

Antioxidant Activity Associated with Lipid and Phenolic Mobilization during Seed Germination of *Pangium edule* Reinw.

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Seeds of the tropical tree *Pangium edule* Reinw. are widely eaten in Southeast Asia after some treatment or processing. Fermented seeds are a specialty in Indonesia and have been used as spices. Because the tree is wild and has not been cultivated commercially, the physiology of germinated seeds of this tree for food uses is not known. This study reports some biochemical changes during seed germination associated with antioxidant activity and the mobilization of lipids and phenolics. Lipid content decreased, whereas the dominant fatty acids did not change significantly. The dominant fatty acids were oleic acid (C_{18:1(n-9)}) and linoleic acid (C_{18:2(n-6)}). During germination, oleic acid decreased while linoleic acid increased proportionally. The hypocotyl synthesized chlorophyll and the tocol composition also changed substantially. The antioxidant activity of phenolic extract increased in proportion to the total phenolics. Guaiacol peroxidase and glucose-6-phosphate dehydrogenase, selected enzymes associated with phenolic metabolism, showed that the increased activities coincided with increased total phenolics and free proline.

Keywords: *Pangium edule*; seed germination; fatty acids; tocols; guaiacol peroxidase; glucose-6-phosphate dehydrogenase; total phenolics; free proline

INTRODUCTION

Pangium edule Reinw. is a tropical tree that grows in Micronesia, Melanesia, and Southeast Asia, including Indonesia. The tree is highly poisonous, mostly because of the presence of cyanogenic glucoside (Burkill, 1935). In Indonesia, seed kernels are edible after some treatment following the removal of the cyanogenic glucoside. "Dage" is a product from boiled seeds after removal of kernels and water soaking for 2–3 days. Dage is utilized by Indonesians (West Java) as a vegetable. Another product is fermented seed product, called "keluwak", which has been used as a spice for soup in Java and South Sulawesi. Keluwak is also a raw material for another product, "kecap pangli" (ketchup, soy sauce like product), and has been used as a spice in Saparua. Sometimes, edible oil is also produced from the seed kernels.

In Indonesian, the name of the plant (fruit) and the oil is now usually spelled "picung", but the (Dutch translation) spelling "pitjoeng" can also be found in the older literature dealing with Java. "Samaun" appears to be the name used in the Philippines for the oil, and pangli is the name for the tree. Some interesting research on fermented seeds has been done by Fardiaz and Romlah (1992) and Puspitasari-Nienaber et al. (1994). Fardiaz and Romlah (1992) found that the

methanol extract of keluwak had antioxidant activity, and Puspitasari-Nienaber et al. (1992) found that keluwak oil did not contain cyclopentenyl fatty acids, a common cyclic fatty acids in the Flacourtiaceae family, and that γ -tocotrienol was a predominant tocol.

Tocotrienols are a series of compounds that are related to the four tocopherols associated with vitamin E, but tocotrienols are less widely distributed in nature. Tocopherols naturally present in foods have been strongly correlated with the polyunsaturated fatty acid because it counteracts the potential oxidative deterioration caused by fats in the diet (Anttolainen et al., 1995). Moreover, tocotrienols have been shown to evoke potent antioxidant, cancer preventive, and cholesterol-lowering properties; in some cases these properties are much stronger than those exhibited by tocopherols. Although tocotrienols were once thought to be of lesser nutritional value than the tocopherols, it is apparent that their activity and importance rank them as one of the most important classes of nutritional compounds for the prevention and treatment of disease (Serbinova et al., 1993; Papas, 1999). Tocotrienol has been known as a predominant tocol in picung seed, and we are interested in the changes or mobilization of this compound relative to lipids during seed germination. Levels of antioxidant compounds in seed are governed by the level of unsaturated fatty acids, and during seed germination, when lipids are used as energy and new synthesis during cellular metabolism (Salisbury and Ross, 1991).

