Chiang Mai J. Sci. 2010; 37(1) : 124-133
www.science.cmu.ac.th/journal-science/josci.html
Contributed Paper

γ-Aminobutyric Acid (GABA) Accumulations in Rice During Germination

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Received: 17 March 2009
Accepted: 10 June 2009

ABSTRACT

GABA (γ-Aminobutyric acid)-enriched rice becomes a popular healthy food nowadays. It has a major inhibitory neurotransmitter function. It inhibits cancer cell proliferation and also reduces blood pressure. GABA concentrations in rice grains and leaves of five well-known rice cultivars were investigated during germination. After germination of whole rice grains, hull and young leaves were removed and grains was used for consumption. Young leaves, waste from rice milling process, and germinated rice grains were collected to determine GABA concentrations by LC-MS after 2-hydroxynaphthaldehyde (HN) derivative formation. Although all of the cultivars have different initial glutamic acid concentrations, GABA concentrations in rice were not different among them (p>0.05). The GABA concentrations in rice grains and leaves were dramatically increased with germination days. However, rice leaves contained more GABA than rice grains by 2-3 folds in all rice cultivars. The highest GABA concentrations in rice grains and young leaves were found at 20 and 30 germination days, respectively. After 20 days of germination, GABA concentrations in rice grains were decreased. GABA concentrations in germinated rice grains were found to be 0.19-1.25 mg/g in Pitsanulok2 (PL2) rice; 0.30-2.01 mg/g in Chainat1 (CN1) rice; 0.51-2.45 mg/g in Kawdokmali 105 (KDML 105) rice; 0.34-1.74 mg/g in Supan 1 (SP1) rice and 0.39-1.59 mg/g in Patum1 (PT1) rice cultivars during germination. In contrast, rice leaves showed increased GABA concentrations until 30 germination days. The GABA concentrations were shown to be 1.45-3.14 mg/g, 1.36-2.85 mg/g, 2.39-2.52 mg/g, 0.82-2.09 mg/g and 1.33-1.50 mg/g in normal rice PL2, CN1, KDML 105, SP1 and PT1, respectively. Data of the GABA accumulation and disappearance in rice produced by germination method were presented. These results support effective uses of germinated rice grains for consumption and rice leaves for pharmaceutical application.

Keywords: GABA, rice, germinated rice, gamma-aminobutyric acid, LC-MS, glutamate decarboxylase.

1. INTRODUCTION

Rice is the major staple food in Thailand and contributes to the human diet since ancient time. It is a good source of dietary fibers, essential amino acids, proteins, carbohydrates, vitamins and other non-nutrient essential phytochemicals, concentrated in the germ and outer layers of the starchy endosperm [1-3]. However, the rice grains contain less nutrition components such as dietary fibers, phytic acids, vitamins and γ-aminobutyric 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 or milling operation [2]. Therefore, nutrition of rice seeds needs to be improved before consumption.

GABA is currently an interesting compound. Many research reported that it presented in the seed of plants during germination [4-11] via protein metabolism of seed components.

Several reports suggested that plant extracts containing high GABA leveled were effective for lowering the blood pressure of experimental animals and human, recovery of alcohol-related symptoms [6-7], being a major neurotransmitter [5-7] and helped improving memory and learning ability of mouse [8].

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 [9].

It is interesting to increase GABA in rice by germination process. This process breakdowns the reserved materials, synthesizes the structural proteins and cell components in seeds after hydration [4, 9].

Germination process activates many enzymes in raw seeds, especially glutamate decarboxylase (GAD) enzyme which catalyzes L-glutamic acid to GABA [5-7].

However, there are many reports using germination method to increase GABA in plants, such as commercial legumes (beans, lentil and pea) [4]. The GABA-enriched legumes are now offered in the market and in the health food shops. Besides these, the germination process was used in many other species including alfalfa [4], mung bean [4], soybean [10], green tea [6] and wheat [11] to increase their nutrition values.

Studies on GABA content in rice have been reported [2, 5-7, 12], but they studied only in brown rice and rice germ but not in whole rice grains.

