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Original Article

GABA content and Antioxidant activity of Thai waxy corn seeds germinated by hypoxia method♣

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Abstract

Germinated seeds have a greater amount of the naturally-occurring γ-aminobutyric acid (GABA) which has many health benefits. Further, colored seeds have higher antioxidant activity. Thai waxy corn is widely consumed after cooking, due to its palatable glutinous texture. However, it is not commonly germinated before use. In this study, two varieties of Thai waxy corn, KKU-KND (purple seed) and KKU-SLE (white seed), were germinated and converted to corn flour with the aim of investigating the effect of germination on GABA content and antioxidant activity. Further, the microstructure of starch granules was also examined. KKU-KND and KKU-SLE were grown and harvested in 2012. The seeds were soaked in distilled water for 6 hrs to attain a moisture content of 31-32%$_{wb}$ and then germinated by employing two methods, i) in an open plastic box, and ii) in a closed plastic box with a headspace of 3 cm for devoid oxygen (hypoxia method); the germination period varied between 12-48 hrs at 35±2°C in both cases. The germinated samples were then dried at 50°C to a moisture content of 10±2%$_{wb}$. The results showed that non-germinated KKU-KND and KKU-SLE contained 2.68±0.77 and 1.58±0.05 mgGABA/100g$_{db}$, respectively, whereas the samples germinated by the hypoxia method contained significantly higher GABA which increased with germination time ($p \leq 0.05$). The highest GABA contents found in KKU-KND and KKU-SLE were 37.20±3.27 and 54.47±2.08 mg/100g$_{db}$, respectively after 48 hrs of germination under the hypoxia method. In addition, the germinated KKU-KND gave ABTS and DPPH values of 388.32±0.53 and 140.29±0.57 mgTrolox/100g$_{db}$, whereas the germinated KKU-SLE gave ABTS and DPPH values of 183.69±1.75 and 38.43±1.64 mgTrolox/100g$_{db}$, respectively. The pictures of starch granules obtained by means of SEM displayed differences in the shape and size of the non-germinated and germinated granules in both verities. In conclusion, the hypoxia method is able to induce higher GABA in both waxy corns. Therefore, germinated waxy corn can be a rich source of GABA and antioxidant activity.

Keywords: waxy corn, germination, GABA, antioxidant activity, hypoxia

1. Introduction

Maize (*Zea mays L.*), commonly known as corn, is a cereal widely used as a source of nutrients, energy and anti-oxidant (Gaboriau *et al.*, 2005).Waxy corn is widely grown in the Northeast of Thailand. It is consumed as a boiled fresh corn on the cob due to its palatable glutinous texture. White waxy corn seed is generally a traditional cultivar and world-wide known while purple waxy corn seed is an important source of anthocyanins with distinctive features regarding coloring of foods as well as the bio-functional components (Yang and Zhai, 2010). However, the utilizations of both white and purple waxy corns are limited and the waxy corn has a lower price compared with other whole grains such as rice. Therefore, increasing the nutritional value of white and purple waxy corn seeds by enhancing the components such as

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γ-aminobutyric acid (GABA) can be an alternative and value added.

GABA has beneficial effects for human health such as decreasing blood pressure and controlling stress (Kayahara and Tsukahara, 2000; Ito and Ishikawa, 2004). Seed germination begins when the dry seeds come into contact with water under favorable conditions. GABA is produced primarily by the decarboxylation of L-glutamic acid and catalyzed by glutamate decarboxylase (GAD) during seed germination (Mayer *et al.*, 1990). GABA found in germinated grains is (68.4 mg/100g_{db}) higher than in non-germinated grains (23.8 mg/100g_{db}) (Moongngarm and Saetung, 2010). It has been reported that GABA synthesis in plants is promoted by various environmental stresses, e.g. hypoxia, salt stress, water stress, darkness, temperature shock, and acidification (Shelp *et al.*,1999; Oh, 2003; Chung *et al.*, 2009). Hypoxia is a stress condition during seed germination, which limits the availability of oxygen for the seed and results in increasing GABA content in plant tissues rapidly (Roberts *et al.*, 1984; Dewar *et al.*, 1997). The grains germinated with normal oxygen levels yield 10.1±1.36 mgGABA/100 g_{db}, but it goes up to 24.9±4.00 mgGABA/100 g_{db} when oxygen levels are low during germination (Komatsuzaki *et al.*, 2007). GABA is also found in corn seeds, which increases during germination (Khampang *et al.*, 2009). However, there have been few reports on germinated corn and products employing it. This study aims to find the GABA content and antioxidant activity in waxy corn kernels, KKU-KND (purple seed) and KKU-SLE (white seed), during germination. The application of the germination will promote the use of germinated corn seeds as a food supplement.