Besides tocols, antioxidant compounds that are phenolic in nature are also found in seeds. Plant cells usually produce these compounds through phenolic secondary metabolism via the shikimic acid pathway (Salisbury and Ross, 1991). Phenolic antioxidants in

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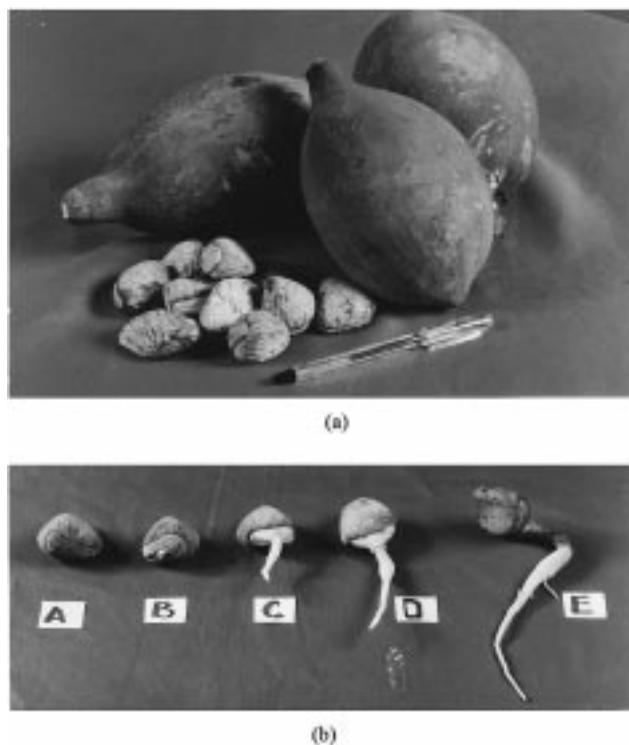


Figure 1. (a) *P. edule* Reinw. fruit and seed; (b) five stages of germinated seeds described under Materials and Methods.

plants are in the free phenolic form and usually are stored in the vacuole. Furthermore, free phenolics are polymerized to lignans and lignins in the plant cell wall. The production of free phenolics is hypothesized to be regulated via the proline-linked pentose phosphate pathway (Shetty, 1997). Furthermore, the free phenolics, once produced, can be polymerized on the cell walls of growing seedlings. Therefore, the objective of this research was primarily to investigate the mobilization of lipids, tocols, and free phenolics and the antioxidant activity associated with seed germination. A secondary objective was to determine the activity of the key enzyme, glucose-6-phosphate dehydrogenase (G-6-PDH), the first committed step of the pentose phosphate pathway, and the correlation to free phenolic and proline levels. Guaiacol peroxidase (GPX) activity was also monitored to obtain preliminary insight into the potential conversion of free phenolics to polymerized derivatives such as lignans and lignins.

MATERIALS AND METHODS

Seeds and Germination. *P. edule* Reinw. seeds were obtained from Bogor, Indonesia. The fruits were harvested October 1, 1996, and placed in the field for 10 days until the fruit was tainted. The seeds were removed and germinated in original potting soil for 4 months. Five groups of germinated seeds were obtained, grouped according to the length of hypocotyl and root (Figure 1). Group A was a dormant seed, group B had a very small root (not measured), group C had 4.24 ± 1.02 cm (mean \pm SD) of root, group D had 11.58 ± 2.03 cm of hypocotyl and root, and group E had 23.96 ± 2.69 cm of hypocotyl and root. The germinated seeds (cotyledon, hypocotyl, and root) were freeze-dried and ground. The freeze-dried seed powders were stored at -20 °C until analysis.

Lipid Content and Fatty Acid Composition. Lipid or oil content was measured gravimetrically after the freeze-dried seed powder was defatted using a Soxhlet extraction method using hexane as the solvent. Oil was obtained after the solvent was evaporated under reduced pressure.

The Soxhlet oil extracts of all freeze-dried seeds were analyzed in duplicate for fatty acid profiles. Methyl ester derivatives were prepared according to AOCS Standard Method Ce 2-66 and IUPAC Standard Method 2.301 with modification. Approximately 1 mg of sample oil (diluted using hexane as the solvent) was placed in a small vial with a Teflon cap. NaOH (0.5–0.7 mL, 2 N; in methanol) was added to each sample. After homogenization, the vial was placed in a heating block at 80 °C for 10 min. The vial was removed, and 1 mL of $\text{BF}_3/\text{methanol}$ reagent (Sigma Chemical Co., St. Louis, MO) was added. After homogenization, the vial was placed in a heating block at 80 °C for 10 min and homogenized every 3 min. One-half milliliter of hexane was added to the reaction mixture after it had cooled. After homogenization, some amount of saturated NaCl solution was added to the mixture, which was followed by centrifugation at 3000 rpm for 1–2 min. The upper phase (hexane phase) was removed and placed in a vial, which contained anhydrous Na_2SO_4 . The hexane phase, which contained fatty acid derivatives, was stored at 4 °C in amber crimp vials wrapped in aluminum foil and was analyzed within a week. Standards of methyl ester derivatives were obtained from Sigma Chemical Co.