The purpose of this work was to study the effect of germination on the content of GABA in five well-known cultivars of whole rice grains and also the young rice leaves. Whole rice grain enriched with GABA could be exported in higher price and the rice leaves can be used as pharmaceuticals and consumed raw in salads or decorative appetizers with high GABA content.

2. MATERIALS AND MATHODS

2.1 Instrument and Chemical Reagents

LC-MS (HP 1100 Binary/G1946A) operated in the positive ion mode was performed. The mass spectrometer with electro-spray ionization (API-EI) source was operated in the positive ion mode. Mass spectrometry experiments were performed to isolate and fragment the targeted ions. The operation conditions of the MS detector were optimized with a solution of GABA with an abundance of m/z 258 $[M+H]^+$ which was determined as follows; Fragmentation range: 70, Mass range: 100-1000, Drying gas flow:12 l/min, Nebulizer pressure: 32 psig, Drying gas temperature: 350°C, Capillary voltage: 3,000 V.

Gamma-aminobutyric acid (GABA) and glutamic acid were purchased from Fluka, China and hydroxy-napthaldehyde (HN) was

purchased from Aldrich, Germany. HPLC grade acetonitrile and ethanol were purchased from BDH Prolabo, EC. PDA agar was purchased from Difco, USA. Formic acid was purchased from Wako Pure Chemical Industries, Japan. Buffer solutions were prepared from sodium tetraborate (Borax) (1 M) and boric acid (1 M) which was purchased from MERCK, Germany.

2.2 Raw Materials

Five whole grain rice (*Oryza sativa* L.ssp. Indica); Kaw Dok Mali 105 (KDML105); Pitsanulok2 (PL2), Supan1 (SP1), Chainat1 (CN1) and Patum 1 (PT1) cultivars were obtained from Pitsanulok rice seed center, Thailand for using in germination experiments. Rice seeds were harvested in March-April 2007. All samples were stored in a refrigerator at 4°C until used for germination.

2.3 Germination

Germination of rice was carried out by the method of Ohtsubo et al. [2] and Komatsuzaki et al. [5] with a slight modification. 250 g of whole rice grains was soaked in 2L of water at a controlled temperature at 30°C for 72 h [2, 5, 12] and water was changed every 24 h. Germinated brown rice grains were sampled after 5, 10,15, 20, 25, 30 days after germination. The hull, root and young leaves were separated from the grains to obtained polished rice grain. The polished germinated grain was analyzed for the levels of GABA. Samples were prepared and analyzed in triplicates.

2.4 Extraction of GABA in Non-Germinated and Germinated rice

The extraction procedure was modified from Oh and Choi [10]. 0.25 g non-germinated and germinated rice powder was placed in eppendorf containing 800 µl of 70% (v/v) ethanol solution. The mixture was vigorously mixed for 1 min at room temperature and then centrifuged at 13,000×*g* at 4°C for 10 min. The supernatant was collected in a glass vial. The same volume of 70% ethanol solution was added to the pellet as described above, and the extraction was repeated. The collected supernatant (3 ml) was filtered through a 0.45 µm Millipore filter and analyzed by LC-MS after hydroxy-napthaldehyde (HN) derivertization (Khuhawar and Rajper [13]).

2.5 Determination of Glutamic Acid and GABA in Non-Germinated and Germinated Rice

The analyses of glutamic acid and GABA in rice extracts were determined by LC-MS. The aliquots of 70% ethanol extracts were derivatised with HN. LC-MS (HP 1100 Binary/G1946A) system and Photodiode Array detector were used for analysis. The linear gradient system with mobile phase A (0.1 % formic acid) and mobile phase B (acetonitrile) ; A; 35-40, 40-55, 55-35 was used at 0-5 min, 5-10 min and 10-20 min, respectively. This system allowed separation of crude extraction in 25 min using a C18 reversed phase column of 330 mm.

