding to polypeptides of 11, 14, 16, 19 and 20 kDa at ST1-ST3, detected by CBB staining (Figure 4.3a1), however, some of these bands disappeared at ST4 and ST5 thereafter. The protein profiles stained with silver showed stronger two bands of 19 and 16 kDa only at ST1-ST3 (Figure 4.3a2). The100 kDa band was observed sharply at ST1 and ST2 while, a 150 kDa showed only at ST2. These two bands were not represented when stained with CBB (Figure 4.3a1).

In the protein profiles of storage roots showed the major polypeptides bands of 14, 15, 18 and 20 kDa similarly when stained with CBB and silver (Figure 4.3b). These bands gradually disappeared from ST3 to ST5.

From the ND-PAGE profiles shown as in Figure 4.3 indicated that the lower molecular polypeptides weight about 14-20 kDa was interested and showed a predominant bands at beginning stage of plant growth (ST1) then it disappeared at flowering (ST4) and dormancy stage (ST5). These proteins may important roles for N storage. Since rhizomes play an important role as a protein storage organ, the characteristics of these rhizomes proteins were investigated further.



Figure 4.3 Non-denaturing- PAGE profiles of proteins compared staining with CBB (a1 and b1) and Silver (a2 and b2) in the rhizome (a) and storage roots (b) of *C*. *alismatifolia* at five stages of growth; ST1: at planting, ST2: at 1 cm of shoot sprouting, ST3: at the second fully-expanded leaf, ST4: at the first floret opened, ST5: at dormancy; M and kDa indicate size marker and kilodalton, respectively.

# 4.4.4 SDS-PAGE profiles in the rhizome comparing stained by silver, PAS, and CBB staining

CBB staining is based on dye binding to the amino groups in proteins (Miller *et al.*, 2006). On the other hand, silver staining of proteins depends on the binding of silver ions to amino acid side chains, primary to the sulphyryl groups in proteins, followed by reduction to free metallic silver (Rabilloud, 1999). Curiously, the 10.6, 12.0 and 16.8 kDa polypeptides did not appear to be stained by silver, although these were the dominant bands stained with CBB (Figure 4.4). These results suggest that rhizome peptides contain few if any sulphur amino acids such as methionine and cysteine. The  $\beta$ -subunit of  $\beta$ -conglycinin, a major storage protein in soybean seed, does not contain sulphur amino acids. As the concentration of the  $\beta$ -subunit was proportional to the concentration of N in seeds, this peptide may function as a form of storage for excess N (Ohtake *et al.*, 2002). The equivalent small peptide in *Curcuma* may play a role in the storage of N, while its composition was not affected by its N status (Ohtake *et al.*, 2006).

The result of periodic acid-Schiff (PAS) staining also revealed that the 10.6, 16.8 and 23.8 kDa peptides in *C. alismatifolia* rhizomes were conjugated with carbohydrates to form glycoproteins. The 29 kDa peptide in *Urginea indica* bulbs was stained with PAS and was identified as an anti-fungal glycoprotein (Deepak *et al.*, 2003).



Figure 4.4 SDS-PAGE protein profiles were stained with silver, periodic acid-Schiff reagent (PAS), or Coomassie Brilliant Blue (CBB).

# 4.4.5 Native-PAGE and SDS-PAGE with CBB-stained proteins purified using Mini Prep Cell on a 15% gel

Every protein fraction eluted by ND-PAGE using the mini-prep cell consisted of the predominant 12–14 kDa polypeptides separated by SDS-PAGE (Figure 4.5a, b). The consistent appearance of these polypeptide bands, together with decreasing N concentrations in the PBS-soluble fraction (storage protein) in rhizomes during the stages of growth (Chapter 3), suggests that these polypeptides were the main storage proteins in the rhizomes of *C. alismatifolia*.



Figure 4.5 Separation and amino acid sequences of six major storage polypeptides in the rhizomes of *C. alismatifolia* fractionations of rhizome proteins using a miniprep cell column. Each fraction (1–6) was separated by non-denaturing-PAGE and stained with Coomassie Brilliant Blue (a), each fraction was separated by SDS-PAGE in a 15% (w/v) polyacrylamide gel (b).