The fatty acid derivatives were analyzed with a Varian Model 3700 gas chromatograph with a flame ionization detector and equipped with an SP 4270 integrator. The column was a Supelco10 fused silica capillary column with dimensions of $30 \text{ m} \times 0.20 \text{ mm}$ and $20 \mu\text{m}$ film thickness (Supelco, Bellefonte, PA). The initial column temperature of 150 °C was increased at a rate of 3 °C/min to a final temperature of 240 °C, held for 10 min. The injector and detector temperatures were 250 and 300 °C, respectively. Fatty acid content was expressed as percent of total fatty acids.

Tocol Analysis. The tocopherols (T) and tocotrienols (T3) were extracted in duplicate from the freeze-dried seed powder in minimal light essentially as described by Budin et al. (1995) and Oomah et al. (1997). Briefly, seed (1 g) was homogenized for 1 min in HPLC grade methanol (20 mL). The sample was filtered through Whatman No. 42 paper. The supernatant was removed and placed in a 25-mL glass vial and evaporated under nitrogen. The residue was resuspended in 15 mL of HPLC grade methanol, and the homogenization and filtration steps were repeated. The supernatant was removed and added to the first extract and dried under nitrogen. The dried extract was dissolved in 2 mL of HPLC grade hexane, mixed briefly in a vortex mixer, and centrifuged at 13000 rpm for 5 min, placed in a 2-mL amber crimp vial, and immediately analyzed.

Extracts were analyzed directly by high-performance liquid chromatography (HPLC) using a Hewlett-Packard Model HP1090 equipped with a LiChrospher RP-18 column ($3.0 \times 250 \text{ mm}$) with guard cartridge and diode array detector at 298 nm. The eluent was HPLC grade acetonitrile/methanol (85:15) at a flow rate of 0.8 mL/min. Identification of tocotrienols (α -, γ -, and δ -tocotrienols) was compared with the retention time and derivative spectrum of each compound peak according to the method of Brzuskiwick (1984). Tocopherol standards (α -, γ -, and δ -tocopherols) were obtained from Sigma Chemical Co. Tocol content was expressed in percent of total tocols.

Total Phenolic Content. Phenolics were extracted from defatted freeze-dried seed powder using the method described by Hammerschmidt and Pratt (1978) with a slight modification in the time of extraction (4 h). A 6 g sample was homogenized in 100 mL of ACS grade OmniSolv methanol (EM Science, Inc., Gibbstown, NJ) for 3 h using a shaker. After the mixture was placed in a water bath at 70 °C for 1 h, it was filtered through Whatman No. 42 paper. The residue was rinsed using methanol, and the supernatant was combined. The solvent was removed by rotary evaporator at 40 °C under reduced pressure. Total phenolics of the extracts were determined from the modified assay described by Chandler and Dodds (1983), which is similar to the method originally developed by Singleton and Rossi (1965) and as used in our laboratory (Shetty et al., 1995). Approximately 1 mL of 5 mg of DW extracts/mL was taken and placed in a test tube to which 1 mL of 95% ethanol (ACS grade) and 5 mL of filtered/deionized water were added. Folin–

Ciocalteu reagent (50%, 0.5 mL; Sigma Chemical Co.) was added to each sample. After 5 min, 1 mL of 5% Na_2CO_3 (Fisher Scientific Co., Pittsburgh, PA) was added and mixed with a vortex mixer, and the reaction mixture was allowed to stand for 60 min in darkness. Samples were again homogenized with a vortex mixer, and absorbance was measured at 725 nm. A standard curve was prepared using gallic acid (Fisher Scientific Co.) in 95% ethanol. Total phenolic content was expressed as milligrams per gram of DW of phenolic extracts.

