CHAPTER 1

INTRODUCTION

1.1 Statement and significance of the problem

Rice is the major staple food in Thailand and contributes to the human diet. It has been in demand in the world market because of the problem of malnutrition worldwide. India and Vietnam used to be the main rice export countries, lowered their exported rice to the world market. Since, they had to stored rice as food storage in their countries. Therefore, Thailand became the main rice export country. The total rice exported during 1 January-31 March 2008 was 3.255 million tons which was an increase from the last year of about 66.75%. The farmers sold the whole grain rice at a high average price range of 10,148-14,551 bath/ton.
(Office of Agricultural economic. 2009. "News." [online] . Available http://www. oae. go.th /main.php?filename=index (30 June 2009).

Not only rice plants are grown for export as described above, but Thai people also cultivated rice for their own consumption. The ordinary polished rice, of which the outer rice shell is removed from whole rice grain, is used to cook as the main form for consumption. The ordinary polished rice contains dietary fibers, essential amino acids, proteins, carbohydrates, vitamins and other non-nutrient essential phytochemicals, which are main concentrated source in rice endosperm (Ohtsubo *et al*., 2005; Kuo *et al*., 2004).

However, ordinary polished rice contains less nutrition components (dietary fibers, phytic acids, vitamins and gamma-amino butyric acid (GABA) than the brown rice. These bio-functional components exist mainly in germ and bran layer which are removed as rice bran during whitening and milling operation (Champagne *et al*., 2004).

Therefore, nutrition of polished rice should be concerned and needs to be improved before consumption.

GABA is currently an interesting compound. Many research reported that it presented in germinated seed via protein metabolism of seed components

Several reports suggested that seedling extracts containing high GABA leveled were effective for lowering the blood pressure of experimental animals, recovery of alcohol-related symptoms, being as a major neurotransmitter (Komatsuzaki *et al*., 2007; Zhang *et al*., 2006; Kuo *et al*., 2004; Oh 2003) and helped improving memory and learning ability of mouse (Miura *et al*., 2006). Recently, brown rice extracts with enhanced levels of GABA had an inhibitory action on leukemia cell proliferation and had a stimulatory action on the cancer cell apoptosis (Komatsuzaki *et al*., 2007).

Many researcher attempted to increase GABA levels in rice, for example by using biotechnology methods such as transferring glutamate decarboxylase (GAD) genes for higher GABA production (Park and Oh 2006), fermentation methods for conversion of L-glutamic acid (L-glu) substrate to GABA (Su *et al*., 2003; Kono and Himeno, 2000; Huang *et al*., 2007) and other methods including seed germination (Komatsuzaki *et al*., 2007; Oh and Choi 2001; Xing *et al*., 2007) as shown in Table 1.1.

However, insufficient data have been collected which would be necessary to effectively study the increase in GABA levels in polished rice grains. Most reports have studied only the increased levels of GABA in brown rice and rice germ, a waste from polished rice production.

It is interesting to increase GABA levels in polished rice grains by germination methods based on the hypothesis that GABA increases nutrients, rice value, price and the income of farmers in Thailand. People can choose the germination methods to increase GABA in rice grain for consumption and for use in the food industry because this method is considered to be economical, efficient and easy to follow.

GAD enzyme activity was also investigated in this study. Because GABA accumulation probably is mediated primarily by GAD enzyme which control rate limiting step for GABA production in rice (Huang *et al*., 2007; Shelp *et al*., 1999).

GAD enzyme is specific for L-glu, PLP-dependent, inhibited by reagents known to react with sulfhydryl groups, possesses a calmodulin-binding domain, and exhibits pH optimum of ~5.8. However, Bautista et al., 1994 suggested that the different rice cultivars effect on the amount of GAD enzyme, activity and properties. Therefore, GAD enzyme from rice seedling will be extracted, determined optimal conditions and activity during germination time.The data from GAD enzyme studied will provide useful information for control GABA production in rice.

Moreover, proteomic analysis technique was employed to study the expressed protein in GABA-rice in this study. Because GABA is a nitrogen source in germinated grain, relationship with other metabolic pathways such as nitrogen and protein metabolism is interesting to investigate (Shelp *et al*., 1999). Change in proteins of GABA-rice after germination will be of help for understanding GABA production from rice because protein is one of the bimolecules that play important role in almost cell functions.

In summary, this study aims to add value to rice by increasing GABA concentration. Although new methods for modifying GABA production were reported, conventional germination will be studied because it showed more economic and easier to apply in daily life. In addition, the GABA concentration will be measured quantitatively and qualitatively by High Performance Liquid Chromatography (HPLC) and Liquid

Chromatography and Mass Spectrometry (LC-MS) analysis, respectively. The relationship between GABA concentration and GAD activity detection will be studied. Investigation of expressed protein in GABA-rice after germination using 2-dimension (2-D) gel electrophoresis will also be done.

Table 1.1 Previous reports of GABA production from rice germination

Authors	Rice cultivars	Methods	GABA contents
Anawachkul and Jiamyangyuen, 2009	Brown rice (*O. sativa* L. *cv*. Munpoo)	Germinated red rice (Munpoo rice) was prepared by soaking in water at rice to water ratio of 1:3 (w/v) for 24 h and leaving in darkness for 48 h to germinate of which the samples were collected every 8 h.	21.32 mg/100 g
		The yogurt formula was developed and 30% germinated red rice flour prepared using the optimum condition was selected to add in formula.	4.09 mg/100 g
Srijessadaruk et al., 2009	Whole rice grain (*O. sativa* L. *cv*. Kaw Mali Dang)	Whole rice grains were soaked in water 10-12 h, and allow to germinate at 35-40 ^{o}C for 30-35 h	12 mg/100 g
Komatsuzaki et al., 2007	Brown rice (*Oryza sativa* L. ssp. Japonica: Haiminori)	Brown rice was soaked in water 3 h and gaseous treatment 21 h	24.9 mg/100 g
Zhang et al., 2006	Rice germ Brown rice (*O. sativa* L. cv Chinese rice)	Rice germ hydrolyzed by protease to obtain supernatant, then adding 4 g of rice germ into supernatant and incubate 40 C for 8 h to accumulate GABA	2260 mg /100g
Patcharee, et al., 2005	Brown rice (*O. sativa* L. *cv*. Kaw Dok Mali 105);	Brown rice was soaked in water 36-72 h	37.2 mg/100 g (normal rice)
	Brown rice (*O. sativa* L. *cv*. RD 258) ;		72.8 mg/100 g (glutinous rice)
Ohsubo et al., 2005	Brown rice (*Oryza sativa* L. ssp. Japonica:Koshihikari)	Soaking brown rice in water 96 h	149.6 mg/100 g
Oh, 2003	Brown rice (*O. sativa* L. cv Korean rice)	Soaking brown rice in water	9.01 mg/100 g
		Soaking brown rice in lactic acid solution	8.11 mg/100 g
		Soaking brown rice in chitosan /lactic acid solution	11.71 mg/100 g
		Soaking brown rice in chitosan /glutamic acid solution	20.71 mg/100 g
		Soaking brown rice in glutamic acid solution	14.42 mg/100 g

1.1.1 Research objectives

1.1.1.1 To determine and compare GABA concentrations of during germination in seven selected commercial rice cultivars in northern Thailand.

1.1.1.2 To study the relationship between GABA concentration and activities of GAD enzymes during germination of selected rice cultivar from step 1.1.1.1.

1.1.1.3 To determine protein expression of GABA enriched-rice

1.1.2 Usefulness of the research

1.1.2.1 Data of the GABA accumulation and disappearance in rice produced by germination method will be obtained.

1.1.2.2 Valued rice with high GABA concentration will be produced.

1.1.3 Research plan, methodology and scope

1.1.3.1 Literature survey

1.1.3.2 The seven famous cultivars of rice in northern Thailand will be obtained from Pitsanulok and Chiang Mai rice seed centers.

1.1.3.3 Germinated rice will be examined, separately, in plastic boxes and GABA concentrations will be determined by HPLC and LC-MS techniques.

1.1.3.4 GAD activity during germination will be studied in a cultivar that showed the highest GABA producing ability by LC-MS and western blotting techniques.

1.1.3.5 Proteome analysis will be surveyed in GABA enriched rice compared to non germinated rice.

1.2 Literature review

1.2.1 Rice (*Oryza Sativa* L.)

1.2.1.1 Definition and characteristic of rice

Rice is a grass (Gramineae) belong to the genus Oryza Linn, which has two species, *Oryza sativa* (*O. sativa*) Linn. and *Oryza glaberrima* (*O. glaberrima*) Steud. The former is more important in term of growing area, *O. glaberrima* being confined to small area in West Africa. The morphological differences are small; *O. glaberrima* having shorter, truncate ligules (6 mm against 15-45 mm.), fertile lemma and palea, and simple undivided panicle-branches.While, *O. sativa* bears short branchlets. Because *O. glaberima* is relatively unimportant, general information will be concerned with *O. sativa*, although much will apply equally to the West African species. (Grist, 1965; Smith and Dilday, 2003)

O. sativa is widely grown in tropical and sub-tropical regions, high moisture, and temperature (22-30^{o}C) as a dry-land crop but usually more in water. Clay is suitable for rice culture according to the ability to retain water and the soil pH 5.0-6.5 with organic matter more than 5% (Grist, 1965).

O. sativa are dividing into three types according to their phenotype appearance described follows: (1) Indica, (2) Japonica, and (3) Javanica type. The physiological differences, especially of grains as shown in Figure 1.1.

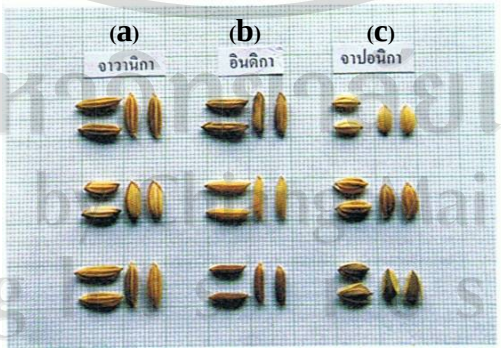

Figure 1.1 Comparative rice grain morphological of sub-species of rice (**a**) Javanica, (**b**) Indica and (**c**) Japonica (On-anong, 2006).

When classification is based on their content of starch and chemical properties, it can be divided into 2 categories as follows (Em-on, 2009);

(1) Non-glutinous rice containing 90% of starch endosperm with 70-90% of amylopectin. The grain is transparent but becomes opaque white rice and loose after streaming.

(2) Glutinous rice contains soluble starch endosperm including dextrin. The main composition in glutinous rice is 95% of amylopectin. The content of amylose is very few or may be none. The rice grain is opaque white and become sticky after streaming.

Table 1.2 Rice classification based on amylose content

Type	Amylose(%)	Appearance	Sample
Glutinious	0-2	Most sticky and yummy	Sanpatong 1 Korkor 6
Low amylose	10-20	Sticky and soft	Kaw dok mali 105
Medium amylose	20-25	Rather not sticky	Patumthani 1
High amylose	25-34	Not sticky and hard	Supanburi 1, Chainat 1 Pitsanulok 2

Source; (Insomphun, 2003; Em-on, 2009)

Amylose content is also used to classify the rice variety. High content of amylose give streamed rice with high water content. Amylose is able to retrograde as an insoluble solid. The property supports why non-glutinous rice can not stick together after streaming. The porosity of steamed non-glutinous rice is because insoluble property give larger volume, classified rice using amylose content as shown in Table 1.2

1.2.1.2 Structure of rice grain

The morphology of the mature rice grain can be used for an understanding of the distribution of protein, fat and vitamins in different parts of the grain.