#### 4.4.6 2D PAGE representative in the rhizome at each growth stages of C. alismatifolia

2D-PAGE showed the 10.6 and 12.0 kDa polypeptides bands were major storage proteins in rhizome of C. alismatifolia, because they were found at almost all stages of growth (ST1-ST5) as shown in Figure 4.6. Although the intensity of these polypeptides was declined gradually throughout growth stage especially, at flowering (ST4) and dormancy (ST5) suggests that storage polypeptides in the rhizomes were used at all stages of plant. In the rhizome of *C. longa*, high intensity spots were observed in the acidic region using pH 4-7 and low abundant proteins (14 kDa) were enriched in the pH range 5.4-10 (Chokchaichamnankit et al. 2009), and the most abundant proteins in the mature tuberous roots of R. asiaticus were shown to have a molecular weight of less than 14 kDa, as observed by separation using SDS-PAGE (Kamenetsky et al. 2005), while a mass range of 14.4-17.0 kDa in the acidic region in C. comosa were manose-binding lectins (Boonmee et al. 2011). This protein in C. zedoary had a molecular weight of 13.4 kDa (Tipthara et al. 2007). In addition, six homologous lectin proteins with various molecular masses (8.8-32.8 kDa) were also found in C. aromatic (Tiptara et al. 2008). The major soluble protein in the Oca tuber (Oxalis tuberose Mol.) was shown to be Ocatin (18 kDa) with a 4.8 isoelectic point (Flores et al. 2002).



**Figure 4.6** Two-dimensional (IEF-SDS-PAGE) gel electrophoresis profile of PBS soluble protein fractions isolated from *C. alismatifolia* rhizomes in each stage of growth; ST1: at planting, ST2: at 1 cm of shoot sprouting, ST3: at the second fully-expanded leaf, ST4: at the first floret opened, ST5: at dormancy.

## 4.4.7 Representative proteins were separated using 2D electrophoresis and amino acids sequence of each spots from the rhizomes of *C. alismatifolia*

Two-dimensional-PAGE showed that the 10.6 kDa band contained more than five peptides (B1 to B5), while the 12.0 kDa polypeptide consisted of an A peptide (labelled A in Figure 4.7). Isoelectric focusing in the pH 4-7 range showed that the less abundant protein in the rhizomes of *C. longa* (14 kDa) was enriched in the pH range 5.4-10.0 (Chokchaichamnankit *et al.*, 2009), and that the most abundant proteins in mature tuberous roots of *R. asiaticus* had molecular weights  $\leq$  14 kDa, as observed using SDS-PAGE (Kamenetsky *et al.*, 2005).

The amino acid sequences of six of the polypeptides identified by 2D-PAGE were not homologous to any known proteins using BLASTP or FASTA analysis (DNA Data Bank of Japan). The sequence data also showed that none of these polypeptides started with a methionine residue. This suggests that these peptides had been post-translationally modified. This was similar to the N-terminal amino acid sequence (SQLKAXIXDF) of the major protein in bulbs of *Urginea indica*, which had no similarity to any anti-fungal protein (Deepak *et al.*, 2003). In *C. comosa*, the abundant protein spots in the acidic range were identified as lectins and cysteine proteases, as in other members of the Zingiberaceae (Boonme *et al.*, 2011). As the complete amino acid sequences of the six polypeptides could not be identified in our study, we suggest that these peptides were unique, based on homology searches. Further investigations should be conducted to provide more information on the function and regulation of these storage polypeptides in the rhizomes and storage roots of *C. alismatifolia* were different, but both were used at all stages of plant.



Figure 4.7 Representative proteins were separated using 2D-PAGE. Protein spot A is the 12.0 kDa protein, and spots B1 – B5 were isomers of the 10.6 kDa protein, amino acid sequences of each spot (A, B1 – B5) as bellows.

