Evaluation of the *In Vivo* Antioxidant Activity of *Mucuna pruriens* DC. var. *utilis* by Using *Caenorhabditis elegans*

Sachie IBE1*, Yoshiharu FUJII2 and Kazunori OTOE3, 4

1 Ohyamatofu Co., Ltd., 575 Shirone, Isehara-shi, Kanagawa 259-1147, Japan  
2 International Environmental Agriculture, Tokyo University of Agriculture and Technology, 3-5-8 Saiwai-cho, Fuchu-shi, Tokyo 183-8509, Japan  
3 National Agricultural Research Center, National Agriculture and Food Research Organization, 3-1-1 Kannondai, Tsukuba, Ibaraki 305-8666, Japan  
4 Graduate School of Life & Environmental Science, University of Tsukuba, 3-1-1 Kannondai, Tsukuba, Ibaraki 305-8666, Japan

Received October 25, 2011; Accepted December 8, 2011

*Mucuna pruriens* (MP) is a legume with seeds that contain substantial amounts of 3, 4-dihydroxy-L-phenylalanine (L-DOPA; 3% to 7% dry weight). We examined the *in vivo* antioxidant activity of distilled-water extracts of steamed or fermented MP seeds in *Caenorhabditis elegans* to evaluate the potential use of MP as a functional food. Paraquat and thermal stress generate reactive oxygen species in *C. elegans*. Worms treated with MP extracts were exposed to acute stress with paraquat or lethal heat shock. The survival rate 3 days after the treatment was used to indicate the antioxidant activity of the MP extracts. Distilled-water extracts of steamed or fermented MP had significantly higher (*P* < 0.01) antioxidant activity than in the control (nematode standard media) *in vivo*. L-DOPA showed high 1,1-diphenyl-2-picrylhydrazyl (DPPH)-radical scavenging activity but did not contribute to the antioxidant activity of MP *in vivo*.

Keywords: *Mucuna pruriens*, antioxidant, L-DOPA, *Caenorhabditis elegans*

Introduction

*Mucuna pruriens* (MP) is a legume cultivated mainly in Africa, South America, and South Asia as a green manure and cover crop. The mature beans contain large amounts of protein (26% to 30%) and starch (34% to 40%), essential amino acids, and fatty acids, and they have a good nutritional mineral composition (i). Plants of the genus *Mucuna* are characterized by an accumulation of the non-protein amino acid 3, 4-dihydroxy-L-phenylalanine (L-DOPA), and MP seeds contain substantial amounts of L-DOPA (3% to 7% dry weight) (Fujii et al., 1991; Teixeira et al., 2003). L-DOPA, a precursor of dopamine, has been the primary and most efficacious medication used to treat Parkinson’s disease (PD) for nearly 40 years. However, L-DOPA is antinutritional compound and there are adverse effects associated with the excessive administration of L-DOPA, including nausea, vomiting, anorexia, paranoia, and unmasking of dementia (Jose-phin and Janardhanan, 1992; Kostrzewa et al., 2002). So the excessive ingestion should be avoided. MP is a potential suppressor of PD symptoms, and recent studies suggest that MP formulations may actually have a higher bioavailability than standard L-DOPA preparations and that it has advantages over conventional L-DOPA preparations in the long-term management of PD (Katzenschlager et al., 2004; Lieu et al., 2010; Pathan et al., 2011).

MP is also a substantial source of natural antioxidants (Rajeshwar et al., 2005a; Sidduraju and Becker, 2003; Tripathi and Upadhyay, 2002), and shows antitumor (Rajeshwar et al., 2005b) and hypoglycemic and hypolipidemic (Bhaskar et al., 2008; Murugan and Reddy, 2009) activity. Kurokawa et al. (2011) recently demonstrated that MP extract stimulates the differentiation of bone marrow cells into dendritic cells and induces apoptosis in cancer cells. Therefore, despite the disadvantages of the L-DOPA it contains, MP is a promising nutritional supplement. When MP is intended for use not for its L-DOPA content but for its nutritional properties, it is necessary to remove the L-DOPA. Vari-
ous methods for preparing MP seeds have been investigated to optimize the method of reducing the l-DOPA content (Iijima et al., 2009; Vijayakumari et al., 1996). Fermentation processing with Bacillus subtilis or Rhizopus oligosporus effectively reduces the l-DOPA content of MP extracts (Egounlety, 2003; Higasa et al., 1996; Mugendi et al., 2010).

