International Food Research Journal 19(4): 1641-1648 (2012)
Journal homepage: *http://www.ifrj.upm.edu.my*

Effects of germination conditions on 5'-phosphodiesterase activity of selected seeds

Pui, L.P., Abdulkarim, S.M. and *Ghazali, H.M.
Department of Food Science, Faculty of Food Science and Technology, Universiti Putra Malaysia, 43400 Serdang, Selangor, Malaysia

Article history

Received: 19 September 2011
Received in revised form: 5 April 2012
Accepted:5 April 2012

Keywords

Germinated seeds
germination
5'-phosphodiesterase
phosphomonesterase

Abstract

5'-Phosphodiesterase (5'-PDE) is an enzyme that hydrolyses RNA to form 5'-inosine monophosphate (5'-IMP) and 5'-guanosine monophosphate (5'-GMP), which function as flavour enhancers. Selection of the best producer of 5'-PDE was made by determining the activity of the enzyme in six seeds that have been germinated, namely mung bean (*Vigna radiate*), soybean (*Glycine max*), adzuki/red bean (*Vigna angularis* L.), chick pea (*Cicer arietinum*), black eye pea (*Vigna unguiculata*) and petai (*Parkia speciosa*). Seeds that were not germinated acted as the control. In order to ensure there is no contamination from potential 5'-PDE-producing microorganisms during germination, microbial growth was reduced by using different surface sterilizing treatments where the seeds were soaked in 100 mL solution containing different concentrations of sodium hypochlorite (with or without 0.05% sodium azide) for 5 minutes before rinsing it five times with sterilized distilled water (total 500 mL). The seeds were observed every day for 3 days and the best surface sterilizing treatment was selected based on absence of mold growth and the effects on hypocotyl length. Sodium hypochlorite at 0.3% (v/v) concentration was able to inhibit mold growth in adzuki bean, soybean and chickpea. On the other hand, only 0.1% (v/v) sodium hypochlorite was needed to inhibit mold growth in black eye pea and petai, while mung bean required 0.05% (v/v) sodium hypochlorite to inhibit mold growth. Under these conditions, the growth of hypocotyl (hypocotyls length) was only slightly affected compared to the control. 5'-PDE was extracted from seeds that have been germinated for 24 hours and their control (ungerminated seeds) by homogenization in a blender with 400 mL of 50 mM acetate buffer, pH 4.5. After that, the homogenates were stirred for 30 min and the centrifuged at 9000 rpm for 15 min at 10°C. 5'-PDE activity was determined using thymidine 5'-monophosphate p-nitrophenyl ester as substrate at pH 7.0 and 55°C. The formation of nucleotide monophosphates, the products of reaction, was determined at 405 nm. As a strong presence of phosphomonoesterase (PME) will reduce the yield of nucleotide monophosphates as the enzyme hydrolyzes these products into nucleosides and orthophosphate, PME activity was also determined using p-nitrophenyl phosphate as the substrate at 60°C and pH 5.0. Thus, the seed with the highest 5'-PDE activity and a low PME activity can be selected. Germinated adzuki bean was found to have the highest 5'-PDE activity (0.59 µmol p-nitrophenol/min/mg protein) among the germinated seeds. A time-course study indicated that the level of 5'-PDE in adzuki bean increased with time of germination until 15 hours (0.69 µmol p-nitrophenol/min/mg protein), after which the acitivity decreased until it reached the basal level (0.44 µmol p-nitrophenol/min/mg protein) at 72 hours. On the other hand, PME in the bean was the highest at 9 h germination (0.98 µmol p-nitrophenol/min/mg protein). In general, controls have very low basal level of 5'-PDE activity (0.18- 0.42 µmol p-nitrophenol/min/mg protein).

Introduction

Germination is a process where the hypocotyl shows growth in length. During this process, metabolic changes occur where stored nutrient reserves in the cotyledons are breakdown and translocated to the growing shoots and roots for utilization. Some germinated seeds or sprouts can be used for food and are popular in dishes. In Malaysia, sprouts from mung bean and soybean are commonly used in cooking. Yet, these sprouts or germinated seeds can be a potential source for useful enzyme meant for industrial purposes. One of these enzymes is 5'-phosphodiesterase (EC 3.1.4.1), an enzyme which produces flavour nucleotides (Dhule *et al*., 2006).

Previous studies on germination of seeds involved the usage of glass wool (Dev, 1985), vermiculite (Nicannuzia dos Prazeres *et al*., 2004) and filter

*Corresponding author.
Email: *hasanah@upm.edu.my*

paper (Vange *et al*., 2004). Besides these, cheese cloth (Kannan *et al*., 2008), cotton cloth (Khalil and Mansour, 1994) and also moist cloth (Khandelwal *et al*., 2010) have also been used in germination studies of different seeds. In addition, the germination involving a seed germinator (Lopez-Amoros *et al*., 2006) and sprout chamber (Khattack *et al*., 2007) as the sprouting bed in the germination process has been reported. Contamination is common during seed germination as common sprouting conditions (2–7 days of sprouting, temperatures of 20–40°C and optimum water activity) create a favourable environment for bacterial growth, while the moist condition is favourable for mold growth. To minimize contamination, extra care is usually given during germination and the water used for germination is pretreated with chemicals such as different percentage of sodium hypochlorite (Fernandes-Orazco *et al*., 2008; Sfaxi-bousbih *et al*., 2010) and ethanol (Khalil and Mansour, 1994).