The rice kernel is composed of a hull and caryopsis (Figure 1.2a). The unpolished caryopsis (number 7) is referred to as brown rice. The hull is comprised of sterile lemmas (number 2), rachilla (number 3), palea (number 5), and lemma (number 4). The lemma covers two-thirds of the seed, with the edges of the palea close tightly

around the seed. The caryopsis contain the embryo and starchy endosperm, surrounded by the seed coat (tegmen) (number 8) and the pericarp (number 7) Figure 1.2b)

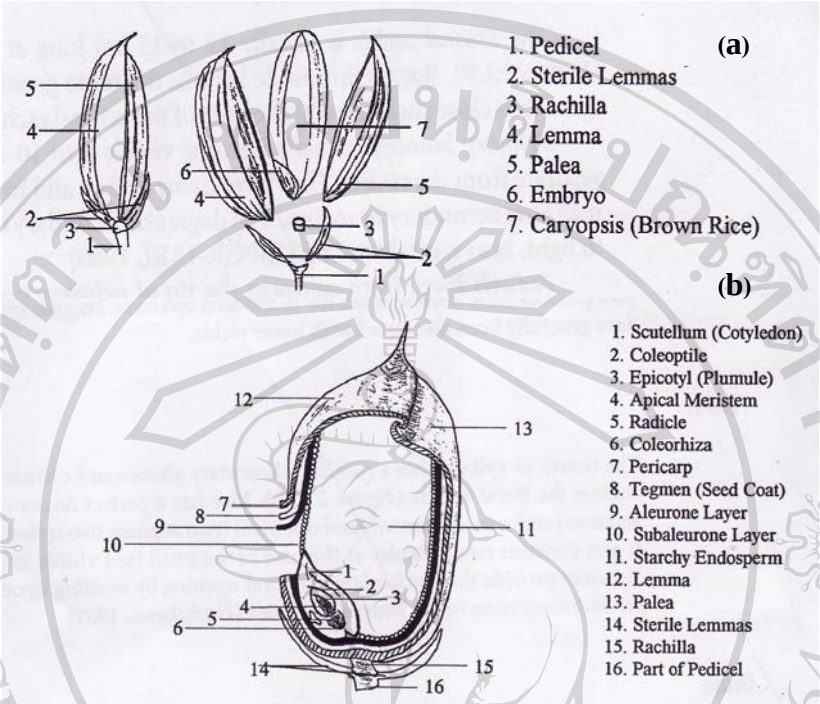

Figure 1.2 Rice grain **(a)** Structure of rice grain and **(b)** Cross section of rice kernel (Smith and Dilday, 2003)

The embryo (number 6, Figure 1.2a) is the rudimentary plant tissue that will develop into the rice plant upon germination. The largest portion is the scutellum (cotyledon) (number 1, Figure 1.2b), which is shaped like a shield around the coleoptile (number 2) and coleorhiza (number 6). The coleoptile encloses the first three leaves (seminal root). All of these cells are very small and swell greatly when water is absorbed during germination.

The endosperm is the tissue formed during the germination period which serves as nutrition for the embryo during germination and early seedling growth. It is comprised of starch storage tissue, filled with starch granules and a small number of protein bodies. It is surrounded by the aleurone layer (number 9, Figure 1.2b) of cell, which are small and almost cubicle and contain protein and lipid bodies but no starch. Sub-aleurone layer (number 10), lying immediately below the aleurone, has characteristics intermediate to the aleurone and stage storage tissue (Juliano, 1972; Smith and Dilday, 2003).

The seed coat or tegmen (number 9) is a thin membrane with broken cell walls, which is a remnant of the inner integument of the ovary (Figure 1.2b).

The pericarp (number 7) is the mature ripened ovary wall, consisting of an epidermis and several layers of parenchyma that surround a vascular brundle. This transports solutes and minerals to the developing seed during ripening. In fully ripe seed, the parenchyma die and become spongy and the vascular bundles lose their functions .

In milling, the germ is usually removed together with the scutellum (number 1), while in the polishing process the pericarp (number 7) of (Figure 1.2a) is removed with the remains of the embryo and the aleurone layers (number 9) .

1.2.1.3 Rice seed germination

Rice seed germinate upon absorption of water at adequate temperature in the presence of oxygen and initiation of the biochemical processes involved in embryo growth. The processes begin with imbibitions and ends with sufficient signs of radical and/or coleoptiles protrusion (Figures 1.3).

Figures 1.3 Rice germinated seed begins with protrusion of (**1**) coleoptiles (**2**) radicle (Smith and Dilday, 2003)

However, in dry rice seed, the radical normally appeared first whereas the water seeded (submerge) rice, the radicle is suppressed and the coleoptiles emerge first.

This appeared to be related to the low oxygen environment of water seeded rice. Takahashi et al, (1995) suggested that the emergence of the suppressed radical is related to the water in that formation of radical.

The rice germination process has been divided into 3 phases, phase 1: imbibitions, phase 2: metabolic activation (respiration and carbohydrate metabolism and phase 3: growth and emergence of root and shoot primordial from the hull.

Water uptake is rapid during phases 1, 3 and controlled by seed coat permeability. Phase 2 is regulated by gases (oxygen, carbon dioxide, and ethylene),

endogenous inhibitors or hormones, and enzymatic activity. Seed coat permeability is also a factor affecting gas exchange during germination.

The seed coat can inhibit germination by reducing permeability to water and gases, amid also for reason related to dormancy. Several dormancy factors, or chemicals, are contained in the seed coat. Therefore, removal of the seed coat will often bring about more rapid germination.

1.2.1.4 Factor affecting rice seed germination

Germination process is major affected by moisture, seeds generally begin to germinate at 15% moisture and attain full germination at 25% moisture (Hoshikawa and Ishi, 1989), seed dormancy, aeration, temperature and many others.

(1) Dormancy

Dormancy refers to many seeds which are unable to germinate when placed in apparently favorable conditions, and they are considered as "dormant seeds" (Kigel and Galili, 2003). It is generally overcome by heat treatment at 40-20^{o}C for 5 days. However, different variety of rice cultivars showed varying levels of dormancy and requirements for heat treatment. U.S. rice cultivars have moderate levels of dormancy, which prevents sprouting in the field. High dormancy in some cultivars allows their seed to remain viable in soil for several years (Srivastava, 2002).

Luo *et al*, (2007) suggested that the influence of dormancy in rice seed depend on temperature, moisture content, oxygen, nitrogen, carbon dioxide and storage conditions.

Storage rice seed in high oxygen accelerates the breaking of dormancy. Carbon dioxide and nitrogen have little or no effect when they exclude oxygen. Variation of moisture content over the range 12.0-14.5%, has little effect at 27°C and no detectable effect at higher temperatures (Luo *et al.,* 2007).

While, Khan and Komatsu, (2004) found the rice seed dormancy would be controlled by nuclear genotype of itself and its maternal parent, sometimes influenced by cytoplasm. When dehulled seeds were subjected to germinate, the rice seed dormancy indices declined dramatically, indicating that hull also played an important role in seed dormancy.

(2) Temperature

Temperature is one of the most important factors affecting germination. Rice germination percentages of 90-70% occurred within 48 h, if temperatures are between 27 and 37°C. Germination drops sharply below these temperatures (Yoshida, 1981).

Counce *et al*, (2000) described four stages of optimal temperature for rice seedling development and indicate that (1) minimum temperature for rice germination and development are between 6-8°C (2) optimum temperature for rice germination and development is 37°C (3) maximum temperature for rice germination is 41°C and for development is 44°C.

However, the majority of temperature studies on rice germination indicate to be optimal at 30-32°C (Takahashi *et al*., 1995).

(3) Oxygen

Aeration determines the order of coleorhiza and coleoptile emergence from the hull (Figures 1.3), under aerobic condition, the coleorhiza emerges first or together with the coleoptile. Under anaerobic conditions, the coleoptile emerges first. Rice showed adaption to hypoxic and anoxic conditions by anaerobic fermentation. The coleoptile is the only organ of the embryo that can emerge from the seed on energy derived solely from anaerobic fermentation. Adaptability to anaerobic germination varies with cultivar.

(3)Water

The first process which occurs during germination is the uptake of the water by the seed. The extension of imbibitions is determined by three factors; (1) the composition of the seed, (2) the permeability of the water to seed coat and (3) the availability of the water in liquid or gaseous from in the environment. (Mayer and Mayber,1982; Yamauchi and Chuong, 1995).

Water absorbed by the surface of the seed coat or mainly through the micropylar/hilar or chalazal region. In a number of taxa with hard-coated impermeable seeds the imbibition is regulated by special structures (Mishira and Salokhe, 2008).

Beside, water uptake is physical process which is related to the properties of colloids. During imbibition, molecules of water enter the substance which is swelling, causing salvation of the colloid particles and occupying the free capillary and intermicellar spaces of the colloid. The swelling of the colloid results in the production of considerable pressures, called imbibition pressure. The imbibition pressure is great importance in the process of germination as it may lead to the breaking of the seed coat and also, to some extent, makes space in the soil for developing seedling (Bouman *et al*., 2005). If imbibition is prevented by impermeable seed coats, as with hard seeds, germination cannot occur (Chahal *et al.,* 2007).

However, when seed were grown on the soil surface, water level had little effect on rice seedling establishment, but it was reduced by raising the water level when seed were sown below the soil surface (Yamauchi and Chuong, 1995).

1.2.1.5 Seedling development

Seedling growth continue after generation with extension of the coleoptiles, coleorhizae, emergence of the prophyll and radical. Plant growth stage can be determined by counting leaves as they emerge. The first leaf emerges from the coleoptiles, the prophyll is not a true leaf because it lacks a blade. It may not be counted as first leaf when describing shoot development. (Counce *et al.,* 2000; Smith and Dilday, 2003).

The first true leaf stage (Figure 1.4d) is defined by Counce *et al*, (2000) as a seedling with a prophyll and a fully emerged first true leaf. This seedling also has five roots from the nodal node (number 5).

The second leaf growth stage (Figure 1.4 e) is defined by full emergence of the second true leaf and is synonymous with the three leaf stage if the prophyll is counted as the first leaf . Seedlings at this stage have roots emerging from the first node. At this stage, plants become autotrophic 100% of the carbohydrate used by the plant. Some researchers consider this stage the end of seedling growth. (Counce *et al.,* 2000; Smith and Dilday, 2003).

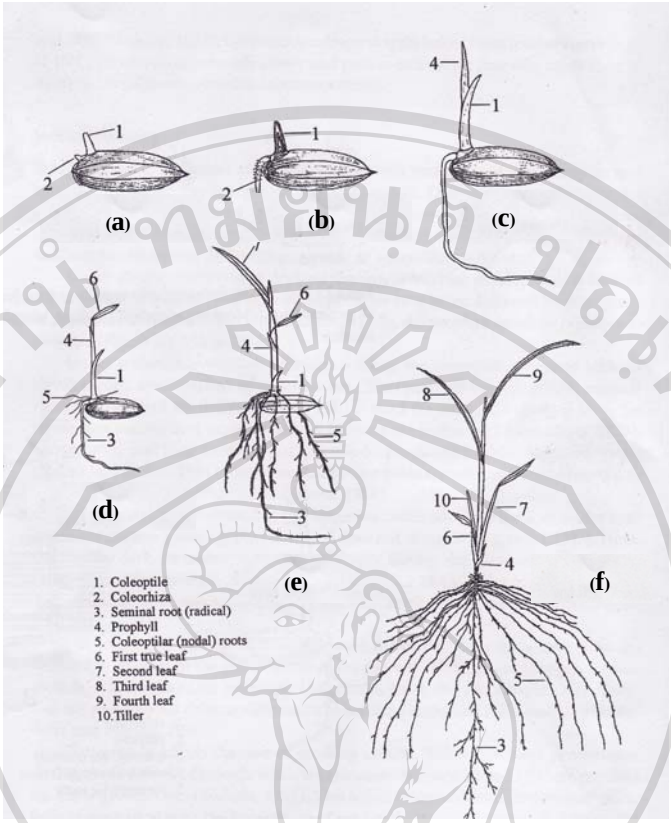

Figure 1.4 Rice seedling development under aerobic and light conditions (**a**) germinating seed (**b**) developing coleorhiza and coleoptile (**c**) emerging prophyll and seminal root (**d**) first true leaf growth stage, (**e**) second leaf growth stage, (f) fourth leaf growth stage. (Counce *et al.,* 2000; Smith and Dilday, 2003).

If seedling has been grown completely in the dark, they will grow beyond the second leaf growth stage. In seedling grown with light, photosynthesis contributes to an increasing proportion of total carbohydrate with time (Counce *et al*., 2000). During the first week of growth, it contributes <30% during the second week, it contribute>84%; and by approximately the third week (second true leaf) it contributes 100% (Yoshida, 1981).