2.6 GAD Activity Assays

GAD activity was modified from the procedure of Zhang, Yao and Chen [6]. The reaction was composed of 200 ul of 50mM sodium phosphate, pH 5.8, 100 ul of 100 mM L-glutamate, 0.2 mM PLP, and 200 ul of rice crude extract. The reaction solution was incubated at 40°C for 60 min, and then terminated by boiling in water for 10 min. The suspension was derivatized with HN solution, filtered through a 0.45 micron filter. The filtrate was analyzed for GABA content by LC-MS, immediately. One unit of GAD activity was defined as release of 1 umol of GABA produced from glutamate within 30

min at 40°C . Specific activity was defined as units of GAD activity per mg of the enzyme.

2.7 Western Blot Analysis

The protein samples were separated on 10 % SDS-PAGE gel, and transferred to nitrocellulose membranes (0.2 μm, Bio-Rad, USA), and the presence of GAD was detected using anti- GAD monoclonal antibody using a chemiluminescence protocol with an ECL kit (Amersham, UK).

2.8 Statistical Analysis

All parameters were analyzed by analysis of variance (ANOVA) using SPSS program version 13. Different value shows significant difference ($p < 0.05$).

3. RESULTS AND DISCUSSION

3.1 GABA Concentration in Whole Rice Grain and Leave During Germination

In this research, whole rice grains were subjected to germination for different days. The germination process leaded to increasing of GABA contents during germination time both in grains and in young leaves (Figure 1), while non-germinated rice seed (control) contained less GABA as shown in Figures 1b and 2a.

According to Figure 1b, GABA concentrations in rice grains of all cultivars increased during germination and decreased after 20 germination days, except SP1 and KDML105 cultivars. However, after 20 germination days, Unchanged GABA contents was presented in KDML105 cultivar. While, GABA contents of SP1 slightly decreased after 25 germination days. The results indicated that GABA was produced during germination and was used as nitrogen source of growing and entered to Kreb's cycle [14].

Although GABA contents in germinating rice seeds at 20-30 days were higher than other germination time, but rice seeds had soft texture. The rice characteristic was thus not acceptable for consuming.

Comparing of GABA concentration in rice grains of each cultivar at the same germination periods, the GABA contents were not different ($p>0.05$) among the cultivars (Figure 1b). However, for SP1 and PL2 cultivars, GABA contents were different ($p<0.05$) from KDML105, CN1 and PT1 cultivars at the periods of 5 days. After 5 days, the GABA concentrations were not different among the cultivars ($p> 0.05$), although they had a varying glutamic acid contents (Table 1) before germination. The CN1 cultivar had glutamic acid content at the amount of 8.79 mg/g, while KDML 105 showed a medium glutamic acid content at 4.28 mg/g. The SP1, PL2 and PT1 had low glutamic acid contents of 1.64, 1.03 and 0.94 mg/g, respectively.

This results indicated that the initial glutamic acid contents had no effect on GABA concentrations in rice grains during germination after the germination period of days in all cultivars.

However, the GABA contents were found in non-germinated rice grains (control) to be 0.19, 0.24, 0.34, 0.39 and 0.51 mg/g in PL2, CN1, SP1, PT1 and KDML 105, respectively (Figures 1b and 2a). The values were not different among the cultivars ($p>0.05$). After germination, the highest levels of GABA were found at 1.25, 1.59, 1.74, 2.01 and 2.45 mg/g in PL2, CN1, SP1, PT1 and KDML 105, respectively. GABA concentrations increased about 6.58, 6.70, 5.11, 4.80, 4.08 folds in PL2, CN1, SP1, PT1 and KDML 105, respectively when compared with the control. This result was the same as the previous reports which studied the effect of germination on GABA accumulation in brown rice and other plant species. For example, Korean and Japanese brown rice prepared by soaking in water, the results showed higher GABA concentrations of 13