Our study focused on the antioxidant activity of water extracts of steamed and fermented MP seeds to evaluate the possibility of utilizing MP as a functional food. In vivo dose-response studies using MP extracts were conducted in Caenorhabditis elegans, a free-living bacteriophagous nematode that has a well-characterized response to oxidative stress and is simple to manipulate (Ibe et al., 2010; Sampayo et al., 2003; Tang and Halliwell, 2010). The in vivo activity of the MP extracts was validated in comparison with the in vitro antioxidant activity of MP extracts or l-DOPA.

Materials and Methods

Chemicals 1,1-diphenyl-2-picrylhydrazyl (DPPH), gallic acid, 5-fluoro-2'-deoxyuridine (FudR), methyl viologen dichloride hydrate, and ampicillin sodium salt were purchased from Sigma (St. Louis, MO, USA). Other chemicals were purchased from Wako (Osaka, Japan).

Preparation of plant materials We used the seeds of two cultivars of MP, Florida velvet bean (FVB) and Hassyo-mame (Japanese velvet bean, JVB); these cultivars are valued in eastern Japan, where they can be harvested after 6 months of cultivation. The seeds were obtained from an experimental farm at the National Institute for Agro-Environmental Sciences in Tsukuba, Japan. Each type of bean was soaked in distilled water (DW) for 24 h. The soaked beans were dehulled and then steamed in an autoclave at 121°C for 15 min. Beans were fermented by using a process similar to that used in natto (fermented soybean) production. The steamed beans were inoculated with Bacillus subtilis natto at approximately 10^6 cells/g and fermented in an incubator at 39°C for 20 h. They were left at 4°C for 24 h after fermentation. Soaked, steamed, and fermented beans were freeze-dried, ground, and diluted to 1:20 (W/V) in DW. Raw beans were firstly ground, then freeze-dried, and diluted in the same way as described above. The mixture was then stirred for 2 h at room temperature. Each extract was centrifuged (9100 × g, 10 min), and the supernatant was used to evaluate the DPPH-radical scavenging activity (see below). For purpose of reference, black soybeans (Kurosengoku) were treated as described above, and their DPPH-radical scavenging activity was compared with those of the MP extracts.

FVBs were used for the in vivo studies, because they showed higher in vivo antioxidant activity in preliminary experiments. Steamed and fermented FVBs were extracted as described, then centrifuged and the supernatant lyophilized. The lyophilized sample was added to S-medium (ii) (for the paraquat-induced oxidative-stress assays) or to nematode growth medium (NGM) agar plates (for the thermotolerance assays) during preparation of agar plates. The extracts from steamed beans and from fermented beans were tested separately.

Quantification of l-DOPA The l-DOPA content of each MP sample was determined by using the method described by Hiradate et al. (2005). Powdered sample (0.25 g) of raw beans and of beans processed to the soaked, steamed, or fermented stages were suspended in 200 volumes of 0.4 M phosphate buffer (pH 4.0) respectively and stirred for 1 h at room temperature. Each extract was then centrifuged (9100 × g, 10 min). The supernatant was filtered through a 0.45-μm pore-size filter membrane and applied to HPLC (Shimadzu LC 10AT with Class VP workstation, Shimadzu Corporation, Kyoto, Japan) in a reversed-phase analytical column (L-column ODS, 5 μm, 4.6 × 150 mm, Chemical Evaluation and Research Institute, Tokyo, Japan). l-DOPA was eluted with a mixed solution of 0.1 M phosphate buffer (pH 2.0) and methanol (9:1) with a flow rate of 1 mL/min and a column temperature of 40°C. The l-DOPA was detected by measuring the absorbance at a wavelength of 200 nm.