The fourth leaf growth stage (Figure 1.13f) is defined by full emergence of the fourth true leaf and is synonymous with the five-leaf stage if the prophyll is counted as first leaf. Seedling height is usually measured at this growth stage.

However, during this growth stage, rice seedling exhibit great morphological response to changes in aeration, light and temperature. Rice is likely a semiaquatic plant and has many characteristics that facilitate

establishment under either aerobic or anaerobic conditions (Hoshikava and Ishi, 1989; Moldenhauer *et al.,*1994; Nemoto *et al,* 1995).

Under aerobic conditions of dry land seedling, coleorhiza development is favored. The coleorhiza develops root hairs, followed by emergence of the radical. Within the coleoptile, the prophyll develops rapidly and emerges.

Under anaerobic conditions of water seedling, the coleoptile elongates without simultaneous development of other tissues. Emergence of the coleorhiza, radicle, and prophyll are delayed until the coleoptile emerges from the water surface and oxygen levels to the root are increased. Oxygenation and root development also can be promoted by draining flood water (Helms and Slaton, 1994)

1.2.2 Germination

1.2.2.1 Definition of germination

Germination begins from dry seed with water uptake (imbibitions) followed by activation of biochemical systems leading to rupture of the outer covering layer and followed by emergence of radical (Bewley and Black 1994; Finch-Savage and Leubner-Metzger, 2006; Khan *et al.,* 2009). The "Germination word" is used for refer to a number of germinated plant species, including germination of seed, spore of bacteria, fungi, fern, and also the process occurring in the pollen grain (Mayer and Mayber, 1982). Although all the processes refer to the germination, this research studied on the seed germination only.

Seed germination can be defined as the process that begin with seed imbibitions and successfully finished with the protrusion of the root or hypocotyls from the seed covering, generally the root first. However, there is no general rule as which part of the embryo first rupture from the seed coat. In many seed is the radicle and followed by root extrusion. However, in some seeds is the shoot which protrudes first, for example in *Salsola* species (herbs, native found in Africa, Asia, and Europe) (Mayer and Mayber, 1982; Bewley and Black, 1994; Schabes and Sigstad, 2006) Sigstad and Prado, 1999)

The processes of germination lead to development of embryo to seedling. The seedling consists of a radicle, pumule or epicotyl, cotyledons and hypocotyl connecting the radicle and the pumule. Seedlings are classified as epigal, which the

cotyledons are above ground and are usually photosynthetic, and hypogal which the cotyledons remain below ground. In this latter type the cotyledon are the source of reserves for seedling. The size, shape and the various tissue of seedling are large morphological variability during germination (Bewley and Black, 1994; Schabes and Sigstad, 2006; Sigstad and Prado, 1999).

In conclusion, the total germination process consists of change in physiological, biochemical system (protein and sub-cellular structural, respiration, macromolecule synthesis including conversion and production new compounds) (Kuo *et al.,* 2004), cell elongation and the other metabolic changes (Bewley and Black, 1994). Moreover, germination causes important change in nutritional and sensory characteristics of germinating seeds. Extensive breakdown of seed storage compounds and synthesis of structural proteins and the other cell components take place during this process (Kuo *et al.,* 2004).

For example, amide, non-protein amino acid, amino acid, phytochemicals compounds, antioxidant compounds, superoxide dismutase (SOD) enzyme were increased by germination (Kuo *et al.,* 2004).

Therefore, germination of seeds for human consumption become to be a simple and effective processing method for achieving desirable change in nutritional quality (Park and Oh, 2006;2007; Komatsuzaki *et al.,* 2005;2007).

1.2.2.2 Change in storage proteins during germination

The storage proteins of seeds are found in the protein bodies. There are small membrane bound particles between 0.5-10 nm in diameter, often contain phytin. It apparently derived from the endoplasmic reticulum during seed formation. The protein bodies in the starchy endosperm was divided into 2 type; one (type I, protein bodies-I) is spherical with a concentric ring structure, whereas the other (type II, protein bodies-II) is stained homogeneously by osmium tetroxide and does not have this structure (Horikoshi and Morita, 1982).

Protein bodies-I contains prolamin, and protein bodies-II is rich in glutelin and globulin. The glutelin in protein bodies-II is composed of two principal subunits, the 22-23 and 37-39 kd complexes, and the prolamin in protein bodies-I is composed

mainly of 13 kd polypeptide. The 16 and 10 kd polypeptides also are located in protein bodies-I. (Yamagata *et al.,* 1982).

However, Osborne's classification of seed proteins based on their solubility and this property is still used today in methods for their separation. Four groups of proteins are defined: (1) Albumins are soluble in water at neutral or slightly acid pH and heat coagulation ability. These are mainly enzyme proteins. (2) Globulins are insoluble in water but dissolve in salt solution (0.4 M NaCl) and do not as readily coagulate upon heating as do albumins. (3) Glutelins are insoluble in water, salt solution and ethanol but can be extracted with fairly strong acidic or alkaline solutions. (4) Prolamins dissolve in ethanol (90%) but not in water.

During germination, the storage proteins in the protein bodies are broken down. There was difference in digestibility between PB-I and PB-II. PB-II did not have a dense core and easily digested from the central portion when germination. At 6 days of germination, PB-II was almost deconstructed. On the other hand, PB-I which displayed concentric rings with a dense core was digested from the outside after 3 days (Horikoshi and Morita, 1982)

Table 1.3 Protein compositions of some types of seeds

Species of strain	Percent of total protein			
	Albumin	Globulin	Glutelin	Prolamin
Avena sultiva	Trace	80	5	15
Zea mays	14	0	31	48
Zea mays (Opaque-2)	25	0	39	24
Oryza sativa	5	10	80	5
Pisum sativum	40	60	0	0
Cucurbita pepo	Trace	92	Small amount	Trace

In rice grains, unlike the proteins of other cereals, the proteins of rice endosperm are insoluble in water, salt solution, and alcohol, and belong to the rather defined class of proteins called glutelins (Jones and Gersdorff, 1927).

Glutelin is the major protein of the starchy rice endosperm (Table 1.3), constituting at least 80% of the total protein, prolamin accounting for less than 5%.

A predominant protein of rice, glutelin, exists in the endosperm, while globulin is mainly localized in the scutellum and aleurone cells (Horikoshi and Morita, 1982).

Source: Mayer and Mayber, 1982

However, two globulins isolated from the endosperm of rice, *O. sativa* were reported. These proteins differ both in the temperatures at which they coagulate (74 and 90^{o}C.) and also in their elementary composition and distribution of nitrogen. (Jones and Gersdorff, 1927).

Not all these groups of protein are found in all species of seed (Table 1.3). The Gramineae contains prolamins (gliadin, zein and hordein of *Triticum*, *Zea* and *Hordeum* respectively), but this group of proteins is uncommon in other seeds. Cereal grains are also rich in glutelins; the gluteins of *Triticum* which is important in giving the structure to bread and oryzenin of *Oryza* are examples. Globulins on the hand, predominate in described as the early classification of Osborne which are arachin, legumin ,vignin, glycinin and vicilin.

1.2.1.3 Protein metabolism

During germination the seed rapidly change to a metabolically active state. Storage protein and starch are hydrolyzed to their consisting amino acids and soluble sugar, respectively. The hydrolysis compounds are then transported to the growing embryonic axis and used for synthesis of protoplasm, structural components and enzyme, new protein and as an energy source (Helel, 1996). The broken down of storage protein in the cotyledons is accompanied by the appearance of new protein and nitrogenous compounds appear in other parts of the seedling (Kigel and Galili, 2003). Protein metabolism during germination is divided into 2 processes; catabolism and anabolism will be described below.

(1) Protein broken down during germination

Dry and germinating seeds contain a variety of proteolysis enzymes, some present already in the dry seeds, while others appear during germination. These enzymes can be classified according to their substrate specificity: (1) endopeptidases: these cleavage the internal bond of polypeptides to yield a smaller polypeptides. (2) aminopeptidases: these sequentially cleavage the internal amino acid

from the free amino end of the polypeptide chain. (3) carboxypeptidases: single amino acid are sequentially cleavage from the carboxyl end of the chain, like aminopeptidases. There are another class of hydrolyzing enzymes to be considered, which hydrolyse various small peptides but not protein, such as the peptide hydrolases (Kigel and Galili, 2003).

As previously reported on the hydrolysis of storage proteins in rice endosperm by rice seed protease into degraded product in the initial step, and followed by peptide with low molecular weights (MW), amino acids and amides (Kigel and Galili, 2003; Horikochi and Morita, 1982). Change in the free amino acid composition in the germinating seed may be indicative the hydrolysis of storage protein as shown in Table 1.4. The shoots of young seedling grow by utilization of the degradation products of storage proteins in the seeds as the nitrogen source (Kigel and Galili, 2003).

Table 1.4 Change in the amino acid content of rice seeds during germination

Amino acid	**Time of germination in days (mg/g)**			
	0	**1**	**2**	**3**
Alanine	5	30	80	220
Threonine	5	20	40	190
Leucine	20	20	60	280
Serine	30	30	60	250
GABA	5	5	15	25
Lysine	15	5	20	40
Tryptophan	5	5	2	-
Glutathione	10	0	0	20
Aspartic acid	40	35	35	40
Glutamic acid	60	80	110	160
Asparagine	30	40	60	240
Glutamine	60	40	360	700

Source: Kigel and Galili, 2003

Related-enzyme systems for the synthesis of amino acids in germinating seeds usually contain enzymes causing hydrolysis of the peptide bond. These are glutaminase and asparaginase. They presented in many developing seeds and their activities depended on K^+ (Sodek *et al*., 1980). Liberation of NH^+_4 from asparagines by asparaginase can presumably lead to amino acids formation processes. Other important enzymes can transfer amino groups from the amide to some keto acid which result in amino acid formation.

Transfer enzyme concerned with protein metabolism is constituted by the transaminases. These enzymes transfer amino group from amino acid to keto acid. The presence of transaminases in a variety of seeds has been shown by Smith and Williams, 1951. They found a marked increase in the activity of the transaminases transferring amino groups from alanine or aspartic acid to alpha-ketoglutaric acid. It is probable that the same enzymes are also aspartic carrying out the reverse reaction. In most cases glutamic-aspartic transminase increased more rapidly than the glutamic-alanine transaminase.

Moreover, Helel, (1996) and Horikoshi and Morita, (1982) investigated change in total protein pattern in germinated rice seedling by Sodium Dodecyl Sulphate-Polycrylamide Gel Electrophoresis (SDS-PAGE) technique as shown in Figure 1.5 a-b. The electrophoretic pattern change of total protein along the period of germination were found. It showed the decreased staining intensity of the bands when the protein content of the major bands declined. Each band differs because the rate of protein hydrolysis and the fastest moving component appears to be the most resistant to hydrolysis.

Helel, (1996) supported the data of Horikoshi and Morita, (1982). The results showed the SDS-PAGE patterns of extracted PB-fractions from germinating grains comparing to the control (lane 1) in Figure 1.5a. Protein bodies fractions were isolated from germinating grains at various stages, then subjected to SDS-PAGE. 13 polypeptides were detected at 13, 15, 16, 17, 20, 20, 21, 24, 31, 35, 36, 37, 58 and 66 kd and decreased after germination after 6 days.

While, Horikoshi and Morita, (1982) supported the data of Helel, (1996). The results showed protein bands at 89.9, 64, 40.7, 25 kd decreased after germination. Exception, 23 kd subunits remained at the later stage of germination (Figure 1.5b).

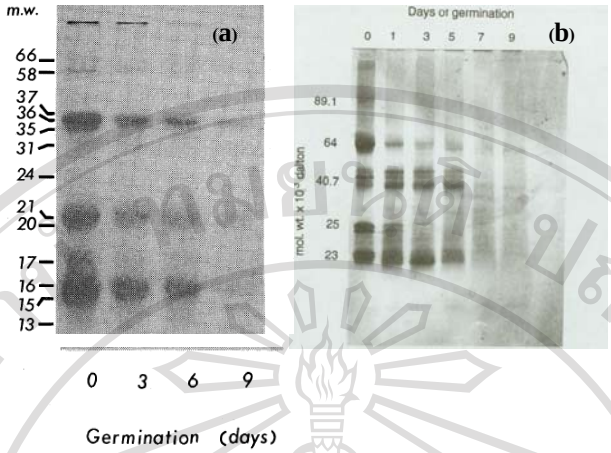

Figure 1.5 SDS-PAGE of total protein of grains at the various germination days (**a**) decreased total protein pattern in rice seedling and (**b**) disappeared protein body after germination (Helel, 1996; Horikoshi and Morita, 1982).