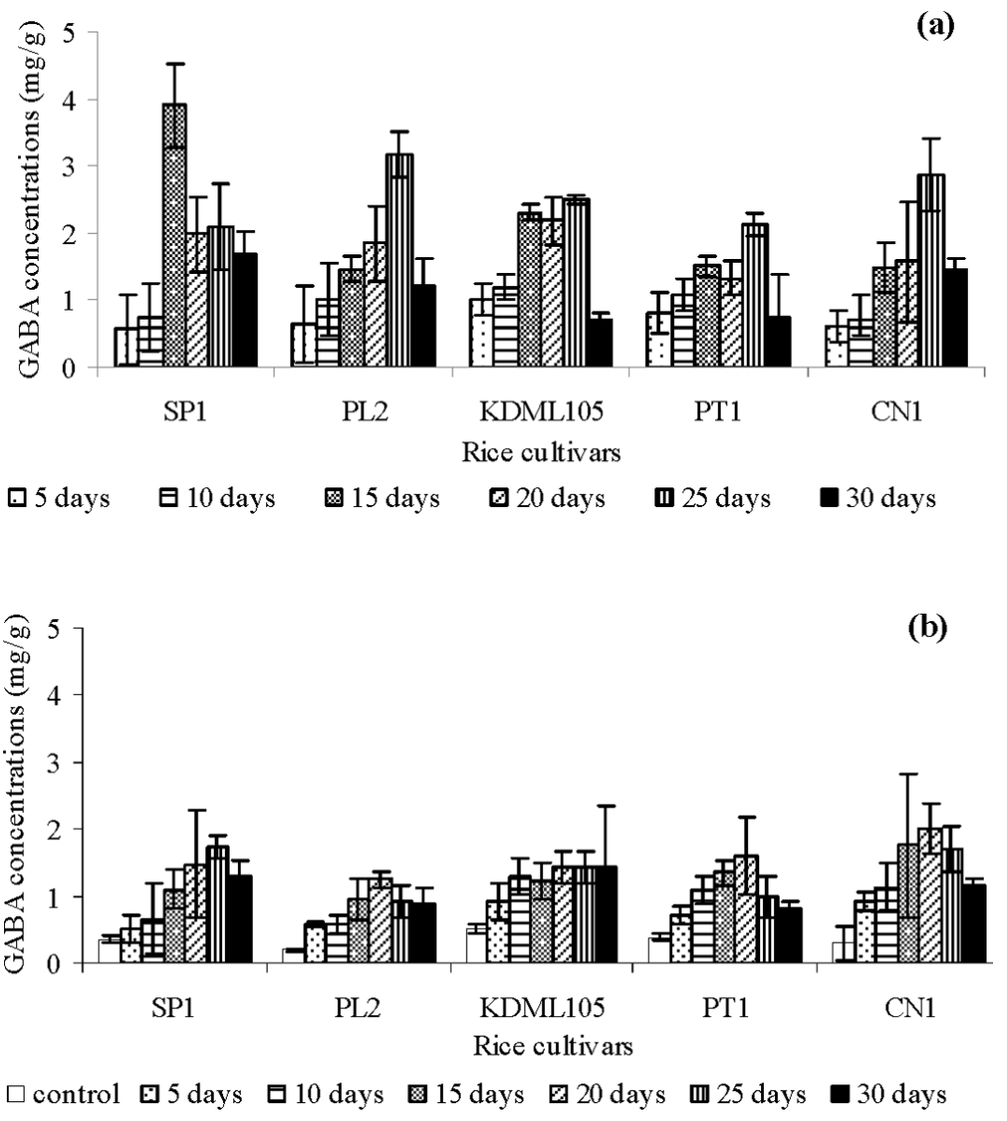

Figure 1. Increased concentrations of GABA in five cultivars of rice in each growing part **(a)** rice leaves **(b)** germinated rice grains compared with non germinated rice (control) at different germination time. Each bar represents the average of three determinations with error bars showing the standard error of the mean.

and 3.41 folds higher than non- germinated brown rice [7-9], Moreover, GABA could accumulate upon soybean [*Glycine max* (L.) Merr] seed growing, about 11-17 folds increase as compared with the control plant [10]. While, germinated wheat had increased GABA content 18 times greater than non-germinated wheat [15].