5'-Phosphodiesterase (5'-PDE) has been reported to be found in different parts of plants such as the leave tissue from oat (Urvardy *et al*., 1970), soybean (Salvucci *et al*., 1995) and sugar beet (Lerch and wolf, 1972), the fruit body of *Flammulina velutipes* (Kurosawa *et al*., 1984), the root from carrot (Harvey *et al*., 1970) and seeds from yellow lupin (Jakubowski et al., 1983). More recent studies have focused on germinated barley seeds (Beluhan *et al*., 2003; Deoda and Singhal, 2003; Dhule *et al*., 2006) and malt roots (Zou *et al*., 2008).

To date, there are only a few reports on 5'-PDE from germinated seeds. It has been reported that the level of the enzyme increased markedly with increasing germination time of barley seeds and the activity of 5'-PDE was maximum after 5 days (Lee and Pyler, 1985; Deoda and Singhal, 2003). The activity in the whole seed extract was higher than that in the rootlets (Lee and Pyler, 1985). 5'-PDE has also been investigated in germinated mung bean (Khutle *et al*., 2011) and germinated yellow soybean and black soybean (Utami *et al*., 2011), however, the substrate used was bis-p-nitrophenyl phosphate which is less specific compared to thymidine 5'-monophosphate nitrophenyl ester.

Different extraction methods have been reported by researchers on 5'-PDE. For instance, barley seeds were soaked for 3-4 h and germinated for 5 days before they were ground at 12-20°C (Deoda and Singhal, 2003) in citrate buffer pH 4.6. On the other hand, Dhule *et al*. (2006) reported on the soaking of barley seeds for 4-5 hours, followed by germination for 4-5 days, and extraction by homogenizing the seeds in buffer. Another extraction method was homogenizing avena leaves with quartz sand and acetate buffer pH 5.5, in a pre-chilled mortar (Udvardy *et al*., 1970).

Different enzyme assays have also been used in the detection and quantification of 5'-PDE activity. Benaiges *et al*. (1989) used 5-monophosphate p-nitrophenyl ester as the substrate to produce the yellow colored p-nitrophenol which was then measured spectrophotometrically at 405 nm to determine the enzyme activity. On the other hand, Bowles *et al*. (1991), Khutle *et al*. (2011) and Utami *et al*. (2011), measured the activity of the enzyme using bis-p-nitrophenyl phosphate as the substrate and determining the amount of p-nitrophenol produced in the reaction at 420 nm. Other methods are the detection of mononucleotides or RNA formed during the hydrolysis of 5'-PDE (Deikus *et al*., 2008; Yang *et al*., 2009).

Phosphomonoesterase (EC 3.1.3.1/ EC 3.1.3.2) or commonly known as alkaline or acid phosphatase, has been reported as a major contaminant enzyme which is produced simultaneously with 5'-PDE (Benaiges *et al*., 1991; Dhule *et al*., 2006). Phosphomonoesterase (PME) hydrolyzes nucleotides which have flavor enhancing properties, into nucleosides and orthophosphatase, thus reducing the overall yiled of nucleotide monophosphates. Although purification work has been attempted in separating PME form 5'-PDE, no successful attempt has been reported (Dhule *et al*., 2006). PME from soybean and corn have been studied and it was found that as the germination time increases, the level of PME also increases (Nicanuzia dos Prazeres *et al*., 2004; Senna *et al*., 2006).

This study was conducted to determine the best source of 5'-PDE among six different seeds and to determine the germination time which will produce the highest level of 5'-PDE. The activity of PME was also determined in order to ascertain which germinating seed has a low level of PME in comparison with 5'-PDE. Different germination conditions, typically surface sterilizing treatments, were also examined in order to select an appropriate sterilizing treatment which will inhibit mold infection yet does not inhibit the growth of the seeds' hypocotyl. It was necessary to ensure no mold growth takes place during the germination process so that there is no contamination of enzyme that could come from microorganisms that grow during germination. Hence, once it can be proven that growth did not take place for at least three days, the study on effect of germination time on 5'-PDE production can be done.

Materials and Methods

Raw materials and chemicals

Mung bean (*Vigna radiate*), soybean (*Glycine max*), adzuki bean (*Vigna angularis* L.), chick pea (*Cicer arietinum*), fresh 'petai' (*Parkia speciosa*) pods and black eye pea (*Vigna unguiculata*) were purchased from a local market in Sri Serdang, Selangor. The seeds were kept at room temperature during the course of the study. However, petai were kept in a refrigerator at 4°C for less than 24 hours before use. Thymidine 5'-monophosphate p-nitrophenyl ester (nitrophenyl–pT) was purchased from Sigma-Aldrich Chemie GmbH, Steinheim, Germany. All other chemical reagents were of general or analytical grade.

Surface sterilization of seeds

Several procedures were used to sterilize the seeds before germination. In one experiment, the seeds (20 g) were first soaked for 5 mins in 100 mL of different concentrations [0, 0.05, 0.1, 0.2, and 0.3% (v/v)] of sodium hypochlorite solution. In this experiment and all other experiments, 'petai' seeds were removed from their pods prior to soaking. After that, the seeds were washed 5 times with a total of 500 mL of sterilized distilled water before placing 25 seeds in separate 250 mL beakers which had been pre-autoclave and containing cotton wool which had been soaked with 15 mL of distilled water, and covered with aluminium foil. The seeds were then kept under dark condition for 3 days at room temperature (28 ± 2°C). There were 4 replicates for each treatment, which consisted of 25 seeds per treatment. The activity of 5'-phosphodiesterase (method described below) was determined at different germination time.