(2) Accumulation of proteins and amide formation

Patterns of protein expression during seed development have been studied intensively in a number of species (Kim *et al*., 1993; Kigel and Galili, 2003; Shimizu and Mazzafera., 1998; Hemalatha and Prasad., 2003).

Previous evidence reported the greatest accumulation of protein in developing rice endosperms at the early stage of germination were shown in Figure 1.6. Changes in individual proteins were examined from the earliest stage of development at endosperms could be harvested. The peptide composition of the starchy endosperm protein was analyzed by SDS-PAGE. The MW of the storage protein subunits ranged from 10-76 kd for more than 15 bands. The increased polypeptide profiles for the 4-6 germination days were similar, but differed from the profile of mature seeds (45 germination days) (Yamagata *et al.,* 1982)

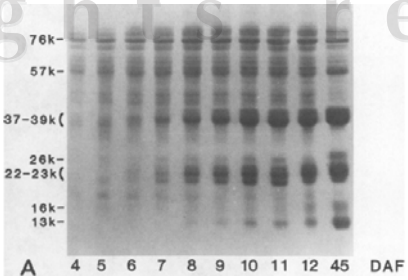

Figure 1.6 Polypeptide composition of the starchy endosperm of rice during germination (Yamagata *et al.,* 1982).

Moreover, germination process possibly induce amide formation (in GABA, γ-methylene glutamic acid, beta-pyrazol-1-alanine, lathyrine, and many others). The review by Clibnall, (1939) has led to understanding on the importance of amide formation during germination. Proteins are broken down to amino acids. Part of these amino acids are oxidatively deaminated and the carbon skeleton enters to various respiratory and carbon cycles. The ammonia formed by deamination is detoxicated by the process of amide formation. The chief amides formed are glutamine and as arginine, depending on the plants.

1.2.3 Gamma-Aminobutyric acid

1.2.3.1 Definition, properties and source

GABA is a four carbon non-protein amino acid (Takahashi *et al*., 2004; Komatsuzaki *et al*., 2005;2007; Huang *et al*., 2007; Lu *et al*., 2008; Su *et al*., 2003; Park and Oh, 2007; Siragusa *et al*., 2007; Aoki *et al*., 2003, Kono and Himeno, 2000; Shelp *et al*., 1999) which is produced primarily by the decarboxylation of L-glu catalyzed by a pyridoxal 5-phosphate (PLP)-dependent GAD enzyme (EC 4.1.1.15) (Komatsuzaki *et al*., 2007; Park *et al*.,2007; Siragusa *et al*., 2007).

GABA has an amino group on the γ-carbon rather than on the *α*-carbon (Figure 1.7) and exists in an unbound form. It is highly soluble in water, a flexibly structural molecule that can assume several conformations in solution, including a cyclic structure that is similar to proline. GABA is zwitterionic (carries both a positive and negative charge) at physiological pH values (p*Ka* values of 4.03 and 10.56) (Shelp *et al*., 1999), MW 103.12 and melting point, 203.7 ^{o}C.

Figure 1.7 GABA structure. (*Enzyme database. 2009.* "GABA structure" *[online]. Available*http://www.brendaenzymes.info/index.php4?page=information/all_enzymes.php4?e no=1.2.1.24 . (3 June 2009)

GABA was first discovered in the extract from a mammalian brain, acting as being inhibitory transmitter in the central nervous system (Takahashi *et al*., 2004), widely distributed among eukaryotes and prokaryotes (Lu *et al*., 2008) and in nature.

In plant, it was found in tea leave, anaerobic green tea or cyclic treatments of tea leaves and shoots, chlorella (Komatsuzaki *et al*., 2007; Park and Oh, 2007; Siragusa *et al*., 2007; Aoki *et al*., 2003), rice germ soaked in water (Takahashi *et al*., 2004; Komatsuzaki *et al*., 2005), germinated soy bean. Microbial source; included lactic acid bacteria (Komatsuzaki *et al*., 2005), fungi; *Monascus* sp (Su *et al*., 2003; Park *et al*., 2007), *Rhizopus, Aspergillus oryzae* (Aoki *et al*., 2003; Kono and Himeno 2000) and yeasts (Takahashi *et al*., 2004). Product from fermented food containing GABA has been reported such as in yoghurt (Park and Oh, 2007), Tempeh (Aoki *et al*., 2003) and dairy product (Komatsuzaki *et al*., 2007).

However, GABA levels in plant tissues are low (ranging from 0.03-2.0 mmol/g fresh weight but increase several folds in response to many diverse stimuli, including heat shock, mechanical stimulation, hypoxia and phytohormones (Komatsuzaki *et al*., 2007; Su *et al*., 2003; Park *et al*., 2007). For example, within 5 min of mechanical or cold stimulation, the GABA concentration in soybean leaves rises to 20-40 folds (Siragusa *et al*., 2007).

1.2.3.2 Pharmacological functions

GABA representated depressive neurotransmitter in the sympathetic nervous system or inhibitory neurotransmitter in the central nervous system (Takahashi *et al*., 2004; Komatsuzaki *et al*., 2005; Huang *et al*., 2007; Komatsuzaki *et al*., 2007; Park and Oh 2007; Aoki *et al*., 2003, Kono and Himeno 2000; Shelp *et al*., 1999). It has been reported that GABA retarded the elevation of systolic blood pressure and improves discrimination learning in mouse. Highly purified GABA is used as medication for amelioration of the brain bloodstream (Aoki *et al*., 2003).

There are currently great interest in several functions of GABA such as antihypertensive for lowering blood pressure in experimental animals and humans and diuretic effects, treatment of epilepsy and tranquilizer effects (Inoue *et al*, 2003; Takahashi *et al*., 2004; Komatsuzaki *et al*., 2005; 2007; Huang *et al*., 2007; Siragusa *et*

al., 2007. Some recent studies showed that GABA caused also a strong secretion of insulin from the pancreas, effectively preventing diabetes (Huang *et al*., 2007, Siragusa *et al*., 2007).

GABA has been reported to reduce blood pressure (BP) in experimental animals and man. The BP-lowering effect of GABA can block peripheral ganglia cell. In spontaneously hypertensive rats, GABA has an antihypertensive effect, possibly through the inhibition of nor-adrenaline release from sympathetic nerve sending (Miura *et al*., 2006). Germinated brown rice (GBR) extracts containing GABA can inhibit cancer cell proliferation (Park and Oh, 2006). Particularly, regard to sleeplessness, depression and automatic disorder observed during menopausal and presenium period.

GABA is involved in the regulation of cardiovascular functions, such as blood pressure and heart rate, and plays a role in the sensations of pain and anxiety, has been reported to depress the elevation of systolic blood pressure in spontaneously hypertensive rats (SHRs) (Park and Oh, 2007).

Treatments for sleeplessness, depression, and autonomic disorders, treatment for chronic alcohol-related symptoms, and stimulation of immune cells have also been the advantage of GABA (Siragusa *et al*., 2007)

1.2.3.3 GABA metabolism

In plants, the GABA shunt was first reported more than half a century ago in potato (Shelp *et al*., 1999). Its functional significance is still not fully understood. The pathway starts with the decarboxylation of L-glutamic acid (L-glu) to produce GABA and CO_2 in the cytosol (Figure 1.8 a-b). GABA is then presumably transported to the mitochondria by an unidentified GABA transporter, where it is converted to succinic semialdehyde (SSA). Subsequently, SSA is converted either to succinate or 4-hydroxybutyrate (GHB) (Shelp *et al*., 1999; Bouché and Fromm, 2004).

The metabolite GABA is proposed to be involved in a processes ranging from neuronal inhibition in animals (Bowery *et al*., 2004) to pollen-tube development such as *Arabidopsis thaliana* (Palanivelu *et al*., 2003). Its rapid accumulation when biotic and abiotic stresses (Kinnersley and Turano 2000) and its high concentration in various tissues as a nitrogen source (Kato-Noguchi and Ohashi, 2005; Brown *et al*., 2002;2006; Fait *et al*., 2006; Reggina *et al*., 2000; Bouche´ *et al*., 2003a; 2003b).

Moreover, the GABA shunt has been shown to be activated by light, during developmental phases and in an N status-dependent manner (Fait *et al*., 2005; Allan and Shelp, 2006; Masclaux-Daubresse, 2002), as well as in parallel to other changes that occur in central metabolism during plant growth (Stitt, 2002).

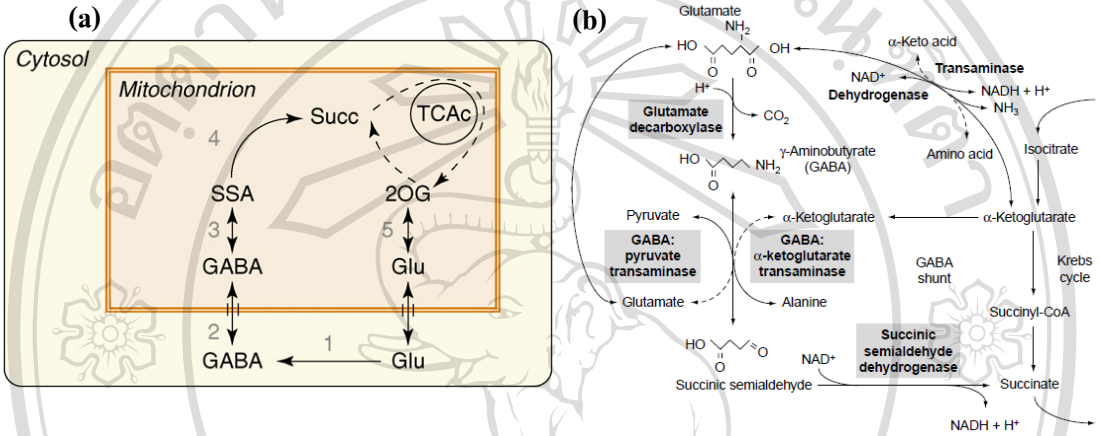

Figure 1.8 Common depiction of the GABA shunt (**a**) the GABA shunt and its relationship to other metabolic pathways (**b**) enzymes specifically associated with the GABA shunt are in bold and highlighted in grey (Shelp *et al*., 1999; Bouche *et al*., 2003a;2003b).

1.2.3.4 GABA shunt related enzyme

The GABA shunt involves three main reactions, catalyzed by GAD, GABA transaminase (GABA-T) and succinic semialdehyde dehydrogenase (SSADH), respectively. (Figure 1.9b) (Shelp *et al*., 1999). Understanding of properties for regulation of these enzymes is relatively well interesting.

In plants, the cytosolic decarboxylation of L-glu to GABA, catalyzed by GAD, is generally controlled by Ca^{2+}-calmodulin binding domain (CaMBD), (Snedden and Fromm, 2001). Removal of the GAD calmodulin-binding domain results in altered GAD structure in GABA metabolism and abherrent plant development in tobacco *(Nicotiana tabacum)* (Fernie *et al*., 2001).

GABA-T can use either pyruvate (Pyr) or 2-oxoglutarate (2OG) as amino acceptor to catalyze the conversion of GABA to SSA. (Figure 1.9). Use of the former leads to alanine (Ala) production, whereas the latter leads to L-glu formation, since at

least part of the Glu recycled by the transamination of GABA would eventually feed back into the GABA shunt (Fernie *et al*., 2001; Geigenberger and Stitt, 1993). This might serve to maintain the mitochondrial GABA/Glu balance as depicted in Figure 1.9.