2. Materials and Methods

2.1 Materials

Two cultivars of waxy corn seeds (*Zea mays L.*) were KKU-SLE (white seeds) and KKU-KND (purple seeds). All were harvested in 2012, and provided by the Plant Breeding Research Center for Sustainable Agriculture (Khon Kaen University, Thailand). The seeds were manually cleaned to remove all foreign materials and broken seeds. 1 kg-samples of waxy corn seeds were vacuum-packed in Nylon/LDPE bags, and stored at 4±2°C, until further experiments (Eneje *et al.*, 2004). The initial moisture content of the corn seeds was determined by drying (AACC, 2000) and was found to be 9-10 $\%_{wb}$.

2.2 Moisture content during soaking

Waxy corn seeds (KKU-SLE and KKU-KND) were soaked in water (a ratio of seed to water of 1: 2) at 35±2°C for 1, 2, 3, 4, 5, 6, 7, 8, 16, and 24 hrs. After each soaking time, the surface water was removed by placing the soaked grains on a tissue paper (Chung *et al.*, 2009). The samples were then weighed and the moisture content was determined by drying the samples in an air convection oven (Memmert, model U30, Schwabach, Germany) at 105±1°C until a constant weight was obtained according to AACC (2000).

2.3 Germination condition

2.3.1 General method

The seeds of two cultivars (100 g) were soaked in 200 ml of distilled water until the moisture content was 30-31$\%_{wb}$. After soaking, the water was drained off the grains, which were washed again and then subjected to germination conditions by placing them on two layers of moist filter paper spread in an uncovered plastic box 12x8x5 cm. The box was put in an incubator at 35±2°C for 12, 24, 36, and 48 hrs. The germinated corn was dried at 50±1°C to approximately 10±2% moisture content and ground by a Perten laboratory mill (model 3100) (Komatsuzaki *et al.*, 2007).

2.3.2 Hypoxia method

This method involves essentially the same steps as above (the general method), except that the plastic box was flushed with nitrogen gas to remove air in the box and then the box was closed immediately with a Nylon/LDPE lid to leave a head space of 3 cm. The head space of the box disconnected from the atmosphere so that the condition employed was lack of oxygen during the germination process. The closed boxes were placed at 35±2°C for 12, 24, 36, and 48 hrs. The germinated seeds from hypoxia conditions were dried at 50±1°C to approximately 10±2% moisture content and ground to be germinated flour (Komatsuzaki *et al.*, 2007).

2.4 Germination percentage

The corn seeds were considered to be germinated when the radicle was 1 mm or longer. The germination percentage was calculated as the number percentage of germinated seeds to total seeds tested (modified from Chung *et al.*, 2009).