Antioxidant Activity Test. The antioxidant activity of the phenolic extract was evaluated using a β -carotene/linoleate model system described by Miller with modifications (Hamerschmidt and Pratt, 1978; Wanasundara et al., 1994). A solution of β -carotene (Sigma Chemical Co.) was prepared by dissolving 2.0 mg of β -carotene in 10 mL of chloroform. One milliliter of this solution was then pipetted into a round-bottom flask. After chloroform was removed under vacuum, using a rotary evaporator at 40 °C, 20 mg of purified linoleic acid, 200 mg of Tween 40 emulsifier (Aldrich Chemical Co., Milwaukee, WI), and 50 mL of aerated distilled water were added to the flask with vigorous shaking. Aliquots (5 mL) of this prepared emulsion were transferred into a series of tubes containing 2 mg dry weight of extract. As soon as the emulsion was added to each tube, the zero time absorbance was read at 470 nm, which is the maximum wavelength for β -carotene. Subsequent absorbance readings were recorded at 30 min by keeping the samples in a water bath at 50 °C. The protection factor (PF) used to express antioxidant activity was determined as the ratio of the sample's absorbance at 30 min to that of the control.

Free Proline Content. Free proline in tissues was determined according to the method of Bates et al. (1973). Approximately 50 mg of freeze-dried seed powder was weighed and placed in 3 mL of 3% sulfosalicylic acid solution (Sigma Chemical Co.). After homogenization and centrifugation (13000 rpm, 10 min) of the sample, 1 mL of supernatant was placed in a reaction test tube with a cap. Glacial acetic acid (1 mL; Fisher Scientific Co.) and acid ninhydrin (1 mL; Sigma Chemical Co.; a mixture of 1.25 g of ninhydrin in 30 mL of glacial acetic acid and 20 mL of 6 M phosphoric acid) were added to each test tube, which was closed and heated in a 100 °C water bath for 1 h. After the sample had cooled in a cold room (5 °C) or in an ice bath for 15 min, 2 mL of ACS grade OmniSolv toluene (EM Science, Inc.) was added to every sample, followed by vortexing for 20 s to ensure thorough mixing. The absorbance of the colored toluene phase (upper phase) was measured at 520 nm, and toluene was used as a blank. A standard curve was prepared using a series of proline (Sigma Chemical Co.) concentrations in 3% sulfosalicylic acid solution. Proline content was expressed as micromoles per gram of fresh weight of freeze-dried seeds.

GPX Activity Assay. This assay is based upon a modification of the assay developed by Laloue et al. (1997) and George (1953). The enzyme was extracted from the freeze-dried seed powder in buffer, under cold conditions. Sample (50 mg) was homogenized with mortar glass in 2.5 mL of extraction buffer at 4 °C, that is, in an ice bath. The extraction buffer consisted of a 0.1 M potassium phosphate (Fisher Scientific Co.) buffer of pH 7.5, containing 2 mM EDTA (Fisher Scientific Co.) and 1% PVP-40 (Fisher Scientific Co.). The homogenate was centrifuged at 13000 rpm for 10 min. The supernatant was utilized for the enzyme and protein assay. The protein content of each enzyme extract was determined according to the modified Bradford method (1976) using the Bio-Rad protein assay (Bio-Rad Laboratories, Hercules, CA).

GPX in the plant extract was quantified in terms of its specific activity. The mixture of 360 μL of 0.056 M guaiacol (Sigma Chemical Co.), 40 μL of 50 mM H_2O_2 (Fisher Scientific, Fair Lawn, NJ), and 600 μL of 0.1 M phosphate buffer (pH 6.8) was taken in a reaction test tube. This gave a 1 mL reaction mixture containing 50 mM potassium phosphate buffer (pH 6.8), 2 mM H_2O_2 , and 20 mM guaiacol. At time $t = 0$, 50 μL of the diluted supernatant ($10\times$ using extraction buffer for dilution) was transferred to the reaction tube and mixed. The oxidation of guaiacol by GPX was followed by

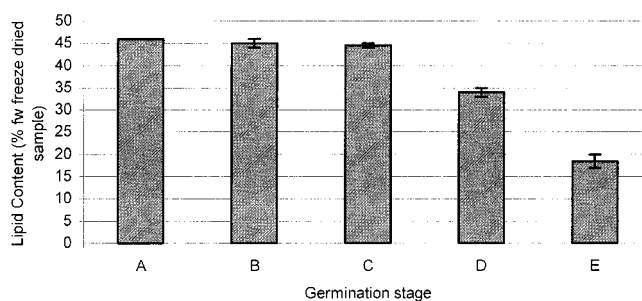


Figure 2. Changes in lipid content in *P. edule* Reinw. seed during various germination stages.

monitoring the increase in absorbance ($\lambda = 470$ nm). The rate of change of absorbance per minute was used to quantify the enzyme in the mixture using the extinction coefficient (ϵ) of the oxidized product (tetraguaiacol) = $26.6 \mu\text{M}^{-1} \text{cm}^{-1}$. The enzyme quantity was reported in micromoles per minute per milligram of protein.