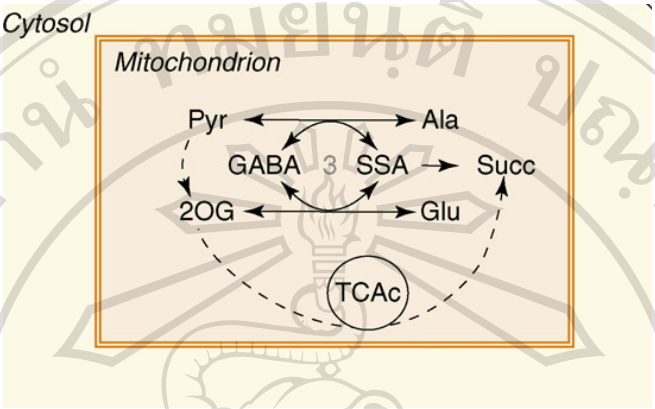

Figure 1.9 Mitochondrial GABA metabolism (Fernie *et al*., 2001)

(1) Glutamate decarboxylase

GAD activity in rice was initially studied in 1964 by Bautista, (1994) who found that GAD activity was a more reliable index for the viability of different rice cultivars. Later, Saikura *et al*, (1994) found that water soaking of rice kernel under a slightly acidic condition resulted in a remarkably increased level of GABA content, which indicated that the GAD of rice activated in slightly acidic condition. Based on this discovery, an efficient and simple method via rice water soaking has been developed (Ohtsubo *et al.,* 2005; Saikura *et al.,* 1994; Komatsuzaki *et al.,* 2007) for production of GABA from rice to develop novel functional food for hypertension prevention.

Akama and Takaiwa, (2007) isolated cDNA clones encoding two distinct GADs gene from rice to more understanding of GAD. They studied; two novel rice GAD genes (OsGAD1 and OsGAD2) and the characterization of deduced proteins. The results strongly suggested that rice has at least two diverged GAD isoforms: structurally, the OsGAD1 protein contains a CaMBD like all dicot GADs, while the OsGAD2 protein is very likely to lack a CaMBD in spite of a presence of the C-terminal extended peptide. The difference between the two isoforms was investigated by in vitro binding assay with a bovine CaMBD. Found that the exon/intron organization of the genes encoding these isoforms differ considerably from each other, and that their expression is differentially regulated, at least in roots and maturing seeds

However, Akama *et al*, (2001) suggested that rice GAD, lacks an authentic CaMBD at the C-terminus, which can functions for catalyzed L-glu to GABA as shown in Figure 1.10 .

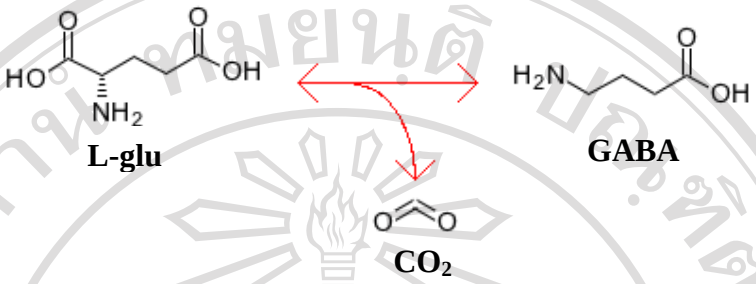

Figure 1.10 GAD enzyme catalyzed L-glu to GABA (GenomeNet Database Resources. *"Glutamate decarboxylase". 2009.* [online]. Available *http://www.genome.jp/dbgetbin/www_bget?K00823+2.6.1.19+R01648 .(3 August 2009).*

GAD enzyme was classified into lyases and the sub-class according to their reaction as shown in below .

EC 4 - Lyases

EC 4.1 - Carbon-Carbon Lyases

EC 4.1.1 - Carboxy-Lyases

EC 4.1.1.15 - Glutamate decarboxylase

When purified GAD from rice germ, it has formed in two homological subunits with approximately equivalent subunit masses of 40 kd. Rice germ GAD has an optimum pH ranging between 5.5 and 5.8, and an optimum temperature of 40°C. Its Km values for L-glu and PLP were determined at 32.3 mM and 1.7 l M, respectively (Zhang *et al*., 2007). However, other molecular properties and form of rice GAD are still not clear. Some evidence reported the MW of GAD in rice was 76 kd (Akama and Takaiwa, 2007).

The other purified plant GAD have been found in different forms. For example, barley embryos GAD had two forms including a 256 and 120 kd form, and barley root GAD was a single species with an MW of 310 kd (Inatomi and Slaughter, 1975). Squash GAD consisted of multiple identical subunits of 58 kd (Matsumoto *et al*., 1986).

(2) GABA transaminase

The first step in GABA catabolism is catalyzed by GABA-T, which localized in the mitochondrial inner matrix (Schousboe *et al.,* 1977). The reaction produces SSA from GABA (Figure 1.19) by two sorts of GABA-Ts that use either α-ketoglutarate (GABA-TK) or pyruvate (GABA-TP) as amino acid acceptors, producing glutamate or alanine (Figure 1.11), respectively (Niranjala *et al.,* 1995).

In mammals, only the GABA-TK seems to be present, whereas both enzyme activities can be detected in crude plant extracts. The GABA-TK enzyme of plants remains to be identified, whereas the GABA-TP was partially purified from tobacco and a homologous Arabidopsis gene was subsequently cloned (Geigenberger and Stitt, 1993).

The recombinant Arabidopsis GABA-TP characterized in vitro uses pyruvate but not α-ketoglutarate, and shares little homology with non plant GABA-Ts. Arabidopsis knockouts disrupted GABA-T (mutant), GABA content elevated 100-fold in flowers compared with the wild type, confirming that GABA-TP is a functional enzyme of the GABA shunt in vivo. While, the increase of GABA levels in other organs (leaves) is limited, implying that the GABA-TP has a specialized function in flowers and that other GABA-Ts might degrade GABA in the rest of the plant.

Therefore, in plants, GABA transamination occur via different types of GABA-T, which probably have specialized functions (Palanivelu *et al*., 2003). GABA-T enzyme was categorized into EC 2.6.1.19 as described below.

EC 2. Transferases

EC 2.6 Transferring nitrogenous groups

EC 2.6.1 Transaminases

EC 2.6.1.19 Aminobutyrate transaminase

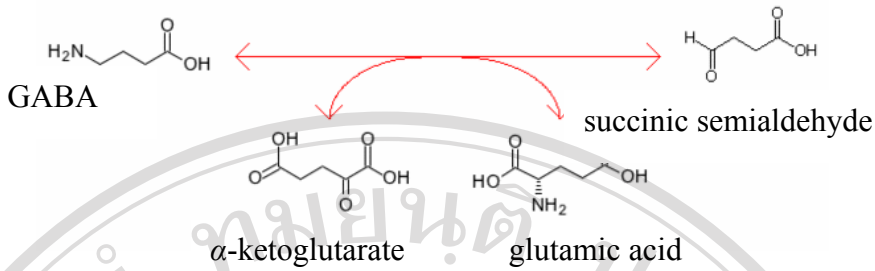

Figure 1.11 GABA-T enzyme catalyzed GABA to SSA.
GenomeNet Database Resources. *2009. "GABA transminase."* [online]. Available *http://www.genome.jp/dbgetbin/www_bget?K00823+2.6.1.19+R01648 .(3 August 2009).*

Optimal pH 8 and temperature at 30°C of this enzyme are found in rice. Its activity is influenced by concentrations of PLP. GABA-T activity is sensitive to inhibition by carbonyl-trapping agents such as 2-aminooxyacetic acid (2-AOAA). Selective GABA-T inhibitors, such as gamma-vinyl GABA (GVG; vigabatrin) are important because increased brain GABA concentrations may be of use in the treatment of epilepsy and control of seizure activity (Tunnicliff and Raess, 1991; Ansari *et al*., 2005).

(3) Succinic semialdehyde dehydrogenase

The final step of GABA catabolism is catalyzed by the NAD^+-dependent SSADH enzyme. SSADH, are known to use SSA as substrate (Niranjala *et al.,* 1995) at optimum temparature 40-42°C (Yamaura *et al.,* 1988) as shown in Figure 1. 12.

It is subcellularly mitochondria localized enzyme, commonly found in various organisms. Exception, in yeast, the enzyme is in the cytosol (Coleman, 2001).

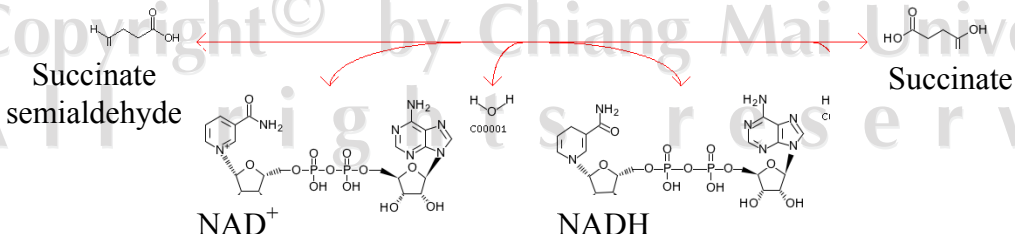

Figure 1.12 SSADH enzyme catalyzed SSA to succinate
GenomeNet Database Resources . *2009. "Succinic dehydrogenase"*. [online]. Available*http://www.genome.jp/dbgetbin/www_bget?K00135+1.2.1.16+R00713+R00714.(3 August 2009).*

The Enzyme Commission number (EC number) of this enzyme was classified as EC 1.2.1.24, based on the chemical reactions and substrate specificity as following;

EC -1 Oxidoreductases

EC -1.2 Acting on the aldehyde or oxo group of donors

EC -1.2.1 With NAD^+ or $NADP^+$ as acceptor

EC -1.2.1.24 succinate-semialdehyde dehydrogenase

The enzyme was maximally active at 100 mM sodium pyrophosphate buffer pH 9 in plant (*Solanum tuberosum, Arabidopsis sp)*, but gave only 20% of the activity found in pyrophosphate buffer and had a shorter linear rate. The other evidence reported, the optimum pH 8 *(Sus scrofa)* (Blaner *et al*., 1979), 8.5-8.7 of *Homo sapient* and *Rattus norreqicus* (Cash *et al*., 1977; 1978) and 9.5-10.5 of *Pseudomonas* sp. (Callewaert *et al*., 1973).

The enzyme specific for SSA as substrate and could not utilize acetaldehyde, glyceraldehydes-3-phosphate, malonaldehyde, lactate, or ethanol as substrates. It was also specific for NAD^+ as cofactor while $NADP^+$ and 3-acetylpyridine adenine dinucleotide could not serve as cofactors. However, the enzyme required exogenous addition of a thiol compound for maximal activity and was inhibited by the thiol-directed reagents *p*-hydroxymercuribenzoate, dithionitrobenzoate, and *N*-ethylmaleimide, by heavy metal ions Hg^{2+}, Cu^{2+}, Cd^{2+}, and Zn^{2+}, and by arsenite. These results indicate a requirement of a SH group for catalytic activity (Narayan and Nair, 1991).

In vitro assays revealed that SSADH is specific for SSA and exclusively uses NAD^+ to produce NADH. Both products (succinate and NADH) of the reaction catalyzed by SSADH are substrates of the mitochondrial respiratory chain, which produces ATP as a final product. Importantly, both ATP and NADH negatively regulate the activity of the enzyme.

Therefore, regulation of SSADH by ATP suggests a tight feedback control of the rate of substrates provided by the GABA shunt to the respiratory chain.

It was suggested that, in Arabidopsis, disruption of the unique SSADH gene results in plants undergoing necrotic cell death when exposed to environmental stresses

because the abnormal accumulation of reactive oxygen intermediates (ROIS). For example, SSADH mutants exposed to white light appear to be dwarfed with necrotic lesions.

Indeed, SSADH mutants are sensitive to at least two types of environmental stresses, both ultraviolet irradiation (particularly ultraviolet B) and heat cause a rapid increase in the levels of H_2O_2 in SSADH mutants, and this is associated with enhanced cell death and necrosis of leaves. The phenotype of the SSADH mutants could be caused by a lack of certain metabolites (loss of NADH or succinate to the respiratory chain), an excess of a metabolite derived from the GABA shunt with a possible toxic effects such as SSA or gamma-hydroxybutyric acid (GHB), or an imbalance in signaling molecules derived from the GABA shunt (including GABA) (Bouche´ *et al.,* 2003).

Consequently, the feedback regulation might also play a role in controlling the steady state levels of GABA and hence possible functions of GABA via pathways other than the TCA cycle.

1.2.4 GABA determination techniques

Many methods of GABA analysis have been developed on the basis of the instrumentation available, the sensitivity required, and the organism or tissue being analyzed.