2.5 GABA content

The ground corn (2g-samples) was extracted by 10 ml of 70% ethanol. The mixture was shaken for 30 min at room temperature and then centrifuged at 12,000×g at 4°C for 15 min. The supernatant was collected and then 5 ml of 70% ethanol was added to the pellet to repeat the extraction once again. The second supernatant was collected and combined with the first one. (Oh and Choi, 2001; Komatsuzaki *et al.*, 2007; Jannoey *et al.*, 2010). The crude extract containing GABA was adjusted to 20 ml with 70% ethanol and then passed through a 0.45 μm filter and analyzed by HPLC after 9-fluorenylmethyl chloroformate (FMOC) derivatization. 1 ml of corn extract sample was added to 1 ml of FMOC (1,000 ppm in acetonitrile) and adjusted to 5 ml by borax buffer

pH 10. The standard curve was constructed from five standard solutions of GABA and amino acid (5, 10, 25, 50, and 100 ppm of GABA) analyzed by the same procedure (Ratahakrut *et al.*, 2007). The HPLC conditions employed were, *fluorescence* detection at 270 nm (lexcited) and 315 nm (lemission) using a flow rate of 0.5 ml/min; the mobile phases consisted of 0.05% TFA (mobile phase A), acetonitrile (mobile phase B), and methanol (mobile phase C).

2.6 Antioxidant activity

2.6.1 Preparation of sample extracts

The extracts of samples were made by grinding 2 g of the sample in 20 ml of 85% methanol. The mixture was shaken for 4 hrs at room temperature and then centrifuged at 12,000×g at 4°C for 15 min (Oh and Choi, 2001).

2.6.2 DPPH assay

The DPPH free radical-scavenging activity of each sample was determined (Leong and Shui, 2002; Hu *et al.*, 2004). Briefly, a 600 mM solution of DPPH solution was prepared. The initial absorbance of the DPPH in methanol was measured at 517 nm and did not change throughout the period of assay. An aliquot (50 µl) of each sample was added to 3.0 ml of DPPH solution. Discolorations were measured at 517 nm after incubation for 30 min at room temperature in the dark.

2.6.3 TEAC assay (ABTS assay)

The ABTS free radical-scavenging activity of each sample was determined according to the method described by Pavel and Vlastimil (2006). The radical cation ABTS was generated by persulfate oxidation of ABTS. A mixture (2:1, v/v) of ABTS (7.0 mM) and potassium persulfate (4.95 mM) was allowed to stand in the dark at 30±2°C for 24 hrs to form radical cations ABTS. A working solution was diluted with methanol to reach absorbance values 0.70±0.02 at 734 nm. An aliquot 50 µl of each sample was mixed with the working solution (3 ml), and the decrease in absorbance was measured at 734 nm after incubation for 10 min in the dark.

2.7 Scanning electron microscope

The starch granules in non-germinated and germinated waxy corn were determined by transversely cutting dry seeds at the cross section. Germinated corn and the control (non-germinated seed) were mounted on stubs with adhesive tape and sputters coated gold, before observation with a scanning electron microscope(LEO 1450VP, LEO Electron Microscopy Ltd.). One micrograph was taken for each starch sample at 1,000X magnification, by modifying the method of Bhattacharyya *et al.* (2007), and Maisont and Narkrugsa (2010).

2.8 Statistical analysis

All measurements in one duplicate were obtained at three replicates which were used to determine mean values. The experiments were performed in duplicate. Statistical analyses were carried out with a Duncan's multiple test ($p \leq 0.05$) using statistical software SPSS V. 17 (SPSS Institute Inc., Cary, NC).

3. Result and Discussion

3.1 Effect of soaking time on moisture contents

Moisture contents of the soaked corn seeds increased with increasing soaking time. It was found that both white and purple corn seeds had similar water uptake profiles. There is rapid water uptake initially, which is followed by a slower rate in the later stages. Soaking of dried corn seeds in water is necessary for corn germination, because moisture content controls the growth of embryos. The critical moisture content for germination in corn seeds has been reported to be 30-35%$_{wb}$. However, different seeds require a different soaking time (Copeland and McDonald, 1997). Figure 1 presents the moisture content of two types of waxy corn seeds soaked in water for 24 hrs. The soaking time of both corn cultivars induced the critical moisture content required for the germination step. A soaking time of6 hrs was used for the soaking step in the further experiment. Data showed 6 hrs for KKU-SLE (31.26%) and KKU-KND (30.92%). Similar finding was reported for corn (Swam 1 variety) seeds where the moisture content was 32.7% (Sobukola *et al.*, 2013).