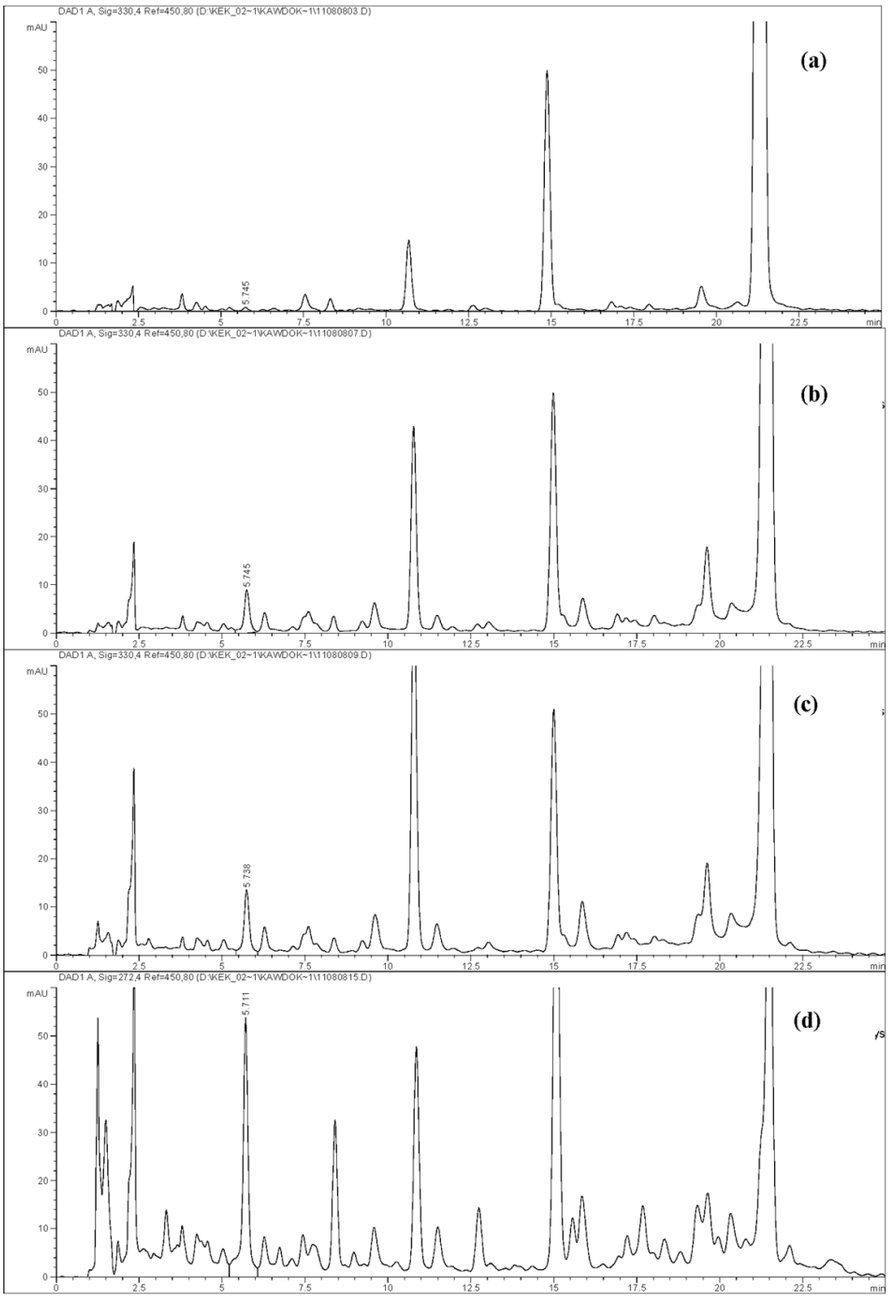

Figure 2. HPLC chromatogram showing GABA levels in rice grains of KDML 105 cultivars at different germination time; **(a)** control (non-germinated grain) **(b)** 5 germination days **(c)** 10 germination days **(d)** 30 germination days. Separated GABA and glutamic acid peaks were shown at retention time (RT) of 5.7 and 7.5 min, respectively in rice grains. Crude extract was separated by HPLC elution with gradient condition of Acetonitrile : 0.1% formic acid with flow-rate of 1 ml /min and UV detection at 330 nm.

Table 1. Glutamic acid contents in different whole rice grain cultivars before germination.