Ackermann *et al,* (1910) and Abderhalden *et al,* (1913) first determined the presence of GABA in bacterial cultures by precipitating GABA by a platinum salt and comparing its melting point with the synthetic GABA platinate. The second report of GABA determination in plant tissue was based on paper chromatography of potato extracts, using a ninhydrin spray to detect GABA and amino acids (Steward *et al*., 1949).

Column chromatography on ion-exchange, starch, or cellulose media was employed by various researchers to isolate GABA from plants and yeast in quantities sufficient for crystallization (Roberts, 1974). From the time of their commercialization, HPLC, gas chromatography (GC), UV-spectrophotometer, Flow injection analysis (FIA) and the other have been developed for GABA determination and will be described below.

1.2.4.1 Liquid chromatography

LC or HPLC are more reported with pre-column derivatization before GABA analysis. Because GABA and amino acids in their native form are generally weak chromophores (do not absorb UV light) and do not possess electrochemical activity. This means that for analytical purposes, they must first be chemically modified (derivatized). These products can be detected at much higher sensitivity by certain types of liquid chromatographic detector (Kehr, 1998).

A number of different reagents have been proposed for derivatization, such as 2,4,6-trinitrobenzenesul (Desiderio *et al.,* 1987), phonic acid (Yamamoto *et al.,* 1985), orthophthalaldehyde (Qu, *et al.,* 1998; Kehr, 1998; Durkin *et al*., 1988), dansyl chloride (Desiderio *et al.,* 1987; Pahuja and Albert, 1984), dabsyl chloride, 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate (Liu *et al.,* 1998), 9-fluorenylmet-thyl chloroformate (Shen *et al.,* 1998) and phenylthiocarbonyl derivatives (Vasanits and Molnar-Perl, 1999). 2- hydroxynaphthaldehyde (Khuhawar and Rajper, 2003).

Considering of an operation of HPLC system, shown in Figure 1.13, which consists of 3 basic main components.

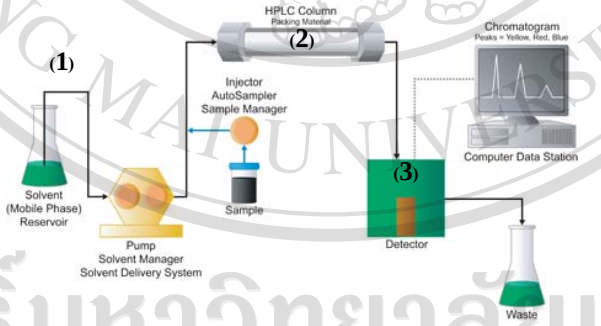

Figure 1.13 High-Performance Liquid Chromatography (HPLC) system. *Chemguide. 2009. "Carrying out HPLC"* [online]. Available *www.chemguide.co.uk/.../chromatography/hplc1.gif* (*25 June 2009*)

(**1**) **Mobile phase**; the mobile phase can be divided into two systems; Isocratic system; the composition of mobile phase is constant during elution time, usually using with reverse phase columns. Previous report showed, the isocratic mobile phase system for GABA determination were methanol:water (62:38 v/v) (Khuhawar and Rajper, 2003), 80% sodium acetate buffer:20% acetonitrile (Rossetti and Lombard,

1996), 35% 0.1M di-sodium hydrogen orthophosphate/50M EDTA (pH 5.6, 1M OPA) and 65% HPLC grade methanol (Clarke *et al*., 2007). However, the eluted GABA in sample is difficult to separate from other component under isocratic system as shown in Figure 1.14a.

The second system is gradient condition; the composition of mobile phase is varied during elution time. Example of gradient system that was used for determination of GABA was the mobile phase of methanol (mobile phase A) and 0.008% triethylamine (mobile phase B), the elution gradient was set as % mobile phase B was 70-70%, 70-60%, 60-50% at 0-25 min, 25-55 min, 55-60 min, respectively (Naval *et al*., 2006). The other system using for separation rice sample containing GABA were 0.1% formic acid (mobile phase A) and acetonitrile (%B). The gradient ratio was %B; 30-40, 40-55, 55-30 was used at 0-5 min, 5-10 min and 10-20 min, respectively. The gradient elution system provided high resolution of GABA at RT 5.78 from the other peaks.

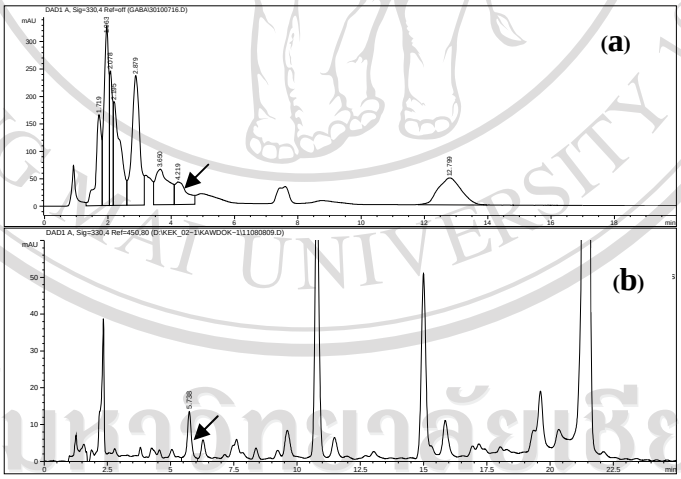

Figure 1.14 HPLC analysis of rice sample consisting of GABA. Separations were carried out on (**a**) isocratic of actonitrile:0.1% formic acid 68:32% v/v and (**b**) gradient condition of mobile phase A (0.1% formic acid) and mobile phase B (acetonitrile);% B; 30-40, 40-55, 55-30 was used at 0-5 min, 5-10 min and 10-20 min, respectively (Panatda *et al*., 2009).

(**2**) **Analytical column**, the sample is introduced through the injector into the system and is then pushed through the analytical column by the constant pumping of mobile phase from the reservoir to the system. It is usually made from stainless steel

and contains the solid packing or stationary phase, it divide into normal phase and reverse phase. Normal phase; the column is filled with a small silica particles, a typical column has an internal diameter of 4.6 mm, and a length of 150-250 mm. Polar compounds in the mixture being passed through the column will stick longer to the polar silica than non-polar compounds. The non-polar will therefore pass more quickly through the column. Reverse phase; the column size is the same, but the silica is modified to make it non-polar by attaching long hydrocarbon chains to its surface typically with either C-8 or C-18. A polar mobile phase is used, for example, a mixture of water and an alcohol such as methanol and acetonitrile.

Non-polar compounds in the mixture will attract with the hydrocarbon groups because of Van Der Waals dispersion forces. They will also be less soluble in the mobile phase because of need to break hydrogen bonds as they squeeze in between the water or methanol molecules, for example. They therefore spend less time in solution in the solvent and this will slow them down on their way through the column. That means that the polar molecules will move through the column more quickly. Reversed phase HPLC is the most commonly used form of HPLC.

(**3) Detector** Following the eluent emergence from the end of column, the uent flow to the detector. Detectors operate on various principles, some monitor the ultraviolet (Khuhawar and Rajper, 2003; Naval *et al.,* 2006), visible, or fluorometric properties of molecules (Clarke *et al*., 2007; Chakraborty *et al*., 1991). Others monitor differences in oxidation-reduction potential and reflective index.

However, it is difficult to assay a large number of samples containing GABA by HPLC because it requires a specific pre and post-column reaction and is time consuming, but it provides high accuracy results.

The summary of GABA determination by HPLC techniques was shown in Table 1.5

Table 1.5 Previous reports for GABA determination by HPLC

Author	Sample	Derivatizing agents	Column	Detector	Flow rate	Mobile phase
Rossetti, V. et al, 1990	GABA standard from Sigma	Phenylthiocarbamyl (PTC)	LiChrospher 100 RP-18 column (250×4 mm particle size 5 um	UV 254 nm.	Isocretic 0.6 ml/min	80% : 20% of solution A:B A: Aqueous solution of sodium acetate, triethylamine, acetic acid, acetonitrile B: acetonitrile-water (60:40)
Chakraborty et al, 1991	homogenate rat brain	O-phthaldialdehyde (OPA)	Altex ultraspere ODS column 4.6×250 mm, 5 um	fluorescent detector excitation 330nm emission 440 nm	Isocretic 1 ml/min	mobile phase containing 0.2M sodium acetate pH 3.8 100 mg/ml EDTA 40% acetronitrile
Khuhawar, MY. et al., 2003	cerebral spinal fluid	2-hydroxynaphthaldehyde (HN)	Phenomenex C 18, 5 mm	UV 330 nm	Isocretic 1 ml/min	methanol: water (62:38 v/v)
Clarke, G. et al, 2006	discrete regions of the rodent brain	naphthalene-2,3-dicarboxaldehyde (NDA)	Luna 5 C18 250×4.6mm column (Phenomenex),	electrochemical detection	Isocretic 0.65 ml/min	mixture of 0.1M di-sodium hydrogen orthophosphate/ 50 mM EDTA (pH 5.6, 1M OPA) and HPLC grade methanol (35:65).
Clarke, G. et al, 2006	discrete regions of the rodent brain	naphthalene-2,3-dicarboxaldehyde (NDA)	reversed phase Luna 3 C18 150×2mm column (Phenomenex)	fluorescent detector excitation 330 nm emission 440 nm	Isocretic 0.1 ml/min	mixture of 0.1Mdi-sodium hydrogen orthophosphate/ 50mM EDTA (pH 5.3, 1M OPA) and HPLC grade methanol (35:65)
Naval, M.V. et al., 2006	Cultured neurons	dansyl chloride	Waters ODS Spherisorb 150mm×4.6mm I	photodiode array detector	Isocretic 1 ml/min	methanol (mobile phase A) 0.6% acetic acid in water–0.008% trietilamine (mobile phase B).

Table 1.5 (continued) Previous reports for GABA determination by HPLC

Author	Sample	Derivatizing agents	Column	Detector	Flow rate	Mobile phase
Iimure et al, 2009	barley bran	AccQ-Fluor reagent	column (3.9 ×150 mm; Waters) by gradient elution at 39 ^{o}C	2475 multi fluorescent detector (Waters) with Ex. 250 nm Em. 395 nm.	Isocretic 1 ml/min	Four eluents were used; A (100 mM sodium acetate+5.6mM triethylamine (pH 5.7); B (100 mM sodium acetate+5.6mM triethylamin (pH 6.8); C (acetonitrile) D (distilled water).
Buck et al, 2009	brain microdialysates	Phonic acid	(ZIC-HILIC, 20×2.1mm i.d., 3.5 µm, SeQuant AB, Ume, Sweden)	fluoresecent detection	gradient 1 ml/min	Mobile phase A: (0.1% formic acid) in water and B:(acetonitrile) 0-0.1 min 15% A, 0.1-1 min 80% A, 1-3.15 min 15% A.
Reisi, P et al., 2009	dentate gyrus of anesthetized streptozotocin-induced diabetic	O-phthaladialdehyde	Column: ODS 4.6×250 mm;	fluorescence detection 330–440 nm	gradient 1 ml/min	Mobile phase A: sodium acetate buffer (pH6.7), methanol and tetrahydrofuran. Mobile phase B: methanol and tetrahydrofuran at 0.0-10 min %A 85, 10-13 min %A 30, 13-13.1min %A30, 13.1-17 min %A 85

35

2.4.2 UV spectrophotometry

GABA has en quantified by UV spectrophotometry using GABase based on the absorbance change caused by the conversion of $NADP^+$ to NADPH at 340 nm.

GABase, which contains two main enzymes GABA-T and SSDH, is known to catalyze the following two reactions:

$$\text{GABA} + \alpha\text{-ketoglutarate} \xrightarrow{\text{GABA-T}} \text{succinic semialdehyde} + \text{glutamate} \quad (1)$$

$$\text{Succinic semialdehyde} + NADP^+ + H_2O \xrightarrow{\text{SSDH}} \text{succinate} + NADPH + H^+ \quad (2)$$

Tsukatani *et al*, (2005) applied this technique to screen GABA-producing lactic acid bacteria in de Man-Rogosa-Sharpe (MRS) medium. The proposed method enabled one to assay 96 samples within an hour without the pre-treatment of samples. These results were highly satisfactory from the point of view of recovery and precision. However, this method allowed high cost for GABase enzyme, but high performance because the volume of the assay system was small. The proposed method met the demands of rapidity, simplicity, precision and recovery for an analytical method to be useful.