3.2 Effect of germination conditions on root length and GABA content

The number of germinated seeds and length of root were highly dependent on the germination conditions. Germination begins with imbibition, the uptake of water (Hopkins, 1999). After the seed absorbs about 30% of its body weight in water through imbibition, metabolism increases

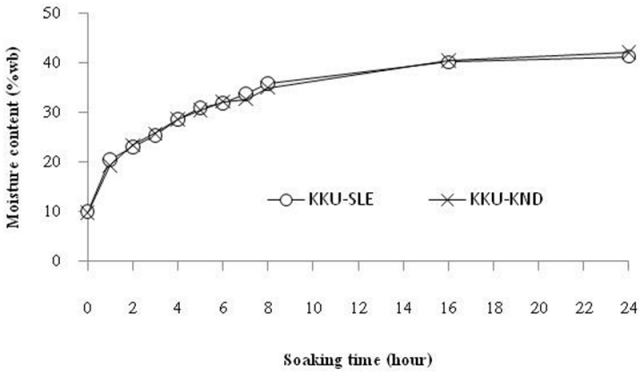

Figure 1. Moisture contents of KKU-SLE and KKU-KND waxy corn seeds soaked in water for 24 hrs.

and the visible stages of germination begin (Hopkins, 1999). Figure 2 shows the percentage of various root length found in germinated KKU-SLE at different germination time; Figure 2a shows the general method and Figure 2b the hypoxia method. 7% germinated seeds were found at 12 hrs germination time in the general method. The root length >10 mm of germinated seeds was observed at 24 hrs and increased with germination time. This is because important external factors which include temperature, water, and oxygen are favorable for KKU-SLE corn seed growth. For the hypoxia method, 1% germinated seeds was observed at 12 hrs germination time. It was remarked that there were only germinated seeds which obtained the embryonic root length of ~1-10 mm during the germination period. Corn seeds germinated by the hypoxia method grew slowly due to the oxygen limit (Copeland and McDonald, 2001). Normally, the embryonic root is revealed at 12 to 24 hrs germination time under the atmosphere. The embryonic root of germinated KKU-SLE can break the surface to reach sunlight and lengthen at 12 hrs germination time. Conversely, for the seeds of the KKU-KND variety which germinated after 12 hrs in the hypoxia condition embryonic root length >10 mm were not found during the germination period (Figure 3b). After 12 hrs germination time, the KKU-KND seed's metabolic rate was much faster than that of KKU-SLE. It was explained by the percentage of germination (Figure 2a and 3a). Some seeds will not germinate until they have undergone a process of after-ripening, defined as metabolic changes that must take place in a seed in order for it to overcome dormancy (Hopkins, 1999). Based on the results of this study it can be noted that the general method of both KKU-SLE and KKU-KND varieties seems to have no effect on the time it takes for corn seeds to germinate ($p>0.05$). However, it seems to have an effect on the percentage of corn seeds that complete germination. Taslak *et al.* (2007) stated that the corn types showed significant differences in terms of germination speed and germination rate. Carpici *et al.* (2009) also indicated that corn types had different responses to environmental factors in germination conditions.

Table 1 shows GABA contents of corn seeds using different germination methods for 48 hrs. It was found that the general method gave lower GABA than the hypoxia method ($p\leq0.05$).The highest GABA contents found in KKU-KND and KKU-SLE were 37.21±3.27 and 54.47±2.08 mg/100g_{db}, respectively, at 48 hrs germination time under the hypoxia method. Changes in oxygen, light, temperature, moisture content, and/or humidity each may play a role in triggering GABA synthesis. It was revealed that both KKU-SLE and KKU-KND varieties had metabolic activity which increased dramatically the GABA content at 24 hrs germination time of the general condition. When protein synthesis begins and endosperm is metabolized, nutrients from the endosperm (sometimes located in the cotyledons) are mobilized and transported to areas of growth. During initial germination stage and proper oxygen, L-glutamic acid was synthesized to GABA which subsequently resulted in an increase