## Amino acid sequences;

### A: DRNVLYGGQILRTDE

N-terminal Asp-Arg-Asn-Val-Leu-Tyr-Gly-Gly-Gln-Ile-Leu-Arg-Thr-Asp-Glu

## **B1: ARNILTGGDRLNADE**

N-terminal Ala-Arg-Asn-Ile-Leu-Thr-Gly-Gly-Asp-Arg-Leu-Asn-Ala-Asp-Glu

## **B2: DRNVLGYNQKLRTDE**

N-terminal Asp-Arg-Asn-Val-Leu-Gly-Tyr-Asn-Gln-Lys-Leu-Arg-Thr-Asp-Glu

### **B3: DRNVLTGGQKLKVGE**

N-terminal Asp-Arg-Asn-Val-Leu-Tyr-Gly-Gly-Gln-Lys-Leu-Lyr-Val-Gly-Glu

## **B4: VTNVLILYAGLKLNT**

N-terminal Val-Thr-Asn-Val-Leu-Ile-Leu-Tyr-Ala-Gly- Leu-Lys-Leu-Asn-Thr

## **B5: DRNVLYGGQKLRTDE**

N-terminal Asp-Arg-Asn-Val-Leu-Tyr-Gly-Gly-Gln-Lys-Leu-Arg-Thr-Asp-Glu

## 4.5 Conclusion

The protein concentrations in both these organs decreased rapidly until the flowering stage in order to support the initial growth of roots and shoots. SDS-PAGE profiles showed that peptides of 10.6 kDa and 12.0 kDa were the two major soluble proteins in rhizomes and were different from the proteins in storage roots. The 10.6 kDa and 12.0 kDa bands contained five peptides and one peptide, respectively, when separated by 2D-PAGE. The amino acid sequences of these six peptides were not homologous to any known proteins using BLASTP or FASTA analysis. The N-terminal amino acid sequences of these two polypeptides did not start with methionine, indicating that they had undergone posttranslational modification. Knowledge of the major storage proteins in the rhizomes and storage roots of *C. alismatifolia* may provide information to improve N-fertilization.
### **CHAPTER 5**

## **General discussion**

Nitrogen (N) is a major essential element for all organisms, and it is compound in amino acids, protein, nucleic acids (DNA, RNA) and other important biochemical substances. Plants generally absorb nitrogen by the roots from soil. However, before developing the root system, they use storage nitrogen in the storage organ of plants, such as seeds, bulbs, rhizomes, tubers, storage roots. In these storage organs, N is reserved mainly in the form of storage protein (Ohyama, 2010).

*Curcuma alismatifolia* Gagnep. consisted of two underground storage organs that are a rhizome and storage roots. A rhizome is a major organ of N storage, and the storage roots mainly reserve carbohydrate with relatively low concentration of storage protein. N stored in the rhizomes and storage roots are used for initial growth and development after planting (Ruamrungsri *et al.*, 2001). From previous research, Khuankaew *et al.* (2010b) reported that most of absorbed N was translocated to leaves, and original N from old rhizomes and old storage roots were transported to aboveground parts to support growth and development. After that a new rhizome formed and N continuously accumulated in this organ. When the *Curcuma* senesced both of N from the nutrient solution and also N accumulated in the previous organ was assimilated and re-mobilized to new storage organs and mainly stored in the new rhizome. Therefore, the study on storage protein profiles in the rhizomes and storage roots of *Curcuma* is important to understand the characteristics of nitrogen storage and nitrogen use after planting in *Curcuma* cultivation.

To success the objective of this study, the experiments were divided into three parts. Firstly, locations of protein in storage organs of *Curcuma* were observed by using plant micro-techniques. Second experiment was studied on the changes of nitrogenous compounds in storage organs at five growth stages of *Curcuma* for understanding N utilization and gave the basic knowledge to investigate change of storage protein. Third parts focused on protein characteristics in storage organ of *Curcuma* in each growth stage and protein identification particular storage protein.

From the first study, the blue color of CBB was present in the cytosol and cell walls in rhizomes, and small particles which may have been protein bodies were heavily stained in the cytosol and also found that in the storage roots but, the intensity of CBB staining was stronger in rhizomes than in storage roots. While, many starch granules were stained with the KI-I<sub>2</sub> solution in both rhizome and storage roots. However, storage roots contains more numerous of larger starch granules than rhizomes. This result similar to Ruamrungsri *et al.* (2001) observed that nitrogen and carbohydrates in both the rhizomes and storage roots of *C. alismatifolia*, with rhizomes being the principal organ for N storage and storage roots being the major organ for C storage. Starch grains were present in cortical cells during the development of tuberous roots in *Ranunculus asiaticus* (Kamenetsky *et al.*, 2005), whereas a cross-section of stem tubers of *Plectranthus esculentus* showed that starch granules were abundant in the inner layer of the storage parenchyma (Allemann *et al.*, 2003).