G-6-PDH Activity Assay. This assay is based upon a modification of the assay developed by Deutsch (1983). The enzyme extract was obtained from the same procedure as the GPX assay and also used for protein estimation. G-6-PDH activity in the plant extract was quantified in terms of its specific activity. Fifty microliters of enzyme extract was added to 1 mL of reaction mixture, which contained 50 mmol/L Tris buffer (pH 7.5), 0.38 mmol/L nicotinamide adenine dinucleotide phosphate (NADP), 6.3 mmol/L MgCl_2 , 3.3 mmol/L glucose-6-phosphate (G-6-P), and 5 mmol/L maleimide in a quartz cuvette. After homogenization, the absorbance of this mixture was measured at 339 nm for a period of 5 min, and the reaction mixture was used as a blank. A change in absorbance of 0.01–0.03 or more is appropriate. The rate of change of absorbance per minute was used to quantify the enzyme in the mixture using the extinction coefficient (ϵ) of the NADPH $6.22 \text{mM}^{-1} \text{cm}^{-1}$. The enzyme quantity was reported in nanomoles per minute per milligram of protein.

RESULTS AND DISCUSSION

Crude Fat. *P. edule* Reinw. seed is high in extractable lipids to be classified as an oilseed. The crude fat content of the seeds during germination decreased from 46.00 to 18.50% on a dry basis (db). Breakdown of fats stored in oleosomes of seed generally releases relatively large amounts of energy. For seeds, this energy is necessary to drive early seedling development before photosynthesis begins (Salisbury and Ross, 1991). The breakdown of fats was more rapid after the hypocotyl emergence (germination stages C–E) than in earlier stages (germination stages A and B) (Figure 2). The hypocotyl and root seldom synthesize fats. Furthermore, both fatty acids and fats are too insoluble in H_2O to be translocated in phloem or xylem, and therefore lipid needs are generally obtained from oleosomes.

Fatty Acid Profiles. The fatty acid profile of *P. edule* Reinw. seed oil (Table 1) showed a predominance of oleic and linoleic acids. The profiles were similar to that of sesame oil (Belitz and Grosch, 1984) and also did not contain cyclopentenoic fatty acids. This study had the same results as the previous study (Puspitasari-Nienaber et al., 1994). The fatty acid composition changed in the dominant fatty acids, oleic and linoleic acids, during seed germination. During seed germination, oleic acid decreased (from 46.53 to 38.46%) and linoleic acid increased (from 38.07 to 46.32%). The decrease in oleic acid was proportional to the increase in linoleic acid, possibly due to the action of esterases. Hydration and temperature decreases do occur during seed germina-

Table 1. Fatty Acid Composition of *P. edule* Reinw. Seed during Various Germination Stages^a

fatty acid (%)	germination stage				
	A	B	C	D	E
C _{14:0}	0.06	0.04	0.05	0.04	0.03
C _{16:0}	8.42	8.14	7.66	8.07	7.72
C _{16:1}	0.11	0.12	0.12	0.11	0.09
C _{18:0}	3.01	2.72	3.07	2.90	2.72
C _{18:1(n9)}	46.53	43.00	41.71	41.03	38.46
C _{18:1(n7)}	1.54	1.55	1.42	1.40	1.33
C _{18:2}	38.07	41.83	43.97	43.87	46.32
C _{18:3}	1.79	2.18	2.19	2.18	2.84
C _{20:0}	0.14	0.13	0.14	0.13	0.14
C _{20:1}	0.29	0.21	0.24	0.25	0.3
C _{22:0}	0.06	0.13	0.02	0.03	0.03

^a Each value is a mean of four independent assays.