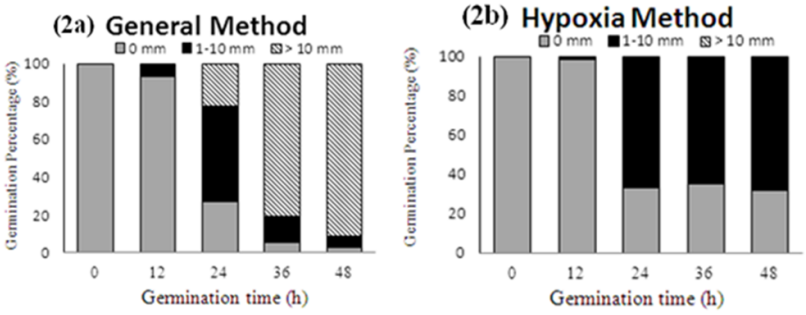

Figure 2. Effect of germination conditions (2a) general method and (2b) hypoxia method on germination percentage of root length in KKU-SLE.

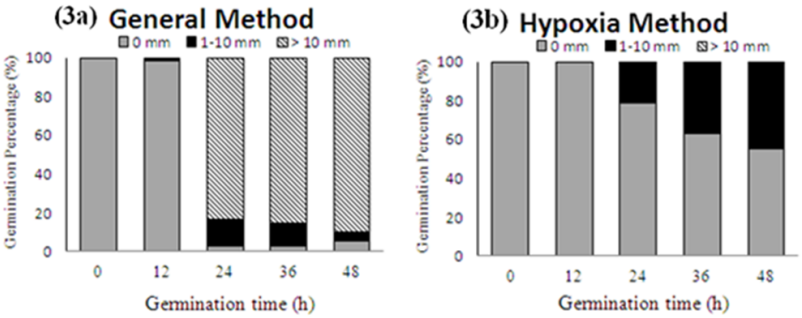

Figure 3. Effect of germination conditions (3a) general method and (3b) method on germination percentage of length root in KKU-KND.

Table 1. Effects of germination conditions (time, method) on GABA contents in KKU-SLE and KKU-KND.

Germination conditions		GABA content(mg/100g db)	
Method	Time (hour)	KKU-SLE	KKU-KND
Control	0	1.58 ± 0.05^{a}	2.68 ± 0.77^{a}
General	12	9.91 ± 1.90^{b}	10.20 ± 2.99^{bc}
	24	10.45 ± 3.02^{b}	10.20 ± 2.36^{bc}
	36	7.72 ± 3.18^{ab}	7.73 ± 0.43^{ab}
	48	5.94 ± 0.02^{ab}	7.78 ± 1.36^{ab}
Hypoxia	12	17.31 ± 3.73^{c}	17.47 ± 4.13^{d}
	24	22.14 ± 5.36^{c}	17.27 ± 6.55^{cd}
	36	42.52 ± 0.46^{d}	32.42 ± 2.04^{e}
	48	54.47 ± 2.08^{e}	37.21 ± 3.27^{e}

Different letters (a-e) in the same column are significantly different ($p \leq 0.05$)

in GABA (Shelp *et al.*, 1999).In addition, low L-glutamic acid content or L-glutamic acid is limited and GABA is also conveyed into the Kreb's cycle that may affect germination rate or germination success (Bown and Shelp, 1997). After 24 hrs germination time of the general condition, the germinated corn seeds showed further embryonic root elongation so that the resulting seed growth decreased the GABA content. However, one variety, KKU-KND, still does. It is related to the number of the embryonic root length >10 mm.