The second parts, significant reductions in the DWs of rhizomes and storage roots from ST1 to ST3 indicated that the reserves of organic compounds were used during the early stages of growth, until 11 WAP. After ST3, the DWs of rhizomes and storage roots did not decrease significantly. The re-mobilization of storage compounds to other organs is essential for Curcuma growth. Khuankaew et al. (2010b) found that the translocation of newly-assimilated N and C to new rhizomes started 12 WAP, when new leaf formation terminated. Total N contents in old rhizomes and old storage roots decreased continuously during growth from ST1-ST5 similar to the N content in PBS-insoluble fraction. This result indicated that the majority of the N in both organs was assimilated into conjugated or structural proteins as components of cell walls, membranes, and organelles, or into salineinsoluble storage proteins rather than soluble proteins. The PBS-insoluble fraction decreased continuously from planting to dormancy, which may have been caused by the senescence and disintegration of cell components in that storage organ. In Narcissus, the percentage of N in the PBS-insoluble fraction was 67% in old bulb scales and increased in new scales at harvest (Ruamrungsri et al., 1997). In terms of soluble protein in PBSsoluble fraction or amino acids and low molecular weight compound in TCA sub-fraction of the PBS-soluble fraction, found that N content was the highest at sprouting stage (ST2) and decreased thereafter. This indicated that some new proteins were synthesized at the shoot sprouting stage (ST2) and that storage proteins in these organs were used during leaf expansion and flowering thereafter. Rapid protein synthesis was also observed in C. longa rhizomes at sprouting (Jayakumar et al., 2001). In Tulipa gesneriana, N stored in both roots and old bulb scales was used rapidly for leaf and stem growth after sprouting in the spring (Ohyama et al., 1988). N in the TCA-soluble sub-fraction increased from ST1to

ST2, then decreased markedly at ST3 similarly observed in the TCA-insoluble sub-fraction in both organs. Nevertheless, the distribution of N in these two sub-fractions was different between rhizomes and storage roots throughout the growth period. The majority of N in the PBS-soluble fraction of rhizomes occurred in the TCA-insoluble sub-fraction, especially at ST3 (84%) suggests that the major form and function of N in the rhizomes is as storage proteins. On the other hand, most N in the PBS-soluble fraction of storage roots was distributed in the TCA-soluble sub-fraction, which consisted of non-protein N compounds such as free amino acids, especially at ST4 (84%) and at ST5 (84%). The initial increase N in the TCA-insoluble and TCA-soluble sub-fractions at the sprouting stage (ST2) indicated the assimilation of N into proteins, amino acids, and other low molecular weight compounds in these organs. Ruamrungsri et al. (1997) reported that about 70% of the N in Narcissus roots was assimilated into the TCA-insoluble fraction, with 8% being TCAsoluble N at the sprouting stage. Increases in protein and RNA contents in C. longa rhizomes were also found at the initial stages of growth, and marked increases were observed at shoot sprouting, while some storage proteins were synthesized then disappeared at later stages (Jayakumar et al., 2001). Osborne (1924) classified protein to albumin, globulin, prolamine and glutelin according to their solubility. Leguminous seeds contain albumin and globulin, so we can extract them by dilute salt water. However wheat, maize and rice contain mainly prolamin and glutelin, so they are not readily extractable by dilute salt water. So, in this storage proteins may be a group of albumin and globulin, which can be extracted by PBS. However, the N content in PBS-insoluble fraction was much higher than PBS-soluble fraction. In addition to storage protein, there are some structural proteins, which are strongly bound to cell wall or cell membranes, but the content may be less than 10%. So we cannot consider the most part of PBS-insoluble proteins are structural proteins. Also structural proteins usually are difficult to degrade, but the N content in PBS-insoluble fraction decreased from ST1 to ST5 in rhizomes and storage roots may be some kind of storage protein like gluteiln in cereal seeds. However, some N remain in PBS-insoluble fraction in rhizome (12.35 mgN) and in storage roots (5.49 mgN) at ST5, they should be the structural proteins.