Another experiment was carried to examine the effect of sodium azide on seed sterilization and germination. Seeds (20 g, about 25 seeds) were first soaked in 100 mL of 0.01% (w/v) sodium azide solution for 5 mins. After that, the solution was discarded and the seeds were soaked in different concentrations of sodium hypochlorite solutions [0, 0.05, 0.1, 0.2 and 0.3% (v/v)] for 5 mins, following which the seeds were washed 5 times, each with 15 mL of sterilised distilled water, before placing into separate sterilized beakers containing cotton wool soaked with distilled water. It was then kept under dark condition for 3 days at room temperature (28 ± 2°C). Four replicates were prepared for this experiment. The activity of 5'-PDE was determined at different germination time.

To test the effect of prolonged exposure of sodium azide on the seeds, sodium azide was incorporated in the cotton wool during germination. The seeds were first soaked in 100 mL of 0.05% (v/v) sodium hypochoride solution for 5 mins and then washed 5 times with sterilized distilled water as described previously. The cotton wool in the beaker contained 15 mL of 0.05% (w/v) sodium azide. It was then placed into a dark place for germination to occur. As a control, 0.05% (w/v) sodium azide was used in the sterilizing treatment solution and seeds were soaked for 5 mins and then washed with 5 times distilled water before germination. All tests on sterilization and germination were done by measuring the hypocotyl length of seeds using a vernier calipher and also to observe the presence of mold on the seeds after 3 days of germination.

Extraction of 5'-phosphodiesterase from germinated seeds

Seeds (100 g) that have germinated were homogenized with 400 mL 50 mM acetate buffer, pH 4.5, using a home blender. A further 200 mL of 50 mM acetate buffer, pH 4.5, was added to the homogenate and the resulting slurry was then stirred for 30 minutes at 4°C, before filtration through a layer of muslin cloth. The filtrate/extract was then centrifuged at 9000 rpm, for 15 min at 10°C (Sartorius Model 3-18 k centrifuge, Sartorius AG, Weender Land Strasse, Gottingen, German). The supernatant (enzyme extract) was used to determine 5'-PDE and PME activities. The activity of the latter enzyme was determined also as it is usually present in seeds, and it is important to select the seed with the highest 5'-PDE activity and a low PME activity. The enzymes were also extracted from ungerminated seeds according to the method described above, and served as the control.

5'-Phosphodiesterase assay

The activity of 5'-PDE was determined according to the method described by Harvey et al. (1970) with some modification. The reaction mixture comprised 0.1 mL of enzyme extract, 0.2 mL of 200 mM phosphate buffer, pH 7.0, and 0.2 mL 1 mg/mL nitrophenyl-pT (substrate). The reaction mixture was incubated at 55°C in a waterbath for 10 min during which time the enzyme converted the substrate into p-nitrophenol and 5'-thymidine monophosphate. The reaction was stopped by adding 0.5 mL 0.1 N NaOH solution. The mixture was then centrifuged at 9000 rpm and 10°C using a Sartorius Model 3-18 k refrigerated benchtop centrifuge (Sartorius AG, Weender Land Strasse, Gottingen, Germany). The yellow color which was produced due to p-nitrophenolate ion was measured spectrophotometrically at 405 nm.

One unit (U) of enzyme was defined as the amount of enzyme required to form 1 µmol of p-nitrophenol per mL enzyme extract in 1 min, at 55°C and pH 7.0. Specific acitivity was expressed as Unit activity per mg protein. The control reaction was prepared by using an aliquot of respective enzyme extract which has been heated in a boiling waterbath for 5 mins.

Phosphomonoesterase activity

The activity of PME was determined according to the method described by Dhule *et al*. (2006) with some modification. The composition of reaction mixture was 0.1 mL of enzyme extract, 0.8 mL of 50 mM acetate buffer, pH 5.0, and 0.1 mL of 10 mM p-nitrophenyl phosphate (substrate). The reaction mixture was incubated at 60°C in a waterbath for 10 mins during which time the enzyme converted the substrate into p-nitrophenol. The reaction was stopped by adding 0.3 mL of 5% (w/v) sodium carbonate solution and then topped up with 1.7 mL of distilled water. The mixture was then vortexed to achieve a homogenous mixture. The yellow color which was produced due to p-nitrophenolate ion was measured spectrophotometrically at 405 nm. One unit (U) of enzyme was defined as the amount of enzyme required to form 1 µmol of p-nitrophenol in 1 min, at 60°C and pH 5.0. Specific acitivity was expressed as Unit activity per mg protein. The control reaction consisted of enzyme extract which had been heated in a boiling waterbath for 5 minutes, buffer and substrate as described above.

Protein measurement

The protein contents of the enzyme extracts were measured using the modified Lowry *et al*. (1951) method (Eggstein and Kreutz, 1955) with bovine serum albumin (BSA) as the standard. The concentration of protein was calculated using a reference constructed with BSA at concentrations ranging from 0-0.6 mg/mL

Effect of germination time on 5'-phosphodiesterase and phosphomonoesterase activity

As adzuki bean showed the highest level of 5'-PDE and lowest level of PME, it was used for further studies. To determine the effect of different germination time on enzyme activity, 5'-PDE activity and PME activity were measured at 0, 9, 15, 24, 48 and 72 hours of germination using the best germination conditions determined earlier. Extraction of both enzymes was conducted as described above.