The synthesis of GABA through glutamate decarboxylase is rapidly stimulated by a variety of stress conditions including hypoxia. The advantage of this process would be the concomitant H^{+}consumption (Crawford *et al.*, 1994; Carroll *et al.*, 1994). It enables to yield the greatest percentage of GABA in both corn seeds. The GABA increased; however, the hypoxia method took longer time to complete seed germination than the general method. It was because the metabolism of cells was slower due to lower oxygen levels in the germination period (Copeland and McDonald, 2001). Many researchers have reported that the hypoxia condition induces cell stress that can sharply increase GABA content in germinated seeds (Roberts *et al.*, 1984; Dewar *et al.*, 1997; Komatsuzak *et al.*, 2007; Chung *et al.*, 2009). Earlier studies found that brown rice germinated under normal oxygen levels yielded 10.1±1.36 mg GABA/$100g_{db}$, but this figure went up to 24.9±4.00 mg GABA/$100g_{db}$ when oxygen levels were lower during the germination period (Komatsuzaki *et al.*, 2007). Chung *et al.* (2009) also reported that the application of anaerobic storage for 12 hrs after germination increased the GABA content in waxy barley grains compared to grains germinated at normal oxygen levels (from 3.7 mg/$100g_{db}$ to 14.3 mg/$100g_{db}$).

3.3 Antioxidant activity

All types of germinated corn have ABTS and DPPH values lower than those of non-germinated seeds (Figure 4 and 5). The germinated KKU-KND gave ABTS and DPPH values of 388.32±0.53 and 140.29±0.57 mgTrolox/$100g_{db}$, respectively, whereas the germinated KKU-SLE gave ABTS and DPPH values of 183.69±1.75 and 38.43±1.64 mgTrolox/$100g_{db}$, respectively. KKU-KND is a purple seed and, as expected, has an antioxidant activity higher than KKU-SLE

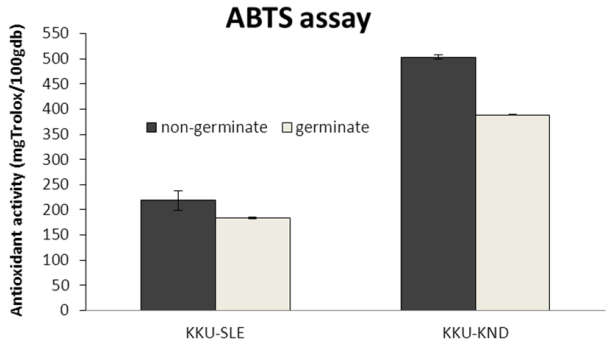

Figure 4. Antioxidant activity (ABTS assay) in non-germinate and germinated corn seed.

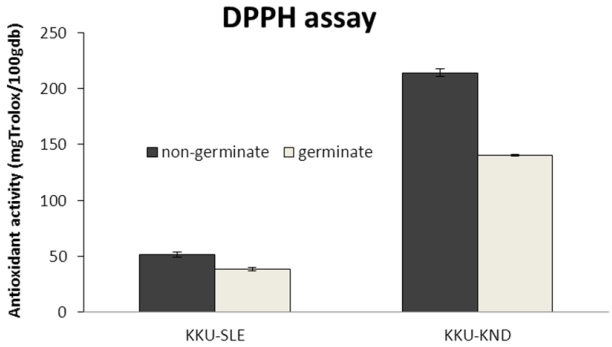

Figure 5. Antioxidant activity (DPPH assay) in non-germinate and germinated corn seed.

(white seed). The results indicated that both soaking step and corn variety affected the reduction in antioxidant activity of germinated seeds, especially KKU-KND ($p \leq 0.05$), which contained high anthocyanins which were water soluble and heat sensitive compounds. For KKU-SLE (white seed), antioxidant activities of germinated and non-germinated seeds were not significant ($p > 0.05$). Corn seed contains ferulic acid in its structure. Ferulic acid is a phenolic acid antioxidant and it is also insoluble in water (Patel and Naik, 2004). Earlier studies have reported that germination effects on reduction of antioxidant activity in the germinated seeds (Oboh, 2006; Lopez *et al.*, 2006). The impact of germination on phenolic content and antioxidant activity in 13 edible seed species was analyzed (Cevallos and Cisneros, 2010). Our study also showed that both germinated corn varieties are an excellent source of dietary phenolic antioxidants, particularly KKU-KND.