Finally part, we tried to separate and identify proteins in each growth stage of *Curcuma*. When protein was extracted by PBS solution in the experiment 2, protein were purified and separated by non-denaturing (ND)-PAGE and SDS-PAGE. The protein profile of rhizomes separated by ND-PAGE showed four strong bands corresponding to

polypeptides of 14 kDa, 16 kDa, 19 kDa, and 20 kDa, with two minor bands of 25 kDa and 75 kDa detected by CBB staining. Since rhizomes play an important role as a protein storage organ, the characteristics of these rhizomes proteins were investigated further. SDS-PAGE showed that the 10.6 kDa and 12.0 kDa polypeptides were the major storage proteins in rhizomes from ST1 to ST5, while proteins of 15–20 kDa disappeared during ST4 and ST5, suggesting that these polypeptides were used during the flowering and dormancy stages. However, the major polypeptide separated by SDS-PAGE from storage roots was found only a 14.3 kDa and different from the two polypeptides in rhizomes. In other bulbous plants, A 29 kDa band was predominant during the early stages of scale culture in *Hyacinthus orientalis* bulbs; however, this polypeptide was almost undetectable after 8 weeks in culture (Yi *et al.*, 2002). Similar results were seen in the tuberous roots of *R. asiaticus*, in which the intensities of the 14 kDa, 23 kDa, 35 kDa, and 44 kDa bands declined during growth and disappeared at the last stage (Kamenetsky *et al.*, 2005).

CBB staining is based on dye binding to the amino groups in proteins (Miller et al., 2006). On the other hand, silver staining of proteins depends on the binding of silver ions to amino acid side chains, primary to the sulphyryl groups in proteins, followed by reduction to free metallic silver (Rabilloud, 1999). Remarkably, the 10.6 kDa, 12.0 kDa, and 16.8 kDa polypeptides did not appear to be stained by silver, although these were the dominant bands stained with CBB. These results suggest that rhizome peptides contain few if any sulphur amino acids such as methionine and cysteine. The  $\beta$ -subunit of  $\beta$ conglycinin, a major storage protein in soybean seed, does not contain sulphur amino acids. As the concentration of the  $\beta$ -subunit was proportional to the concentration of N in seeds, this peptide may function as a form of storage for excess N (Ohtake et al., 2002). The equivalent small peptide in *Curcuma* may play a role in the storage of N, while its composition was not affected by its N status (Ohtake et al., 2006). The result of periodic acid-Schiff (PAS) staining also revealed that the 10.6 kDa, 16.8 kDa, and 23.8 kDa peptides in C. alismatifolia rhizomes were conjugated with carbohydrates to form glycoproteins. The 29 kDa peptide in Urginea indica bulbs was stained with PAS and was identified as an anti-fungal glycoprotein (Deepak et al., 2003). Every protein fraction eluted by ND-PAGE using the mini-prep cell consisted of the predominant 12-14 kDa polypeptides separated by SDS-PAGE. The consistent appearance of these polypeptide bands, together with decreasing N concentrations in the PBS-soluble fraction (storage protein) in rhizomes during the stages of growth, suggests that these polypeptides were the main storage proteins in the rhizomes of C. alismatifolia.