Rice cultivars	Glutamic acid concentrations (mg/g)
Chainat1 (CN1)	$8.79^{c} \pm 1.19$
Kawdokmali (KDML 105)	$4.28^{b} \pm 1.03$
Supan 1 (SP1)	$1.64^{ab} \pm 1.04$
Pitsanulok2 (PL2)	$1.03^{a} \pm 0.91$
Patum1 (PT1)	$0.94^{a} \pm 0.49$

The results indicated that germination process can increase GABA concentrations in many seed species, possibly because the plant germination has a metabolic change in seed and reserved materials were breakdown after hydration , which results in an accumulation of a soluble nitrogen compound such as GABA [14]. A related reason is that extensive breakdown of seed-storage compounds occurs and protein and other cell components are synthesized during this process [4,9]. Moreover, Komatsuzaki et al [5] suggested that, storage proteins were decomposed by water absorption during germination, changed into transportable amide and supplied to the growing parts of rice seedling as a nitrogen source for embryo growth during germination [7]. Another reason concerns an increase of glutamic acid during germination. It can be converted to higher GABA than non germination condition. The higher glutamic acid might be resulted from activating of enzymes during germination, for example, a variety of proteolytic enzymes. As a result, protein breakdown into free amino acids especially glutamic acid can be changed into GABA compound [16]. Another type of enzymes involving in glutamic acid formation is aminotransferase. The previous report showed glutamic acid produced in seed during germination by the action of glutamine-oxyglutarate aminotransferase [14]. This enzyme reaction leads to glutamic acid formation and subsequent transformation to higher GABA during germination by GAD enzyme [10]. However, some evidence suggested that, glutamic acid was increased because it was synthesized by the glutamate synthase (GOGAT) and glutamine synthase (GS). The GS/GOGAT system plays an important role in glutamate accumulation during germination [17].

The GABA content in this research was compared with other data, such as Japanese rice prepared by soaking and gas treatment during germination contained GABA at 2.49 mg/g [5] and GABA contents of 0.207 mg/g for Korean rice [7]. But when Japanese rice soaked in water for 96 hours at 30°C, 1.49 mg/g of GABA were reported [2]. After soaking with gaseous treatment, the content of GABA was higher than that by the conventional soaking method. It is possible that, rice treated by gas during germination can reduce microorganisms and prevent fermentation during rice soaking. Fermentation process leads to decrease amount of GABA during germination [5]. The highest of GABA content of our germinated rice was 3.16 mg/g, which was higher than the previous reports because the different methods (germination and soaking time, temperature and variety of rice cultivars) used for treatment during germination which had the effect on GABA contents. Different cultivars showed the varying of embryo weights. There was

a report that, GABA in the brown rice with giant embryo drastically increased after germination when soaked in water at 30°C for 24 hours [18]. This result related with the previous report that *O. sativa* mutant with giant embryo was enhanced in GABA concentration and several japonica rice (*O. sativa* var *japonica*.) mutants with giant embryo were found to be nutritionally enhanced [19]. Saikura et al. [20] suggested that the amount and pattern of GABA accumulation varied with each cultivar. This variation might be due to the different GAD activity and purity of the rice cultivars.

Beside these, the GABA accumulation in rice leaves was investigated (Figure 1a). Young leaves are considered as waste from GABA-enriched rice production, they were removed from grains before milling. Young leaves contained high amount of GABA after rice germination, it increased upon germination time the same as rice grains (Figure 1a). The GABA concentrations found in young leaves were 1.68-3.91 mg/g of SP1, 1.45-3.16 mg/g of PL2, 0.69-2.50 mg/g of KDML105, 0.23-2.12 mg/g of PT1 and 0.72-2.86 mg/g of CN1.

3.2 Comparison of GABA Concentrations Between Rice Grains and Young Leaves

From results in Figure 1a, rice leaves showed the higher GABA concentrations than in rice grains (Figure 1b) in all cultivars during germination time. The higher GABA contents of leaves were 2.25, 2.53, 1.02, 1.33 and 1.42 folds of those in grains of SP1, PL2, KDML105, PT1 and CN1, respectively. The difference in the level of GABA in each seedling part was seen in previous report [14, 15]. The GABA concentrations were detected depending on the stage of tissue development with the highest level found in the apical regions such as root, and leave [15].