Determination of DPPH-radical scavenging activity DPPH-radical scavenging activity was assayed by using a modification of the method described by Suda (2000). A 0.25-ml sample of 400 μM DPPH in ethanol was mixed with equal volumes of 0.2 M 2-(N-morpholino)ethanesulfonic acid (MES) buffer (pH 6.0) and ethanol in a 2-ml microtube. A 0.25-ml aliquot of the MP sample solution (in water) was added, mixed for 20 min, and the absorbance at 517 nm was measured with a spectrophotometer (Hitachi U-2800, Hitachi Ltd, Tokyo, Japan). The EC_{50} (half-maximal effective concentration) value was defined as the concentration (in mg/mL) of extract that decreased the initial concentration of DPPH radicals by 50%. Gallic acid was used as a standard control. The gallic-acid-equivalent antioxidant capacity (i.e. the EC_{50} of the sample as a ratio of the EC_{50} of gallic acid) was used to compare antioxidant activities.

Determination of in vivo antioxidant activity using C. elegans Two strains of C. elegans, N2 (wild type) and mev-1 (methyl-viologen-sensitive mutant) (Ishii et al., 1990), were obtained from the Caenorhabditis Genetics Center (University of Minnesota, Minneapolis, USA). Worms were maintained at 20°C in appropriate incubators (MEE Cool Incubator, Mitsubishi Electric Engineering Co. Ltd, Tokyo, Japan) on NGM agar plates (90 mm diameter) seeded with Escherichia coli strain OP50 as a food source. Worms treated with MP extract (see below) were exposed to acute...
stress with the herbicide paraquat (methyl viologen) or to lethal thermal stress. Paraquat causes oxidative stress by a metabolically catalyzed reaction, resulting in depletion of cellular NADPH and production of reactive oxygen species (ROS), primarily superoxide anions (Bus et al., 1974, 1976). The toxicity of thermal stress is due partially to the increased production of ROS (Kampkotter et al., 2007). Nematode development was synchronized for both the paraquat-induced oxidative stress assays and thermal stress assays, according to a method adapted by Yanase and Ishii (2003). Embryos (eggs) were collected from gravid hermaphrodites using alkaline sodium hypochlorite. The released eggs were allowed to hatch by overnight incubation at 20°C in S basal buffer (100 mM NaCl, 50 mM potassium phosphate (pH 6.0)). The newly hatched larvae (L1-stage larvae) were cultured in S-medium or on NGM plates.

Paraquat-induced oxidative stress assays were performed with mev-1 hermaphrodites at 20°C. L1 larvae were grown to the L4/young adult stage in S-medium seeded with OP50 (5 × 10⁸ cells/mL) in 6-well tissue culture plates, with each well containing 2.5 mL S-medium without cholesterol (Sampayo et al., 2003). The young adult worms were transferred onto fresh medium plus 50 μM FudR, the sample (i.e. FVB extract, mev-1/DOPA, 0 to 1mg/mL), ampicillin 100 μg/mL, and OP50 bacteria (5 × 10⁸ cells/mL), and then cultured at 20°C for 3 days. FudR was used to block the development of progeny (Hosono, 1978). The worms were then transferred to fresh medium containing 50 mM paraquat and OP50 (5 × 10⁸ cells/mL) and viability was determined every 24 h for 3 days. Worms were considered dead when they showed no response to a gentle touch with a platinum wire. Experiments were usually performed 3 times. Approximately 90 worms were used per experiment to evaluate the effect of each concentration of FVB extract or l-DOPA.