Statistical analysis

The mean ± S.D. data for groups of all experiments were analyzed by ANOVA using MINITAB 13.1.1 Software. Values of $p < 0.05$ were considered significant.

Results and Discussion

Preparation of seeds for germination: effect of surface sterilisation

It is important to ensure that seeds germinate successfully without microbial contamination, since, 5'-PDE has a higher activity in germinated seeds, and also there is no contribution in 5'-PDE activity from the microorganisms. From Table 1, it can be observed that the effects of different chemicals used in the surface sterilization treatments on the length of hypocotyl at Day 3 of germination varied. Third day sprouts of mung bean and soybean had hypocotyls lengths of about 48-42 mm and 39-32 mm, respectively. On the other hand, adzuki bean, black eye pea, chick pea and petai sprouts which are not produced commercially also germinated, with the longest length of hypocotyls of 20, 46, 35 and 46 mm, respectively.

In general, the surface sterilizing treatments were found to decrease the length of hypocotyls. The application of sodium azide (0.01% and 0.05%) under any condition severely affected germination as the length of hypocotyls decreased by about 5-10 mm which is between 10-25% of overall length of a sprout that did not undergo any treatment. This may be caused by the nature of sodium azide itself, as it is toxic and when the compound penetrates into the seeds, it affects seed hypocotyl growth (Kleinhofs *et al*., 1978).

The respiratory toxic nature of sodium azide has led to its use as a fungicide and also herbicide in agriculture (Kleinhofs *et al*., 1978). Pearson *et al*. (1975) reported that although sodium azide can be used to induce germination, it can also cause a delay in mitosis in barley to produce shoot. The addition of sodium azide in the germinating solution (Table 1) portrays the strong inhibitory effect where there was a 69-98.5% decrease in hypocotyl length compared to the control. Mold growth at Day 3 was observed for different seeds that were surface sterilized and the results are shown in Table 2 where different surface sterilizing treatment had different effects on mold growth for different seeds. It can be seen that different seed requires different concentration of either sodium hypochlorite or sodium azide or their combinations to inhibit the growth of mold.

The study shows that the addition of sodium azide did not assist in the process of sterilizing the seeds as mold contamination still occurred with its addition.

Table 1. Effect of different surface sterilization treatments on hypocotyl length of different seeds germinated for three days

Sterilization solutions	Mean length of hypocotyl (mm) of different seeds					
	Mung bean	Adzuki bean	Soybean	Black eye pea	Chick pea	Petai
No treatment (Control)	48.0 ± 0.6^{f}	20.9 ± 1.0^{d}	38.6 ± 1.5^{d}	41.5 ± 14.7^{bc}	33.4 ± 1.2^{e}	46.2 ± 1.0^{g}
0.05% (v/v) SH	44.6 ± 1.1^{de}	18.1 ± 1.6^{cd}	33.3 ± 3.1^{c}	44.2 ± 1.7^{c}	24.3 ± 2.0^{c}	33.6 ± 1.4^{c}
0.10% (v/v) SH	42.8 ± 1.0^{d}	17.6 ± 2.4^{c}	33.2 ± 2.0^{c}	43.3 ± 2.2^{bc}	22.4 ± 2.0^{b}	31.5 ± 0.9^{c}
0.20% (v/v) SH	43.0 ± 1.4^{d}	16.4 ± 1.2^{c}	32.0 ± 2.7^{c}	41.6 ± 0.8^{bc}	21.4 ± 1.1^{b}	28.8 ± 0.5^{b}
0.30% (v/v)SH	42.4 ± 1.9^{d}	17.2 ± 1.2^{cd}	32.2 ± 2.1^{c}	40.9 ± 1.7^{bc}	21.3 ± 1.1^{b}	28.7 ± 0.7^{b}
0.01% (w/v) SA	37.0 ± 0.7^{bc}	19.1 ± 0.6^{d}	28.0 ± 1.1^{c}	37.8 ± 1.3^{b}	35.0 ± 0.4^{f}	38.0 ± 0.8^{d}
0.01% (w/v) SA + 0.05% (v/v) SH	35.9 ± 0.4^{b}	17.7 ± 0.7^{cd}	26.1 ± 0.3^{bc}	36.6 ± 0.5^{b}	33.6 ± 0.8^{ef}	35.9 ± 0.8^{d}
0.01% (w/v) SA + 0.10% (v/v) SH	35.2 ± 0.3^{b}	17.1 ± 0.9^{cd}	24.3 ± 1.3^{bc}	35.5 ± 0.5^{b}	32.7 ± 0.6^{ef}	34.9 ± 1.1^{cd}
0.01% (w/v) SA + 0.20% (v/v) SH	33.9 ± 0.5^{b}	16.8 ± 0.8^{c}	23.1 ± 1.7^{b}	35.3 ± 0.4^{b}	32.1 ± 0.6^{e}	33.4 ± 1.1^{c}
0.01% (w/v) SA + 0.30% (v/v) SH	33.4 ± 1.1^{b}	16.0 ± 1.4^{c}	21.9 ± 2.6^{b}	34.2 ± 0.9^{b}	31.8 ± 0.9^{e}	31.1 ± 0.8^{b}
0.5% (v/v) SH + 0.05% (w/v) SA	38.4 ± 0.9^{b}	11.7 ± 0.5^{b}	28.4 ± 0.6^{c}	36.8 ± 1.4^{b}	36.8 ± 1.4^{g}	33.3 ± 3.6^{c}
0.5% (v/v) SH + 0.05% (w/v) SA in germinating solution	13.1 ± 0.9^{a}	0.3 ± 0.6^{a}	12.2 ± 0.6^{a}	10.5 ± 1.0^{a}	5.9 ± 0.4^{a}	9.7 ± 0.5^{a}