1.2.4.3 Gas chromatography

GC has been used to detect GABA. The published HPLC and GC methods of GABA analysis were often different in the procedure used to derivatize GABA. While derivatization for HPLC usually involves adding a chromophore that allows GABA to be detected by fluorescence or UV spectroscopy. Derivatization for GC usually involves forming a volatile compound. Structural confirmation of the derivatized GABA can then be achieved via chromatography coupled to mass spectrometry, GC-MS.

The GC-MS data show highly repeatable mass spectral fragmentation on electron impact (EI) ionization, and unsurpassed chromatographic reproducibility and resolution.

In addition, the GC-MS are relatively less expensive than other instrumentation modalities such as CE-MS, LC-MS, or LC-NMR.

In the past, GC and GC-MS were normally used for determination of GABA in biological sample such as grass herbage (Kagan *et al*., 2008), fresh mouse whole brain synaptosomes (Palaty *et al*., 1994), subregions of rat brain (Holdiness, 1981).

Sensitive and efficient assay for GABA was applied to fresh mouse whole brain synaptosomes where the extracted GABA was analyzed as its di(tert-butyl-(dimethylsilyl) derivative and using GABA-d6 as an internal standard (Palaty *et al*., 1994). However, GABA extraction method is problematic when biological sample will be applied to the GC-MS system.

A solid phase extraction method was developed by Richard *et al*., (2005) to extract GABA and all its metabolites simultaneously in each tissue type. Because GC techniques presented in the earlier literature did not allow the simultaneous analysis of GABA and the other metabolites (GHB, 1,4-BD, GBL) in animal or human tissues. It was found this technique allowed the limit of detection (LOD) of target compounds to be significantly lowered (LOD: 0.027, 0.025, and 5.7 μg/mL for GHB, 1,4-BD, and GABA, respectively, in 200 μL or μg of sample.

While, Kagan *et al*, (2008) developed a rapid GC-FID method for analyzing GABA in grass herbage by a derivatization kit EZ:faast™ method. It was found, limits of quantification and detection were 2.00 and 1.00 nmol/100 μL, respectively. Method recoveries at two different spike levels were 96.4 and 94.2%, with coefficients of variation of 7.3 and 7.2%, respectively)

Generally, the optimum condition used for GABA detection in mass spectrometric (MS) part were carried out by electronic impact under an electric field of 70 eV. MS detection was operated in SIM mode using a 5% phenyl/95% dimethylpolysiloxane capillary column (VF-5ms, 30 m 0.25 mm, 0.25 μm film thickness, Varian, Inc.). The following parameters were used: injector temperature, 250 C; helium flow, 1.0 ml/min; makeup gas flow, 23 ml/min; hydrogen flow, 30 ml/min; air flow, 300 ml/ min (Kagan *et al*., 2008).

1.2.4.4 Capillary electrophoresis

Capillary electrophoresis (CE) with laser-induced fluorescence (LIF) provides the advantages of high resolution, short analysis time, high sensitivity, and

small sample size for GABA determination. It quite suitable for the routine determination of a great variety of compounds in biological samples (Lujan *et al*., 2005)

Derivatization is necessary to enhance detection signals of this technique, when the analytes such as most amino acids do not possess intrinsic fluorescence. Many fluorophores have been tested to form highly fluorescent derivative compounds with amino acids. Among these, naphthalene-2,3- dicarboxaldehyde (NDA) is one of the most common reagent for labeling amino acids. NDA reacts with primary amines in the presence of cyanide to produce cyano benzoisoindole (CBI) products with high quantum yields (Horie and Rechnitz, 1995). CBI products exhibit two weaker excitation maxima in the visible region at approximately 420 and 440 nm and fluorescence at 490 nm after excitation.

1.2.4.5 Flow-injection system

The sequential quantification of GABA and L-glu by FIA using immobilized-enzyme reactors and fluorescence detection was developed. A co-immobilized L-glutamate oxidase (L-GOD), catalase (CAT) and an immobilized GABase reactor were introduced into the flowline in series as shown in Figure 1.15. The principle of this method is used for quantification of GABA and L-glu by using of immobilized-enzyme reactors in a single line, the NADPH will be produced and monitored by fluorometrically. This method for the sequential quantification of GABA and L-glu is based on the following reactions; (Lacerda *et al*., 2003).

$$\text{GABA} + \alpha\text{-ketoglutarate} + \text{NADP}^+ + \text{H}_2\text{O} \xrightarrow{\text{GABAase}} \text{L-glutamate} + \text{succinate} + \text{NADPH} + \text{H}^+ \quad (1)$$

$$\text{L-glu} + \text{O}_2 \xrightarrow{\text{L-GOD}} \alpha\text{-ketoglutarate} + \text{NH}_4^+ + \text{H}_2\text{O}_2 \quad (2)$$

$$2\text{H}_2\text{O}_2 \xrightarrow{\text{CAT}} \text{O}_2 + 2\text{H}_2\text{O} \quad (3)$$

GABA was selectively detected by enzymatic reaction (1) when α-ketoglutarate at a high concentration and $NADP^+$ were injected as reagents. The amount of NADPH produced depends on the concentration of GABA in the presence of α-ketoglutarate in the GABase reaction. When GABA and $NADP^+$ were

injected as the reagents with a sample, L-glu only was measured by the series of enzymatic reactions (2) and (1).

The amount of NADPH produced depends on the concentration of α-ketoglutarate produced from L-glu by L-GOD in the presence of GABA at a high concentration in the GABase reaction. Hydrogen peroxide produced by L-GOD slightly inhibited the GABase reaction. Therefore, CAT was co-immobilized with L-GOD in order to eliminate hydrogen peroxide reaction (3).

A schematic diagram of the FIA system for the sequential quantification of GABA and L-glu is shown in Figure 1.15.

The sample and the reagents ($NADP^+$ and α-ketoglutarate were injected by an open sandwich method in order to save expensive reagents, such as coenzymes. In this injection mode, sample and reagent were injected into zones next to each other.

Then, the sample and reagent were transported to the immobilized enzyme reactors while being mixed. The line for the reagent was switched between reagent 1 (GABA and $NADP^+$) and reagent 2 (α-ketoglutarate and $NADP^+$) by a six-way switching valve. Fluorescence intensity was measured at an excitation wavelength of 340 nm and emission wavelength of 455 nm with a spectrofluorometer. The flow system was operated at room temperature. (Kiba *et al*, 2002; Pasco *et al*, 1999, Hayakawa *et al*., 2004).

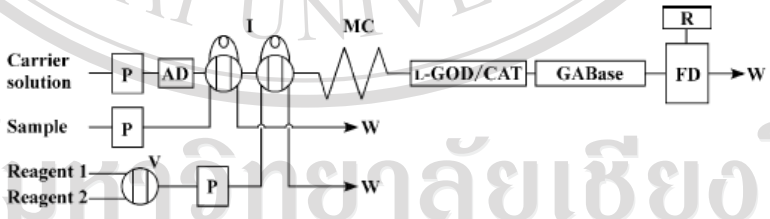

Figure 1.15 Schematic diagram of the flow-injection system for the sequential quantification of GABA and L-glu. P: micro-tube pump, AD: air damper; I: injector, MC: mixing coil; L-GOD/CAT: co-immobilized L-GOD and CAT reactor; GABase: immobilized GABase reactor; FD: fluorescence detector; R: recorder; W: waste (Lacerda *et al*., 2003).

1.2.4.6 Other techniques

The applicability of any published methods depends on the laboratory resources and the sample which GABA is to be extracted. The summary of GABA determination techniques were collected in Table 1.6

Table 1.6 Various methods for GABA determination

Authors	Reaction requirement		Techniques	Advantage	Drawback
Rossetti, V., et al, 1990 Chakraborty et al, 1991 Khuhawar et al, 2003 Clarke, G et al, 2006 Naval, M.V. et al, 2006 Iimure, T et al., 2009 Buck, K et al., 2009 Reisi, P et al., 2009 Chung, H.J et al., 2009	Derivatization		HPLC with UV or fluorescence detector	High sensitivity High accuracy High precision	Time-consuming High -cost instrument
Zhang, G and Brown, A.W., 1997 Tsukatani et al, 2005	GABA +L-glu SSA + NADP	SSA NADPH + H^+	UV spectrophotometer for NADPH detection at 340 nm	Non-specific Rapidity Simplicity, Moderate Precision Moderate Recovery	High cost for enzyme (GABA-T, SSDH) Low -cost instrument
Holdiness, 1981 Palaty et al., 1994 Richard et al., 2005 Kagan et al, 2008	Derivatized volatile		GC	High sensitivity High accuracy High precision	Time-consuming High -cost instrument
Horie and Rechnitz, 1995 Lujan et al., 2005	Derivatization →		CE with laser-induced fluorescence detector	High resolution Short analysis time High sensitivity Small sample	High -cost instrument
Kiba et al, 2002 Lacerda et al., 2003	GABA + α-ke + $NADP^+$ L-glu + O_2 $2H_2O_2$	NADPH + L-glu α-ke + $NH4^+$ + H_2O_2 $O_2 + 2H_2O$	Flow-injection system	Rapid and continuous Small size instrument	High cost for immobilized enzyme

40

1.2.5 Glutamate decarboxylase acitivity and GAD protein assays

1.2.5.1 Liquid chromatrography

The development of HPLC method to assay enzyme activities was easier to assay a single enzyme activity in the presence of others. The assay of enzyme activity is composed of several discrete steps.

The first is preparation of both the reaction mixture and the enzyme. The reaction mixture usually contains components of the buffer used to establish the correct pH, the substrate, and any cofactor (metal, PLP and the others) that may be required for catalysis. Preparation of the reaction mixtures involves mixing these ingredients in a reaction vessel such as a test tube, cuvette or eppendorf. Some cases the reaction mixture is brought to the required temperature prior to initiation of the reaction. In most case, the second step in the assays comprises initiation and incubation. (Rossomando, 1998) as shown in Figure 1.16.

A reaction can be initiated by the addition of the enzyme preparation to the substrate in the reaction mixture. This step is considered to start the reaction, and all subsequent time points are related to this time.

Termination can be achieved in several ways, most often separation involves isolating the substrate from the reaction product, but usually inactivation of the enzyme rather than the substrate removal.

Detection, the fifth step, refers to the process by which the amount of product formed by the enzyme during a specific incubation interval is determined.

The last step is an assay involves reduction of the data. This step includes all procedures in which the data are analyzed and graphed to determine initial rate as well as kinetic constants. Not all steps are involved in all assay methods, and in some methods one or more of the steps may be complex.

HPLC method for the determination of GAD activity has been investigated by Rossetti and Lombard, (1996). The standard enzyme obtained from Sigma was evaluated by incubation with L-glu in the presence of PLP. GABA formed was derivatized to phenylisothiocyanyl (PTC-GABA). The latter was subsequently separated and assayed by isocratic HPLC (LiChrospher RP-18 column; isocratic elution with pH 5.8 acetate buffer in acetonitrile-water) with UV absorbance detection at 254 nm, immediately.

Another evidences for assays GAD activity in rice germ using the reaction mixture consisted sodium phosphate, pH 5.6, 100 mM L-glutamate, PLP, and enzyme liquid. The reaction solution was incubated at 40°C for 60 min, and then terminated by addition of 32% (w/v) trichloroacetic acid (TCA). The suspension was filtered through membrane filter. The filtrate was analyzed for its GABA derivative form content by Agilent 1100 HPLC (Zhang *et al.,* 2007a; Zhang *et al.,* 2007b; 2006).