Two-dimensional-PAGE showed that the 10.6 kDa band contained more than five peptides (B1 to B5), while the 12.0 kDa polypeptide consisted of an A peptide. Isoelectric focusing in the pH 4-7 range showed that the less abundant protein in the rhizomes of C. longa (14 kDa) was enriched in the pH range 5.4-10.0 (Chokchaichamnankit et al., 2009), and that the most abundant proteins in mature tuberous roots of R. asiaticus had molecular weights  $\leq$  14 kDa, as observed using SDS-PAGE (Kamenetsky et al., 2005). The amino acid sequences of six of the polypeptides identified by 2D-PAGE were not homologous to any known proteins using BLASTP or FASTA analysis (DNA Data Bank of Japan). The sequence data also showed that none of these polypeptides started with a methionine residue. This suggests that these peptides had been post-translationally modified. This was similar to the N-terminal amino acid sequence (SQLKAXIXDF) of the major protein in bulbs of *Urginea indica*, which had no similarity to any anti-fungal protein (Deepak et al., 2003). In C. comosa, the abundant protein spots in the acidic range were identified as lectins and cysteine proteases, as in other members of the Zingiberaceae (Boonme et al., 2011). Recently, a Cystatin (cysteine proteases inhibitors) was isolated from a cDNA library from flower tissue of C. alismatifolia. The deduced amino acid sequence consists of a putative N-terminal secretory signal peptide of 22 amino acids and measured molecular mass of 11.2 kDa of the mature protein and about 12 kDa using SDS-PAGE. There are highly conserved blocks included Gly-Gly, the reactive site motif QXVXG and PW residues located in the C-terminal region of protein (Porruana *et al.*, 2013).

As the complete amino acid sequences of the six polypeptides could not be identified in our study, we suggest that these peptides were unique, based on homology searches. Further investigations should be conducted to provide more information on the function and regulation of these storage proteins during the growth of *C. alismatifolia* Gagnep. This research revealed that storage polypeptides in the rhizomes and storage roots of *C. alismatifolia* were different, but both were used at all stages of plant growth and development, especially during the early stages (ST2 to ST3). This information may help to optimize the efficient supply of N fertilizer during *Curcuma* cultivation. The mainly results were concluded as follow;

1. It was confirmed that the rhizome of *C. alismatifolia* is a greater source of N storage than storage roots, while the storage roots mainly store starch that demonstrated by using freezing microtome and free-hand section techniques together with CBB and KI-I<sub>2</sub> staining. The intensity of CBB staining of tissues was stronger in rhizomes than in storage

roots. Protein staining was present in the cytosol and in the cell walls in rhizomes, and was intense in small particle-like protein bodies in the cytosol.

2. N was assimilated into conjugated or structural proteins and insoluble storage protein rather than soluble protein all growth stages. The PBS-insoluble fraction was higher than the PBS-soluble fraction both in rhizome and storage roots. There were decreased sharply when plant become to dormancy. After sprouting stage, PBS-soluble including TCA-insoluble and TCA-soluble fraction was the highest and it was decreased thereafter, the fluctuation of the fraction associated the storage protein or amino acids and low molecular weight compound were utilized during leaf unfolding and flowering stage. Moreover, it might be synthesized a new proteins at sprouting stage.

3. The protein concentrations in both these organs decreased rapidly until the flowering stage in order to support the initial growth of roots and shoots.

4. SDS-PAGE profiles showed that peptides of 10.6 kDa and 12.0 kDa were the two major soluble proteins in rhizomes and were different from the proteins in storage roots. The 10.6 kDa and 12.0 kDa bands contained five peptides and one peptide, respectively, when separated by 2D-PAGE.

5. The amino acid sequences of these six peptides were not homologous to any known proteins using BLASTP or FASTA analysis. The N-terminal amino acid sequences of these two polypeptides did not start with methionine, indicating that they had undergone posttranslational modification.

6. The understanding of the major storage proteins in the rhizomes and storage roots of *C. alismatifolia* may provide information to improve N-fertilization during theirs cultivation.

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# **Abstract in English**

Curcuma alismatifolia Gagnep. consisted of two underground storage organs that are a rhizome and storage roots. Both organs support the initial growth of *Curcuma* plants, and become senescent at the end of growth. A rhizome is a major organ of N storage, and the storage roots mainly reserve carbohydrate with relatively low concentration of storage protein. N stored in the rhizomes and storage roots are used for initial growth and development after planting (Ruamrungsri et al., 2001). From previous research, Khuankaew et al. (2010) reported that most of absorbed N was translocated to the leaves, and original N from old rhizomes and old storage roots were transported to aboveground parts to support growth and development. After that a new rhizome formed and N continuously accumulated in this organ. When the Curcuma senesced both of N from the nutrient solution and also N accumulated in the previous organ was assimilated and remobilized to new storage organs and mainly stored in the new rhizome. Therefore, the study on storage protein profiles in the rhizomes and storage roots of *Curcuma* is important to understand the characteristics of nitrogen storage and nitrogen use after planting in Curcuma cultivation. To success the objective of this study, the experiments were divided into three parts. First, localizations of protein in storage organs of *Curcuma* were observed by using plant micro-techniques. Second, experiment was studied on the changes of soluble and insoluble nitrogenous compounds in storage organs at five growth stages of Curcuma for understanding N utilization and gave the basic knowledge to investigate change of storage protein. Third parts focused on the protein characteristics in storage organ of Curcuma in each growth stage and protein identification particular storage protein.