Note: SH and SA indicate sodium hypochlorite and sodium azide, respectively
Standard deviation from 4 replicates were done, where different alphabet indicates significant differences (P< 0.05)

Table 2. Effect of different surface sterilization treatments on mold growth for different seeds during germination for three days

Treatment solutions	Mold growth of different seeds					
	Mung bean	Adzuki bean	Soybean	Black eye pea	Chick pea	Petai
No treatment (Control)	Yes	Yes	Yes	Yes	Yes	Yes
0.05% (v/v) SH	No	Yes	Yes	Yes	Yes	Yes
0.10% (v/v) SH	No	Yes	Yes	No	Yes	No
0.20% (v/v) SH	No	Yes	Yes	No	Yes	No
0.30% (v/v)SH	No	No	No	No	No	No
0.01% (w/v) SA	Yes	Yes	Yes	Yes	Yes	Yes
0.01% (w/v) SA + 0.05% (v/v) SH	Yes	No	Yes	Yes	No	Yes
0.01% (w/v) SA + 0.10% (v/v) SH	No	No	Yes	Yes	No	No
0.01% (w/v) SA + 0.20% (v/v) SH	No	No	Yes	Yes	Yes	No
0.01% (w/v) SA + 0.30% (v/v) SH	No	No	No	Yes	Yes	No
0.5% (v/v) SH + 0.05% (w/v) SA	Yes	No	Yes	Yes	No	Yes
0.5% (v/v) SH + 0.05% (w/v) SA in germinating solution	Yes	No	Yes	Yes	No	Yes

Note: SH and SA indicate sodium hypochlorite and sodium azide, respectively

In fact, there was even a decrease of sterilization effect compared to the absence of sodium azide in treatments containing the same amount of sodium hypochlorite. Therefore, the addition of sodium azide in the germinating solution did not inhibit mold growth, as mold growth could be observed both in germinating solution and those with sodium azide in the washing solution. There are different types of mold that are present on the surfaces of seeds such as *Aspergillus* and *Trichorderma* that are found in maize where even after surface sterilization, mold growth was still observed (Maity *et al*., 2008). This indicates that surface sterilization alone may not be enough to ensure the seeds are free from mold due to the fact that mold also can be found beneath the seed coat as well (Maity *et al*., 2008).

With sodium hypochloride, it can be concluded that as the level increased, the length of hypocotyls of the seeds being examined decreased. This chemical is commonly used in surface sterilization methods, however, the concentration to be applied and the time of exposure differ. In this study, the range of sodium hypochlorite concentrations used was from 0 (control) to 0.3% (v/v), which is generally lower compared to those used by other researchers. For the germination of soybean, seeds were soaked in 2% (v/v) sodium hypochlorite solution for 15 minutes and rinsed with sterile water 5 times (Nicanuzia dos Prazeres *et al*., 2004). Lopez-Amoros *et al*. (2006) soaked beans, lentils and peas in 0.7% (v/v) sodium hypochlorite solution for 30 minutes before undergoing the germination proces. In other researches, lower concentrations of sodium hypochlorite were used. For example, maize grains were soaked in 0.5% (v/v) sodium hypochlorite for 30 second before rinsing four times with water (Martha *et al*., 1987). In the work of Fernandez-Orazco *et al*. (2008), 0.07% (v/v) sodium hypochlorite were used for surface sterilizing treatment of mung bean and soybean.

Thus, the choice of best surface sterilizing treatment not only requires considerations of hypocotyl length which denotes the growth of germinated seeds, but also the absence of mold growth. As sodium azide is not helpful in both prevention of mold and germination (hypocotyls growth), the surface sterilizing with sodium azide was discontinued. Since treatments involving different concentrations of sodium hypochlorite have minimal effects on germination, it is important to consider the minimum level of sodium hypochlorite used for effective mold prevention. Thus, it appears that the most appropriate approach is the treatment of seeds with up to 0.3% (v/v) sodium hypochlorite. Thus, the surface sterilizing treatment with sodium hypochlorite is 0.05% (v/v) for mung bean, 0.1% (v/v) sodium hypochlorite for petai and black eye pea and 0.3% (v/v) for the rest of the seeds. It can also be observed that as the percentage of sodium hypochlorite increase, the length of hypcotyl decreased (Table 1).

5'-Phosphodiesterase and phosphomonoesterase activities in germinated seeds

Figure 1 shows the level of 5'-PDE and PME from ungerminated seeds (Control) and seeds that have been germinated for 24 hours. It can be seen that generally germinated seeds have higher enzyme activity (5'-PDE and PME) compared to ungerminated seeds. This is especially the case with the latter enzyme, where there was a significant difference in activity in germinated seeds.