3.4 SEM results

The pictures of starch granules obtained by means of SEM displayed differences between the shape and size of the non-germinated and germinated granules (Figure 6). SEM reveals the morphology of starch granules of germinated and non-germinated corn. The germinated corn has fewer radicles which causes the starch granules to be more separated after germination, and facilitates enzyme degradation (Mayer *et al.*, 1990). Corn seeds, as well as other cereal seeds, produce the enzyme α-amylase during germination. The substrate for α-amylase is stored starch and the end product is free sugar, which is needed for the growth of emerging embryo. Waxy corn has spherical or spherical polygonal shape with diameter in the range 2-30 microns (Beynum and Roels, 1985). Figure 6 shows that the shape of starch granules in all non-germinated corn (KKU-SLE and KKU-KND) was spherical and the granule surface was also smooth. Moreover, non-germinated corn, the starch granules were close together and the structure appeared to resemble fiber cladding (Figure 6a and c). However, after germination (Figure 6b and d) the shape of starch granules was more spherical and the surface was not smooth but it contained pinholes. This may be due to enzyme degradation, especially the action of amylase on the starch granules (Mayer *et al.*, 1990). During seed germination, the embryo will use its stored food to grow into a mature plant. In most seeds, the food is stored as starch. Although starch is a storage form of glucose, it cannot pass through living membranes due to the large molecular structure, so before it can be used for energy it needs to be broken down to its constituent sugars (Bewley and Black, 1994). The catabolism of starch was done by alpha-amylase, an enzyme released by the embryo in the seed germination. Amylase and invertases are the important hydrolytic enzymes which are found in plants (Rahman *et al.*, 2007).

4. Conclusion

The results indicated that the hypoxia germination process causes various changes in the antioxidant activity and modifies GABA content. The soaking time which gave the critical moisture content (31-32%) for waxy corn germination was the same for KKU-SLE and KKU-KND, i.e. 6 hrs. The germination conditions (time, method) influenced the GABA contents. The limited availability of oxygen in the hypoxia method tended to beneficially increase the GABA content in waxy corn seeds. However, the optimal germination time and the final GABA content depended on the corn

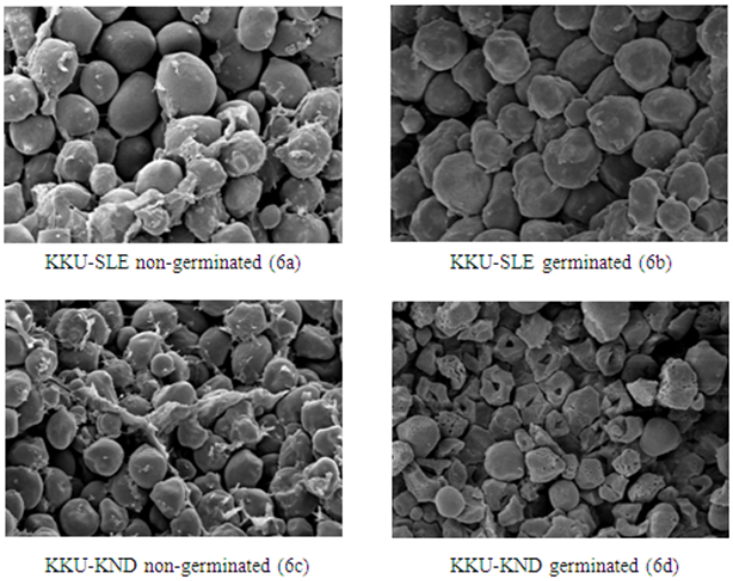

Figure 6. Scanning electron micrographs of starch granules; 6a) KKU-SLE non-germinated 6b) KKU-SLE germinated 6c) KKU-KND non-germinated and 6d) KKU-KND germinated.

cultivar. Both KKU-SLE and KKU-KND varieties of Thai waxy corn seeds can potentially gain GABA and remain the bioactive compounds as well as became value added products. Germinated KKU-SLE and KKU-KND seeds may be used as a source of natural antioxidants in functional foods.

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