#### I. Localization of protein in storage organs of C. alismatifolia

From the first study, it was confirmed that the rhizome of *C. alismatifolia* is a greater source of N storage than storage roots, while the storage roots mainly store starch that demonstrated by using freezing microtome and free-hand section techniques together with CBB and KI-I<sub>2</sub> staining. The intensity of CBB staining of tissues was stronger in rhizomes than in storage roots. Protein staining was present in the cytosol and in the cell walls in rhizomes, and was intense in small particle-like protein bodies in the cytosol.

#### II. Changes of nitrogenous compound in storage organs of C. alismatifolia

Freeze-dried powder of old rhizomes and old storage roots harvested at 5 stages were extracted with dilute phosphate buffer (PBS), and the N concentrations of PBS-soluble and PBS-insoluble fractions are analyzed. PBS soluble fraction contains water-soluble compounds, such as soluble proteins and amino acid. On the other hand, PBS-insoluble fraction contains mainly insoluble protein, nucleic acid and lipids. At every growth stage, the amount of N in the PBS-insoluble fraction was 3-4 times higher than the PBS-soluble fraction in both rhizomes and storage roots. Along with growth stages, both PBS-soluble and PBS-insoluble N in rhizome and storage roots were decreased sharply when plants become to dormancy, which indicates that most of N stored in rhizome and storage roots are a kind of storage proteins supplying N for plant growth. PBS-soluble fractions are further fractionated into trichloroacetic acid (TCA) soluble and insoluble fractions. TCAinsoluble fraction contains proteins, and TCA-soluble fraction contains amino acids and low molecular weight compounds. From planting to sprouting stage, the N in the TCAinsoluble tentatively increased both in rhizomes and storage roots, suggesting that protein synthesis was active using amino acids in this stage for degradation of storage compounds such as starch and storage nitrogen.

#### III. Protein characteristics in storage organs of C. alismatifolia

Protein profiles in the PBS soluble fraction of rhizomes and storage roots were further analyzed by SDS-PAGE. The results of protein profiles showed that peptides of 10.6 kDa and 12.0 kDa were the two major soluble proteins in rhizomes and were different from the proteins in storage roots. The major polypeptide separated by SDS-PAGE from storage roots was found only a 14.3 kDa. In rhizomes, the 10.6 kDa and 12.0 kDa bands contained five peptides and one peptide, respectively, when separated by 2D-PAGE. The amino acid sequences of these six peptides were not homologous to any known proteins using BLASTP or FASTA analysis. The N-terminal amino acid sequences of these two polypeptides did not start with methionine, indicating that they had undergone posttranslational modification. Knowledge of the major storage proteins in the rhizomes and storage roots of *C. alismatifolia* may provide information to improve N-fertilization.