In comparison with other germinated seeds, germinated adzuki bean has the highest level of 5'-PDE activity (0.59 µmol p-nitrophenol/min/mg

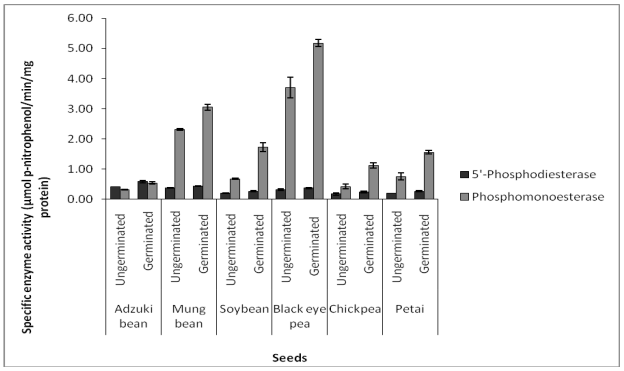

Figure 1. Specific enzyme activities of 5'-phosphodiesterase and phosphomonoesterase from ungerminated seeds (Control) and germinated seeds after 24h of germination. The error bars represent mean ± standard deviation of three replicates

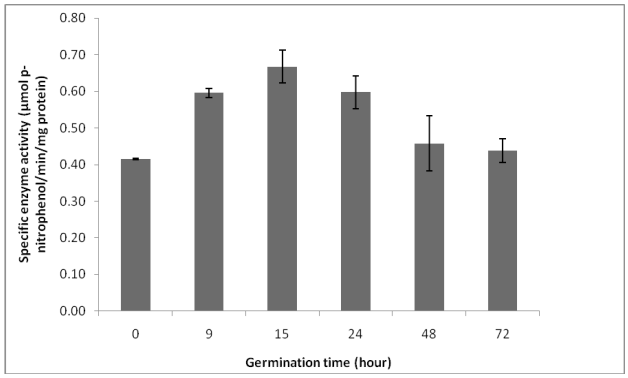

Figure 2. 5'-Phosphodiesterase activity of adzuki bean at different germination time. The error bars represent mean ± standard deviation of three replicates

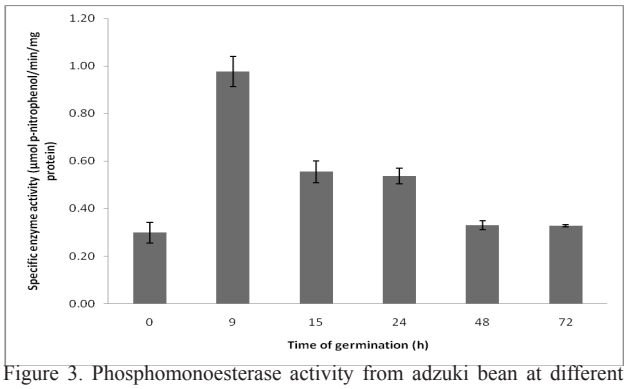

Figure 3. Phosphomonoesterase activity from adzuki bean at different germination time. The error bars represent mean ± standard deviation of three replicates

protein), followed by germinated mung bean and germinated black eye pea with specific enzyme activities of 0.44 and 0.37 µmol p-nitrophenol/min/mg protein, respectively. Germinated 'petai' seeds and germinated soybean both had 0.27 µmol p-nitrophenol/min/mg protein of 5'-PDE in its seed, respectively. Germinated chickpea have 0.24 µmol p-nitrophenol/min/mg protein, which had the lowest value of 5'-PDE among the six seeds used in this study.

The 5'-PDE level measured among the six germinated seeds were in agreement with work by other researches on plant or seed 5'-PDE. Avena leave tissue was reported to have a 5'-PDE activity of 0.18 µmol p-nitrophenol/min/mg protein (Udvardy *et al*., 1970). On the other hand, yellow lupin seeds had a 5'-PDE activity of 0.9 µmol p-nitrophenol/min/mg protein (Jakubowski *et al*., 1983). It has been reported also that germinated mung bean have higher 5'-PDE content compared to germinated soybean (Utami *et al*., 2011).

Figure 1 also shows the level of PME in the six germinated seeds. Among the germinated seeds, germinated black eye pea contained the highest level of PME (5.18 µmol p-nitrophenol/min/mg protein), followed by germinated mung bean (3.05 µmol p-nitrophenol/min/mg protein). Soybean, petai and chickpea contained less than half of the activity present in black eye pea. Adzuki bean possessed the lowest level of PME activity, with the specific enzyme activity of 0.54 µmol p-nitrophenol/min/mg protein.

Leelapon *et al*. (2004) have reported that soybean contained PME activity at 3.2 µmol p-nitrophenol/min/mg protein using 4-nitrophenol as the substrate. Kidney bean, on the other hand, contained 1.3 µmol p-nitrophenol/min/mg protein of PME activity (Yoneyama *et al*., 2007), while garlic seedlings had 168 µmol p-nitrophenol/min/mg protein of activity (Yenigün *et al*., 2003). Thus, possessing the highest level of 5'-PDE activity and the lowest level of PME activity at 24 hours germination, made adzuki bean the best candidate for further studies on 5'-PDE.

Effect of germination time on 5'-phosphodiesterase activity in adzuki bean

Figure 2 shows 5'-PDE activity in adzuki bean at different germination time. As can be seen, the level of the enzyme increased from 0.42 (Control) to 0.67 µmo/l min/mg protein, the highest level, within 15 hours of germination. Thereafter, the level of 5'-PDE decreased. After 72 hours (3 days) of germination, the level of the enzyme decreased to 0.44 µmol p-nitrophenol/min/mg protein, the basal level present in ungerminated seeds. Hence, the germination time used for further studies on adzuki bean 5'-PDE was 15 hours, as it has the highest level of 5'-PDE activity.