Thermal stress assays were performed with wild-type N2 worms. An 800-μL of 0.5-mg/mL FudR was added to the NGM plates to block progeny development 3 days after synchronization culture of L1 larvae on NGM plates, and the worms were cultured overnight. Then the young adult worms were transferred by platinum wire onto 60-mm-diameter NGM plates seeded with FVB extract (0 to 2mg/mL), OP50, and incubated at 20°C for 3 days. The plates were then incubated at 35°C for 7 h and returned to 20°C. The plates were evaluated for survival every 24 h for 3 days. Approximately 90 worms (allocated over 3 plates) were used per experiment to evaluate the effect of each concentration of FVB extract.

Statistical analysis Independent groups were statistically compared by using t-tests (Table 2). The Bonferroni test was used when multiple groups were compared (Table 1, Fig. 3, and Fig. 5). Statistical significance between multiple dose groups and a control was determined by using ANOVA followed by a Williams multiple comparison test (Fig. 4). The statistical significance of a correlation coefficient was determined by using a t-test (Fig. 2). A value of P < 0.05 was considered to be significant.

Results
l-DOPA content of MP, and in vitro antioxidant activity
FVB maintained higher l-DOPA levels than JVB, although the levels were significantly decreased (P < 0.01) by the soaking process (Table 1). The fermentation process was associated with a negligible decrease in the level of l-DOPA (Table 1).

We examined the DPPH-radical scavenging activity of water extracts of MP beans (Fig. 1). FVB extracts had greater activity than JVB extracts and much greater activity than black soybean extracts at any stage of treatment. Furuta et al. (2003) reported that black soybeans had the greatest DPPH-radical scavenging activity among tested soybeans with pigmented seed coats. Both types of MP had markedly higher activity than the black soybean. l-DOPA exhibits high DPPH-radical scavenging activity (Gulcin, 2007). The actual activity of the l-DOPA reagent was 7412 ± 468 m mol gallic acid equivalent/g. The DPPH-radical scavenging activity of MP extracts was closely correlated with the l-DOPA content of the MP seeds (Fig. 2). The activity of the l-DOPA reagent, which was converted to that of the equivalent of a 4% l-DOPA solution, was also plotted near the approximation curve (Fig. 2).

Effects of MP extract on paraquat resistance Among worms exposed to 50 mM paraquat for 3 days, those treated with 2 mg/mL of extract from fermented FVBs showed significantly greater viability than untreated worms or those treated with 0.5 or 1 mg/mL extract from the fermented beans. A 2-mg/mL extract from steamed FVBs gave significantly greater viability than in untreated controls (Fig. 3). At each extract concentration, there were no significant differences in viability between worms treated with extracts

<table>
<thead>
<tr>
<th>l-DOPA Content in MP Seeds (%)</th>
<th>JVB</th>
<th>FVB</th>
</tr>
</thead>
<tbody>
<tr>
<td>Raw</td>
<td>4.9 ± 0.13 a</td>
<td>5.9 ± 0.09 d</td>
</tr>
<tr>
<td>Soaked</td>
<td>3.6 ± 0.06 bc</td>
<td>4.6 ± 0.06 ae</td>
</tr>
<tr>
<td>Steamed</td>
<td>3.6 ± 0.07 bc</td>
<td>4.7 ± 0.27 ae</td>
</tr>
<tr>
<td>Fermented</td>
<td>3.4 ± 0.07 c</td>
<td>4.2 ± 0.12 bc</td>
</tr>
</tbody>
</table>

Means ± SE, n = 3

Means with different characters are significantly different by Bonferroni test (P < 0.01)
from steamed beans and worms treated with extracts from fermented beans. However, the yield of DW extract differed significantly between processing stages \((P < 0.001; \text{Table 2})\). The amount of DW extract from fermented beans was greater than that from steamed beans; thus the total antioxidant activity of fermented beans was higher than that of steamed beans.