It might be due to the mobility of various compounds of seed to leave during germination. It was reported that, after the breakdown of materials in seed, the transport of materials from seed to another can occur. For example, from the endosperm (seed) to the embryo or from the cotyledon to growing parts and lastly the synthesis of new materials from the breakdown product occurs [14]. It was reported that, a loss of nitrogenous compounds such as GABA in seed was observed together with its increase in seedling and pumule of seedling [16].

GABA contents in leave were not different among the cultivars during germination ($p > 0.05$). This result suggested that the application of rice leaves for pharmaceutical uses. In addition, seedlings are often consumed fresh in salads or as decorative appetizers. However, other nutritional values of seedlings is needed to be considered.

The results of GAD activity in developing rice seedling of KDML105 were shown in Figure 3. The GAD activities increased in young leave and germinated grains during germination, but different levels of GAD were observed in both tissues. GAD activity was very low in the germinated rice grains, while 3.30 fold higher was found in young leaves.

Besides, GAD protein expression patterns in young leave and grain were compared using western blotting technique. The expression of GAD protein in young leave (lane 3) and germinated grains (lane 2) higher than the control (lane 1) at 70.8 Kdal [21] as shown in Figure 3b.

Interestingly, the GAD activity of rice grains before germination (control) not found Figure 3a, but GAD protein in control was detected (lane 1) when using western blotting technique. Komatsuzaki et al. [5] suggested that upon water absorption during soaking and germination, GAD enzyme be activated after germination.

The results in Figure 3a and 3b supported the data of GABA content (Figure1), which were described above. It is suggested that young leaves is a good source of GAD enzyme than grains, it may be possible to use GAD enzyme in other applications.

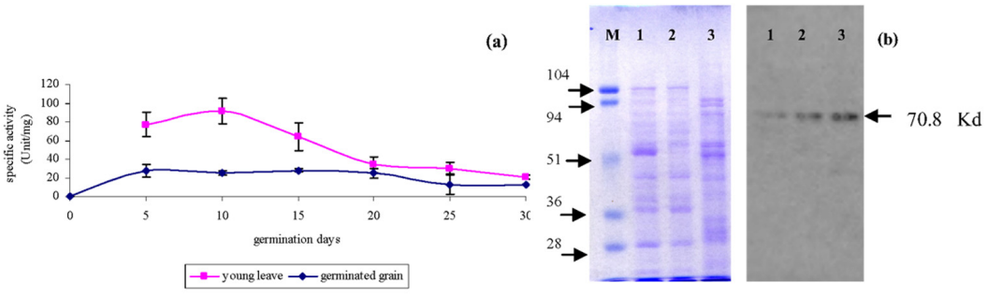

Figure 3. Specific activity of GAD enzyme of KDML105 rice seedling and Western-blot analyses of Rice GAD-encoded protein expression: **(a)** GAD specific activity in young leave and germinated rice grain **(b)** Western-blot, detected with an anti-GAD monoclonal antibody. The arrows indicate the positions of the rice GAD protein expressed at 70.8 Kd; Lanes: 1, non-germinated grains; 2, germinated grain; 3, young leave.

4. CONCLUSIONS

This results imply that rice, both grains and leaves, is a good resource for GABA accumulated during germination. With regard of GABA function, consumers will be of health benefits. This research also gave the data about the different amount of initial glutamic acid contents in each cultivars which had no effect to GABA levels. However, GAD enzyme level correlated with GABA content.

ACKNOWLEDGEMENTS

We gratefully acknowledge the full financial support from The Commission for Higher Education; Strategic Consortia for Capacity Building of University Faculties and Staff Scholarships, Ministry of education Thailand, partial support from Pibulsongkram Rajabhat University, Pitsanulok, Thailand and The Center for Innovation in Chemistry: Postgraduate Education and Research Program in Chemistry (PERCH-CIC), Thailand.

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