Table 2. Tocopherol (T) and Tocotrienol (T3) Composition of *P. edule* Reinw. Seed during Various Germination Stages^a

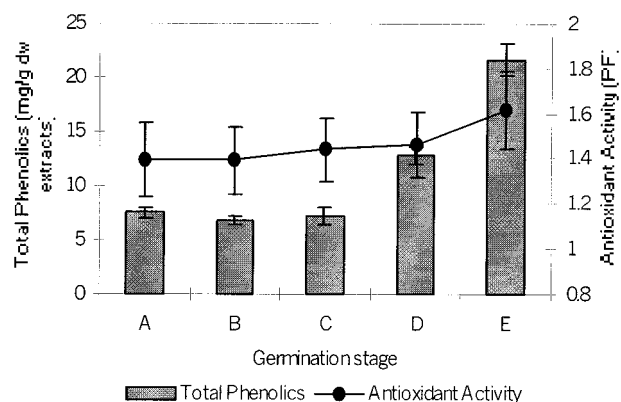
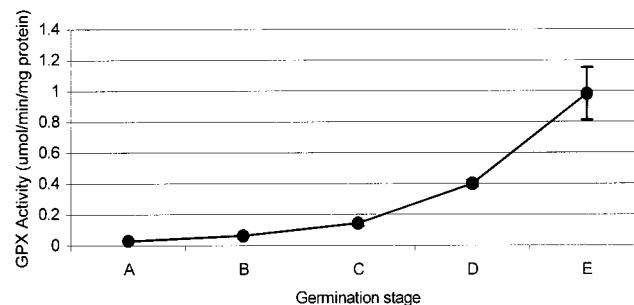
germination stage	δ -T3 (%)	γ -T3 (%)	γ -T3 isomer (%)	α -T3 (%)	α -T (%)	total (%)
A	13.51	58.74	nd ^b	27.78	nd	100
B	50.35	49.65	nd	nd	nd	100
C	34.60	65.40	nd	nd	nd	100
D	59.57	40.48	nd	nd	nd	100
E	57.71	27.98	12.25	nd	3.29	100

^a Each value is a mean of two independent assays. ^b Not detected.

tion. The increasing oxygen solubility in water, while the temperature decreased, could supply oxygen as an essential hydrogen atom receptor for unsaturated process in the endoplasmic reticulum. In higher plants, linoleic acid is synthesized from oleic acid (Salisbury and Ross, 1991).

Tocol Profiles. The tocol or tocopherol (tocopherols and tocotrienols) level in plants is governed by the level of unsaturated fatty acids and a simple increase in unsaturation results in the formation of higher amounts of antioxidants to protect the oil (Eskin et al., 1996). Antioxidant activities of tocopherols have been known, but only little research has been undertaken for tocotrienols. Furthermore, a tocol profile of vegetable oil is specific between different species and is used for identification of oil profiles of different species. As in previous research (Puspitasari-Nienaber et al., 1994), we predominantly found γ -tocotrienol in the tocol fraction during seed germination in the early stage. During seed germination, this compound had changed little compared to the other tocols (Table 2). The tocol profiles had changed substantially in stage E, in which the hypocotyl synthesized chlorophyll. In this stage, α -tocopherol was synthesized. In higher plants, α -tocopherol is synthesized in chloroplast and proplastid (Hess, 1993).

Phenolic Content and Antioxidant Activity. Phenolics or phenolic acids, intermediates in phenylpropanoid metabolism, play many important roles in plant cells, tissue, and organs (Dixon and Paiva, 1995). They are also known to be involved in growth regulation and in the process of differentiation and organogenesis. Recent discoveries have revealed that phenolic compounds are involved in plant development during seed germination and in plant-microbe recognition and signal transduction (Lynn and Chang, 1990). During seed germination, the phenolic content increased corresponding to the increased antioxidant activity (Figure 3). The increasing phenolic content indicates that the

**Figure 3.** Total phenolics and antioxidant activity of phenolic extracts of *P. edule* Reinw. seed during various germination stages.**Figure 4.** GPX activity of *P. edule* Reinw. seed during various germination stages.

plant produced precursors for the potential synthesis of lignin (Lewis and Yamamoto, 1990), and the antioxidant activity of the phenolics extract showed that phenolics in seed are also important as antioxidants when oxygen demand during germination is high. The antioxidant might be protecting the cell from potential oxidation-induced deterioration.

GPX Activity. Peroxidases, belonging to the oxidoreductive group of enzymes, are scavengers of H₂O₂, a byproduct of photosynthetic activity in plants (Laloue et al., 1997). They are also involved in mediating the stress-sensitive lignin biosynthesis in plants (Lewis, 1993). The GPX activity in seed during germination increased (Figure 4) corresponding to the increases in phenolic content (Figure 3). This indicates that the plant's need of phenolics for lignification and structure development is being mobilized during germination. Lignin content and partially polymerized phenolics from germinated seeds could also serve as a source of valuable functional metabolites for disease-preventive applications and dietary nutraceutical.