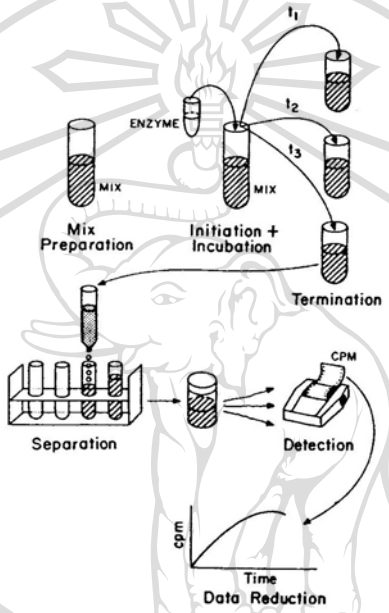

Figure 1.16 Schematic of a representative enzymatic assay by HPLC

The reaction mixture is prepared and the reaction can be initiated by the addition of the enzyme. During the reaction (incubation), sample are removed at the intervals labeled t_1, t_2 and t_3 and the reaction is stopped (termination) by inactivating the enzyme. One unit of GAD activity was defined as release of 1 µmol of GABA produced from L-glu per 30 min at 40°C. Specific activity was defined as units of GAD activity per mg of the enzyme.

The method described is a sensitive, reproducible and specific assay useful for following variations of GAD activity in vitro.

1.2.5.2 Western blotting analysis

Western blotting or Immunoblotting, which are used to detect a protein immobilized on a membrane. Before employing this procedure, it is necessary to have monoclonal or polyclonal antibody capable of recognizing the interested protein, either a crude extract or a more purified preparation.

It is an extremely powerful technique for identifying a single protein (or epitope) in a complex mixture following separation based on its MW, size, charge, and pI. In addition, when immunoblotting combined with immunoprecipitation permits the quantitative analysis of minor antigens. The immobilization of proteins on a membrane is preferred to working directly with SDS-PAGE because the proteins are more accessible, membranes are easier to handle than gels. Smaller amount of reagents are needed, and processing times are shorter.

In summary, western blotting can be devided into two steps: transfer of protein from SDS-PAGE gel to the membrane, and protein detection with the specific antibody.

(1)Transfer of protein from SDS-PAGE gel to the membrane

Protein transfer is most commonly accomplished by SDS-PAGE. First, two kinds of membranes which are most commonly used for transferring protein from gels should be considered.

- Nitrocellulose; nitrocellulose is preferred because it is relatively inexpensive and blocking nitrocellulose from nonspecific antibody binding is fast and simple.
- Nylon; nylon can be useful if a high protein binding capacity is required, especially high molecular weight or acidic proteins. The use of nylon provide greater resistance to mechanical stress. It has been limited because it is more expensive than nitrocellulose, blocking is cumbersome, and staining for total protein with anionic dyes is not possible.

Two common blotting methods are shown in below;

- Semi-dry blotting, in which the gel and immobilizing membrane are sandwiched between buffer-wetted filter papers through which a current is applied for 10-30 minutes.
- Wet tank blotting, in which the gel-membrane sandwich is submerged in

transfer buffer for electrotransfer, which may take as little as 45 minutes or may be allowed to continue overnight.

(2) Protein detection by specific antibody

Detection of protein will be described in two steps following;

- Transferred membrane is incubated with the primary antibody for several hours or overnight.
- Next, a second antibody, which recognizes an epitope on the first antibody (usually using goat antibodies raised against rabbit immunoglobulins for rabbit-generated first antibody) is purchased already conjugated to a labeling agent such as the enzyme horseradish peroxides. This marker is then visualized by a colorimetric reaction catalyzed by the enzyme which yields a color product that remains fixed to membrane. Other detection system including alkaline phosphatase or immunogold conjugates and I^{125}.

There are previous reports of Yun and Oh, (1998) using western blotting for GAD detection in *E. coli* cells, when transformed with pVUCH-RicGAD and induced with IPTG. The molecular weight of the GAD protein recognized by the antibody was approximately 56-58 kd (Figure 1.17), larger than that of the *E. coli* GAD, which is 53 kd. These data clearly suggest that RiceGAD encodes for a GAD protein using western blotting analysis.

While, Akama and Takaiwa, (2007) using western blotting analysis of the recombinant extracted GADs from *E.Coli* after two rounds of purification by nickel-affinity chromatography and anion-exchange chromatography. The band sizes of these two recombinant proteins, calculated with a molecular size marker, were 78 kd and 74 kd for OsGAD1 and OsGAD2, respectively as shown in Figure 1.18. Lanes 1, 2, and 3 represent protein size marker, wild-type OsGAD2 and the other was based on wild-type OsGAD2, but lacked the coding region for the C-terminal extension.

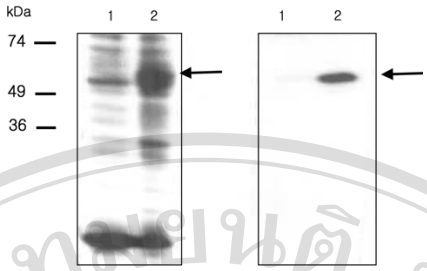

Figure 1.17 SDS-PAGE and Western-blot analyses of RiceGAD-encoded protein expression (Yun and Oh, 1998). **(a)** Coomassie blue-stained gel of lanes 1 and 2, protein extracts of *E. coli* transformed with pVUCH-RiceGAD before and after induction by IPTG, respectively **(b)** Western-blot, detected with an anti-GAD monoclonal antibody. The arrows indicate the positions of the RiceGAD protein expressed in *E. coli.*

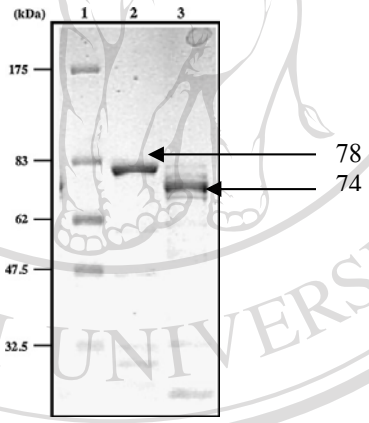

Figure 1.18 Rice GAD introduced into *E. coli* BL21 for overexpression of the recombinant proteins using western blotting analysis Akama and Takaiwa, (2007)

1.2.5.3 Gas chromatographic-mass spectrometry

A quantitative GC-MS method has been developed for the determination of GAD activity in biological sample. For example, subregions of rat brain, globus pallidus, entopeduncular nucleus, ventromedial thalamus, and substantia nigra medical and lateral. The activity of the GAD enzyme has been determined indirectly by measurement of GABA using gamma-[2,2-$2H_2$] aminobutyric acid as the internal standard. GABA were quantitatively converted to derivatized trimethylsilyl-GABA and trimethylsilyl-[$2H_2$] GABA with hexamethylchlorosilane, trimethylchlorosilane, pyridine, respectively (Holdiness *et al*., 1981). (N,O bis(trimethylsilyl)

trifluoroacetamide silylating is the commonly used deverivating agent in the.GABA work (Palaty, 1994). The instrument normally using selective ion monitoring and electron impact ionization at 70 eV has the detection limit of 15 ng GABA /mg various tissue.

1.2.5.4 pH indicator method

pH indicator method (PIM) using for rapid screening of GAD activities in many samples. The qualitative analysis by the complex pH indicator, methyl red and methylene blue dissolved in methanol, pH 5.4, was added to the sample, and the color change was observed. The color will change from magenta to green, if the sample have GAD activity.

This method provides a more rapid, less expensive and less laborious GAD qualitative detection method when compared to the methods previously mentioned. However, the quantitative analysis of GAD activity should be together performed with the other techniques (Yang *et al*., 2006)

1.2.5.5 Radiometric method

The GAD assay can be performed via a radiometric technique, which is predicated on L-[^{14}C]Glu-dependent $^{14}CO_2$ production. The reaction medium consisting of main components such as, sodium hydroxide or potassium hydroxide, buffer, cofactor (PLP), L-[^{14}C] glutamic acid in sealed side-arm flask containing $^{14}CO_2$ trap. All reactions were initiated by the addition of the GAD enzyme into the reaction medium, via injection through the rubber stopper with a microsyringe in a shaking water bath (Spink *et al*., 1985; Satyanarayan and Madhusudanan,1985).

The samples were then incubated, and the reactions were terminated. The reaction flasks were then allowed to stand at 4^{o}C overnight, in order to ensure the complete evolution of the $^{14}CO_2$ and absorption by the sodium or potassium hydroxide trap, prior to the determination of the ^{14}C content of the $^{14}CO_2$ trap, which was accomplished by liquid scintillation counting (Spink *et al*., 1985; Satyanarayan and Mudhusudana, 1985).

Beside, GAD activity can be assayed for detection of the product ^{14}C-GABA in *Trichroderma viride* conidia of developing mycelia by using U-^{14}C-glutamic acid as substrate (Strigáčová *et al.,* 2001).

1.2.5.6 Manometric Warberg technique

GAD activity was assayed by determined the rate of carbon dioxide with conventional Warburg techniques. Measurements were made in the main chamber contained buffered substrate solution; after thermal equilibration at optimal time, enzyme solution was tipped in from the side arm. Under these conditions, gas evolution continued at an essentially linear rate for several minutes (Cozzani *et al*., 1970)

Specific activity was defined as micro liters of CO_2 evolved in 10 minutes by 1 mg of protein.

1.2.5.7 Non-denaturing gel electrophoresis

Non-denaturing gel electrophoresis, also called native-gel electrophoresis, that separate proteins based on their size and charge properties. While the acrylamide pore sizes serve to sieve molecules of different sizes, proteins which are more highly charge at the pH of the separating gel have a greater mobility. This method is capable of separating molecules which differ by a single unit charge. The conditions for non-denaturing gel electrophoresis minimize protein de-naturation without SDS, in contrast to SDS-PAGE (Hames and Rickwood, 1990).

For example, this technique can be applied useful for GAD detection in native-gel electrophoresis by incubating the native gel contains activated GAD enzyme in solution A containing L-glu at 37^{o}C for 30 min. Then, solution B (the mixture of NADP, phenazine methosulphate (PMS), nitro-blue tetrazolium (NBT), GABA-T and SSDH enzyme) was added and placed in the dark at 37^{o}C until dark blue band appeared Visible formazan, a water-insoluble coloured azo compound, which formed by reduction of a NBT. Stained gel was washed in water and fixed in 50% ethanol (**Manchenko, 1996**)

The described methods for GAD activity determination was summarized in Table 1.7

Table 1.7 Method for GAD activity determination

Authors	Techniques	Method	Unit calculation	Advantage/Drawback
Rossetti and Lombard, 1996 Rossomando,1998 Zhang et al., 2007a;, 2007b; 2006	HPLC	The reaction consisted of sodium phosphate, pH 5.6, L-glu, PLP, and enzyme. Incubate at 40°C for 60 min, and then terminated by 32% (w/v) TCA. Analysis for GABA content by HPLC	1 µmol of GABA produced from L-glu per 30 min at 40°	Time-consuming High accuracy
Holdiness et al., 1981	GC/GC-MS	Derivatived volatile was trapped and directly injected to GC/GC-MS port	1 µmol of GABA produced from L-glu per 30 min at 40°C.	Time-consuming High accuracy
Yang et al., 2006	pH indicator	Qualitative analysis by the complex pH indicator (methyl red and methylene blue dissolved in methanol, pH 5.4 was added to the sample, and the color change was observed.	-	Rapid and Low cost Many sample can be applied Should be together performed with the other techniques
Spink et al., 1985 Satyanarayan and Madhusudanan,1985	Radiometric	Predicated on L-[^{14}C]Glu-dependent $^{14}CO_2$ production. The reaction medium consisting of main components such as, sodium hydroxide or potassium hydroxide, buffer, enzyme, cofactor (PLP), L-[^{14}C] glutamic acid in sealed side arm flask containing $^{14}CO_2$ trap.	One unit of GAD activity is defined as 1 ,µmol of CO_2 evolved per min at 37 °C	Harmful radiometric chemical
Strigáčová *et al.,* 2001	Radiometric	Assayed for the product ^{14}C-GABA by using U-^{14}C-glutamic acid	One unit of GAD activity is defined as 1 ,µmol of CO_2 evolved per min at 37 °C	
Cozzani *et al*., 1970	Warburg manometric	Measurements chamber contained buffered substrate solution; after thermal equilibration at optimal time, enzyme solution was tipped in from the side arm. Gas evolution continued at an essentially linear rate for several minutes	One unit was defined µl of CO_2 evolved in 10 min.	Contaminated non-specify CO_2 Traditional method
Manchenko, 1996	Native gel	Incubate the native gel contains activated GAD enzyme in colored substrate solution	-	High cost of reagents using for color emission