It has also been reported that germinating barley also showed an increase of enzyme activity with an increase of germination time (Deoda and Singhal 2003). The highest increase in 5'-PDE activity occurred between Day 2 and Day 3 of germination. Thereafter, the activity showed a slight increase until the end of Day 5. Khutle *et al*. (2011) found that 5'-PDE activity increased as the germination time of mung bean increased from Day 1 to Day 3. On the other hand, Utami *et al*. (2011) reported that 5'-PDE activity from mung bean and yellow soybean increased up to 4 days of germination and

decreased in the fifth day of germination, while 5'-PDE from black soybean increased up to 5 days of germination.

Phosphomonoesterase activity in adzuki beans during germination

The activity of PME in adzuki bean was found to be affected by germination time (Figure 3) where the enzyme activity increased dramatically until 9 hours of germination, After that, the level of the enzyme decreased until it reached the basal/ungerminated level (33 µmol p-nitrophenol/min/mg protein) after 30 hours of germination. At 15 hours of germination when 5'-PDE activity was the highest, PME activity was relatively lower than the activity after 9 hours of germination. The increase in PME activity with increasing germination time and subsequent decrease is in agreement with the study on maize seeds by Senna *et al*. (2006). PME from soybean also showed an increasing pattern from Day 1 until it reached its peak at Day 6 of germination, followed by decreasing pattern (Nicanuzia dos Prazeres *et al*., 2004).

Conclusion

Surface sterilizing treatment of seeds is very important in seed germination study so as to avoid microbial contamination. Surface sterilization was done by soaking the seeds in different surface sterilizing solutions for 5 mins before rinsing them 5 times with sterilized distilled water. It was found only 0.05% (v/v) sodium hypochlorite was needed to inhibit mold growth in germinated mung bean,while 0.1% (v/v) sodium hypochlorite was needed to inhibit mold growth in germinated black eye pea and germinated petai. A higher concentration, 0.3% sodium hypochlorite, was needed to inhibit mold growth in germinated adzuki bean, soybean and chickpea. The study showed that the usage of sodium hypochlorite caused minimal effect on the ability of the seeds to germinate based on growth of hypocotyl. The use of sodium hypochlorite is discouraged as the presence of sodium azide brought a significant growth inhibition. Results obtained show that among the germinated seeds, adzuki bean had the highest 5'-PDE activity and the lowest PME activity, an enzyme which is not favored in 5'-PDE study, after 24 hours of germination. Both 5'-PDE and PME activities of adzuki bean changed during germination, where 15 hours of germination yielded the highest 5'-PDE content, while the highest PME activity was highest after 9 hours of germination.

Acknowledgements

The author would like to express her deepest gratitude to Universiti Putra Malaysia for the Graduate Research Scholarship (GRF) in supporting her study.

References

Beluhan, S., Karmelic, I., Novak, S. and Maric, V. 2003. Partial purification and biochemical characterization of alkaline 5'-phosphodiesterase from barley malt sprouts. Biotechnology Letters 25: 1099-1103.

Benaiges, M. D., Lopez-Santin, J. and Sola, C. 1989. Partial purification of 5'-phosphodiesterase activity from barley rootlets. Enzyme and Microbial Technology 11: 444-451.

Bowles, L. K. 1991. 5'-Phosphodiesterase enzyme preparation and methods for its production. US Patent 5034325.

Deikus, G., Condon, C. and Bechhofer, D. H. 2008. Role of Bacillus subtilis RNase J1 endonuclease and 5'-exonuclease activities in trp leader RNA turnover. Journal of Biological Chemistry 283: 17158-17167.

Deoda, A. J. and Singhal, R. S. 2003. 5'-Phosphodiesterase (5'-PDE) from germinated barley for hydrolysis of RNA to produce flavour nucleotides. Bioresource Technology 88: 245-250.

Dev, P. M. 1985. Changes in the form of invertase during germination of mung bean seeds. Phytochemistry 25: 51-53

Dhule, S. S., Shetty, P. R., Iyer, J. L. and Singhal, R. S. 2006. Purification and characterization of 5'-phosphodiesterase from germinated barley. Process Biochemistry 41: 1899-1902.

Eggstein, M. and Kreutz, F. H. 1955. Vergleichende unterschungen zur quantitaven Eiweissbestimmung im Liquor und eiwessarmen Losnungun. Klinikal Wochenschr 33: 879-884.

Fernandez-Oroczo, R., Frias, J., Zielinski, H., Piskula, M. K., Kozlowska, H. and Vidal-Valverde, C. 2008. Kinetic study of antioxidant compounds and antioxidant capacity during germination of *Vigna radiata* cv. Emerald, Glycine max cv. Jutro and Glycine max cv. Merit. Food Chemistry111: 622-630.

Harvey, C., Olson, K. C. and Wright, R. 1970. Purification and properties of phosphodiesterase from the carrot. Biochemistry 9: 921-925.

Jakubowski, H. and Guranowski, A. 1983. Enzymes hydrolyzing ApppA and/or AppppA in higher plants. Purification and some properties of diadenosine triphosphatase, diadenosine tetraphosphatase, and phosphodiesterase from yellow lupin (*Lupinus luteus*) seeds. Journal of Biological Chemistry 258: 9982-9989.