G-6-PDH Activity and Free Proline Content. In many plants, free proline accumulates in response to the imposition of a wide range of biotic and abiotic stresses. Proline has the ability to mediate osmotic adjustment, stabilize subcellular structures, and scavenge free radicals. However, often the cytoplasmic pool of free proline even after the imposition of stress is insufficient in size to account for pronounced biophysical effects (Hare and Cress, 1997). During seed germination, free proline content in the seed increased (Figure 5). High levels of proline synthesis may maintain NAD(P)⁺/NAD(P)H ratios at values compatible with metabolism under normal condition. The increased NAD(P)⁺/NAD(P)H ratio mediated by proline biosynthesis is likely to enhance the activity of the oxidative pentose

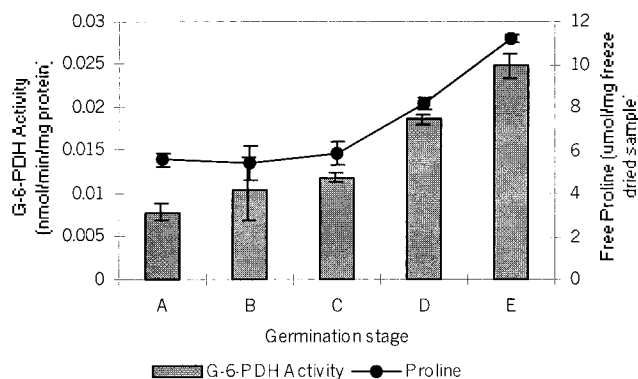


Figure 5. G-6-PDH activity and free proline content of *P. edule* Reinw. seed during various germination stages.

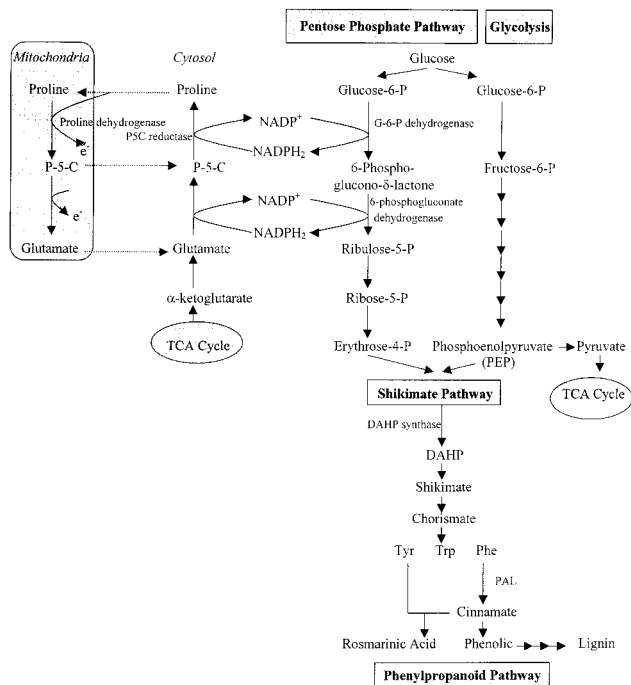


Figure 6. Proline-linked pentose phosphate pathway.

phosphate pathway. The increase in G-6-PDH activity in this study (Figure 5) supports this concept. The enzyme activity as well as free proline content increased during seed germination. The increased proline-linked stimulation of the pentose phosphate pathway may make available NADPH₂ and phosphorylated sugars for the phenylpropanoid pathway (phenolic antioxidants and lignin) and other anabolic pathways (Figure 6) (Shetty, 1997). Proline oxidation during the germination process could serve as an alternative source of reductant for oxidative phosphorylation and at the same time supply and maintain the needs of NADPH₂ and phosphorylated sugar for anabolic pathways.

Implications. There are two major implications of this study. At the fundamental level the role of proline-linked phenolic synthesis and regulation of antioxidant function in relation to lipid mobilization as well as development-linked lignification can be further investigated. At the application level, the germination process and the regulation of phenolic metabolites can be used as sources of valuable functional phenolics for functional foods, nutraceuticals, and prevention of oxidation-linked diseases via a dietary approach.

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