# **Abstract in Japanese**

# クルクマにおける貯蔵タンパク質の性質と利用に関する研究

クルクマ(Curcuma alismatifolia Gagnep.)は、地下部に根茎と貯蔵根の二つの 貯蔵器官を持つ。どちらの器官もクルクマの初期生育に養分を供給して生育期間 の終わりには枯死する。これまでの研究で、根茎は主に窒素貯蔵の働きをするが、 一方、貯蔵根は、貯蔵タンパク質濃度が低く、主に炭水化物を貯蔵することが報 告されている。根茎および貯蔵根中の貯蔵タンパク質は、クルクマの生育成長と 分化に利用されることが報告されている(Ruamrungsri. et al., 2010)。さらに Khuankaew ら(2010)は、根から吸収した窒素と根茎、貯蔵根由来の窒素は、大 部分が葉の生長に伴い葉へ移行し、その後、新器官の分化や生長に再利用される ことを報告した。生育後期には、数本の花茎の下部に新しい根茎と貯蔵根が形成 され窒素とデンプンが貯蔵される。最終的にクルクマが成熟枯死するときに、培 地から吸収した窒素も、古い貯蔵器官由来の窒素も大部分が新しい根茎に再移動 し貯蔵される。したがって、根茎と貯蔵根の貯蔵タンパク質の性質と利用に関す る研究はクルクマ栽培上も重要であると考えられる。本研究は、次の3つを課題 とした。1) 顕微鏡観察によるクルクマの貯蔵器官におけるタンパク質の集積部 位、2) クルクマの生育期間の5ステージにおける貯蔵器官中の可溶性窒素、不 溶性窒素の変化、3) クルクマの貯蔵タンパク質の性質と同定について研究を実 施した。

#### I. クルクマの貯蔵器官におけるタンパク質の集積部位

貯蔵器官の徒手切片を作成し、タンパク質染色にクマシーブリリアントブルー (CBB)を、デンプンの染色にヨウ素・ヨウ化カリウム溶液を用いて、タンパク質 とデンプンの蓄積を顕微鏡観察した。CBB 染色では、根茎の方が貯蔵根よりも強 く染まり、細胞質のプロテインボディ様の粒子が強く染色された。 Ⅱ. クルクマの生育期間における貯蔵器官中の可溶性窒素、不溶性窒素の変化

植付け時の根茎と貯蔵根を生育時期別に5つのステージで採取し、凍結乾燥後 粉砕した。粉末を低濃度のリン酸緩衝液(PBS)で抽出し、PBS 可溶性画分とPBS 不溶性画分に分画した。PBS 可溶性画分は、主として水溶性タンパク質および遊 離アミノ酸などを含み、PBS 不溶性画分は、不溶性タンパク質や核酸、脂質など をふくむ。根茎、貯蔵根ともに、PBS 不溶性画分が PBS 可溶性画分の 3~4 倍多く の窒素を含んでいた。生育ステージが進むに連れて、両器官ともに、PBS 可溶性 画分も PBS 不溶性画分も急激に減少した。このことは、どちらの画分もある種の 貯蔵タンパク質を含んでおり、生育器官中に分解されて植物に窒素を供給すると 考えられる。PBS 可溶性画分は、さらに、トリクロロ酢酸(TCA)を加えて、TCA 可 溶性画分と TCA 不溶性画分に分けた。TCA 不溶性画分は主にタンパク質を、TCA 可 溶性画分は遊離アミノ酸などを含む。球根植え込み後から萌芽までの間に、根茎 も貯蔵根も一時的に TCA 可溶性画分が減少し TCA 不溶性画分が増加した。このこ とは、萌芽までに貯蔵組織でタンパク質合成が起こったことを示唆する。

#### III. クルクマの貯蔵タンパク質の性質と同定

根茎と貯蔵根の PBS 可溶性画分に含まれる可溶性タンパク質をポリアクリルア ミドゲル電気泳動 (SDS-PAGE) で分離し、貯蔵タンパク質の生育時期別変化と同 定を試みた。SDS-PAGE の結果、根茎に含まれる主要な貯蔵タンパク質は、10.6 kDa と 12.0kDa の二つであり、これらは貯蔵根の主要な貯蔵タンパク質バンド (14.3kDa)と分子量が異なっていた。根茎のタンパク質を二次元電気泳動でさらに 分離したところ、10.6 kDa のバンドには、5 種のペプチドが、12.0kDa のバンド には1 種類のペプチドが含まれた。6 種のペプチドのアミノ酸配列を調べて、 BLASTP および FASTA 検索をしたところ、既知のタンパク質との相同性は認められ なかった。また、どのペプチドもN 末端にメチオニンを含んでいなかったので、 翻訳後修飾が行われていると考えられた。クルクマの貯蔵タンパク質に関する研 究は、窒素施肥の改善に役立つことが期待される。

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