Kannan, A. and Upreti, R. K. 2008. Influence of distillery effluent on germination and growth of mung bean (*Vigna radiata*) seeds. Journal of Hazardous Materials 53:609-615.

Khalil, A. H. and Mansour, E. H. 1995. The effect of cooking, autoclaving and germination on the nutritional quality of faba beans. Food Chemistry 54: 177-182.

Khandelwal, S., Udipi, S. A. and Ghugre, P. 2010. Polyphenols and tannins in Indian pulses: Effect of soaking, germination and pressure cooking. Food Research International 43:526-530.

Khattack, A. B., Zeb, A., Bibi, N., Khalil, S. A. and Khattack, M. S. 2007. Influence of germination techniquespn phytic acid and polyphenols content of chickpea (*Cicer arietinum* L.) sprouts. Food Chemistry 104: 1074-1079.

Khutle, N. M, Vijaya, D. C., Pawar, H., Jodhe, P. and Gaikar, A. 2011. Extraction, partial purification and characterization of 5'-phosphodiesterase from germinated *Phaseolus mungo* (mung bean). International Journal of Pharmaceutical and Phytopharmacological Research 1: 55-56.

Kleinhofs, A., Owais, W. M. and Nilan, R. A. 1978. Azide. Mutation Research 55: 165-195.

Kurosawa, S. I., Hayashi, M. and Ishizawa, H. 1984. Purification and properties of alkaline phosphodiesterase from the fruit body of *Flammulina velutipes*. Agricultural and Biological Chemistry 48: 299-305.

Lee, W. J. and Pyler, R. E. 1985. Nucleic acid degrading enzymes of barley malt. I. Nucleases and phosphatases. Journal of the American Society of Brewing Chemists 43: 1-6.

Leelapon, O., Sarath, G. and Staswick, P. E. 2004. A single amino acid substitution in soybean VSPa increases its acid phosphatase activity nearly 20-fold. Planta 219: 1071-1079.

Lerch, B. and Wolf, G. 1972. Isolation of phosphodiesterase from sugar beet leaves. Biochimica et Biophysica Acta 258: 206-218.

Lopez-Amoroz, M. L., Hernandes, T. and Estrella, I. 2006. Effect of germination on legume phenolic compounds and their antioxidant activity. Journal of Food Composition and Analysis 19: 277-283.

Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. 1951. Protein measurement with the Folin Phenol Reagent. The Journal of Biological Chemistry 193: 265-275.

Maity, J. P., Chakraborty, A., Chanda, S. and Santra, S. C. 2008. Effect of gamma radiation on growth and survival of common seed-born fungi in India. Radiation Physics and Chemistry 77: 907-912.

Martha, Z. H., Irma, B. L. and Jorge, M. V. R. 1987. Effect of benzyladenine on the DNA synthesis during early germination of maize embryo axes. Mutation Research 181: 103-110.

Nicannuzia dos Prazeres, J., Ferirreira, C. V. and Aoyama, H. 2004. Acid phosphatase acitivities during the germination of *Glycine max* seeds. Plant Physiology and Biochemistry 42: 15-20.

Pearson, O. W., Sander, C. and Nilan, R. A. 1975. The effect of sodium azide in cell processes in the embryonic barley shoot. Radiation Botany 15: 315-322.

Salvucci, M. E. and Crafts-Brandner, S. J. 1995. Purification and properties of a unique nucleotide pyrophosphatase/ phosphodiesterase I that accumulates in soybean leaves in response to fruit removal. Plant Physiology 108: 1269-1276.

Senna, R., Simonin, V., Silva-Neto, M. A. C. and Fialho, E. 2006. Induction of acid phosphatase activity during germination of maize (*Zea mays*) seeds. Plant Physiology and Biochemistry 44: 467-473.

Sfaxi-Bousbih, A., Chaoui, A. and El Ferjani, E. 2010. Cadmium impairs mineral and carbohydrate mobilization during the germination of bean seeds. Ecotoxicology and Environmental Safety 73: 1123-1129.

Urvardy, J., Marre, E. and Farkas, G. L. 1970. Purification and properties of a phosphodiesterase from avena leaf tissues. Biochimica et Biophysica Acta 206: 392-403.

Utami, T., Maharani, S. and Nur Chahyanto, M. 2011. Activity of 5'-Phosphodiesterase Isolated from Various Germinated Beans. The 12th ASEAN FOOD CONFERENCE, p. 639-642. Bangkok, Thailand.

Yang, X. C., Sullivan, K. D., Marzluff, W. F. and Dominski, Z. 2009. Studies of the 5' exonuclease and endonuclease activities of CPSF-73 in histone pre-mRNA processing. Molecular and Cellular Biology 29: 31-42.

Yenigün, B. and Güvenilir, Y. 2003. Partial purification and kinetic characterization of acid phosphatase from garlic seedling. Applied Biochemistry and Biotechnology 105 -108: 677-687.

Yoneyama, T., Taira, M., Suzuki, T., Nakamura, M., Niwa, K., Watanabe, T. and Ohyama, T. 2007. Expression and characterization of a recombinant unique acid phosphatase from kidney bean hypocotyl exhibiting chloroperoxidase activity in the yeast pichia pastoris. Protein Expression and Purification 53: 31-39.

Zou, H. C., Cai, G. Y., Cai, W., Li, H. L., Gu, Y., Park, Y. D. and Meng, F. G. 2008. Extraction and DNA digestion of 5'-phosphodiesterase from malt root. Tsinghua Science and Technology 13: 480-484.