The data in Table 2 demonstrates that the L-DOPA content of a 2-mg/mL solution derived from steamed FVBs was 0.28 mg/mL; that of the same solution from fermented beans was 0.15 mg/mL. Comparison of the \textit{in vivo} antioxidant activity of L-DOPA solution (0 to 1 mg/mL) (Fig. 4) revealed that L-DOPA 0 to 0.5 mg/mL resulted in no change in worm viability in worms exposed to 50 mM paraquat for 3 days. However, L-DOPA 1 mg/mL caused a significant reduction in viability compared with that in the controls (0 mg/mL).

\textbf{Effects of MP extract on thermal tolerance} Among thermally stressed worms, treatment with extract from FVBs

\begin{table}[h]
\centering
\begin{tabular}{lcc}
\hline
 & DW extract (g/100 g dry seed) & L-DOPA (mg/mL) \\
\hline
steamed & 23 ± 0.58 & 14.5 ± 0.52 \\
fermented & 34 ± 0.58 & 7.4 ± 0.23 \\
\hline
\end{tabular}
\caption{Yield of DW extract and L-DOPA content.}
\end{table}
fermented soybean, as estimated by an in vivo assay using *C. elegans*, with the extracts’ DPPH radical scavenging activity and superoxide dismutase-like activity. We showed that the antioxidant effects of these extracts in vivo were not related to their effects in vitro. Similarly, in our current study, the in vitro antioxidant activity of l-DOPA was not a predictor of its benefits in vivo in whole animals.

L-DOPA causes cytotoxicity by generating ROS during its oxidative polymerization into melanin (Hachinohe and Mat-
sumoto, 2007; Vatassery et al., 2006). Kawaii et al. (1993) found that \( \alpha \)-DOPA is lethal in \( C.\ elegans \) at high concentrations (> 625 \( \mu \)g/mL) owing to a rapid increase in intracellular free \( \text{Ca}^{2+} \), whereas our preliminary experiment showed that incubation for 3 days in 1 mg/mL \( \alpha \)-DOPA had no adverse effects on \( C.\ elegans \). However, 3 days of incubation in paraquat decreased viability of worms (Fig. 5), and under the oxidative stress induced by the paraquat (and possibly in combination with other synergistic effects) \( \alpha \)-DOPA at 1 mg/mL became lethal.

Our results showed that MP extracts of both steamed and fermented beans had \textit{in vivo} antioxidant activity. In terms of both paraquat resistance and thermal tolerance, there were no significant differences in worm viability between the treatments (i.e. extracts from steamed or fermented beans) at each dose rate. However, the yield of DW extract from fermented beans was significantly higher (\( P < 0.001 \)) than that of DW extract from steamed beans (Table 2). Thus fermentation increased the yield of water-soluble materials, which had antioxidant activity \textit{in vivo}. These results suggest that unidentified antioxidants were produced during fermentation.

Steamed MP seeds were light brown but turned blackish during fermentation, implying that \( \alpha \)-DOPA was converted chemically to refractory, inactive melanin (Mugendi et al., 2010; Teixeira and Rich, 2003). Intact MP seeds contain abundant antioxidants other than \( \alpha \)-DOPA, including polyphenol, glutathione, and gallic acid (iii), although no reports on the antioxidants produced during fermentation of the seeds are available. Fermentation processing of soybeans by \( B.\ subtilis \) increases the content of peptides (Fan et al., 2009) or total phenolic compounds (Fernandez-Orozco et al., 2007; Hu et al., 2010). In our previous study, similar fermentation processing of soybeans to that used in the present study resulted in \textit{in vivo} antioxidant activity higher than that from simple steaming (Ibe et al., 2010). These results with soybean fermentation suggest that similar increases in the abundance of antioxidant compounds occur in the processing of MP seeds.

If MP is to be useful as a functional food, because of the adverse effects of excessive \( \alpha \)-DOPA consumption it will be essential to control the \( \alpha \)-DOPA content of the food. Our results suggest that the \textit{in vivo} antioxidant activity of processed MP is independent of the \( \alpha \)-DOPA concentration. Meticulous investigations of the activity of MP treated to reduce \( \alpha \)-DOPA concentrations will be essential in further studies.

References


Antioxidant activity of Mucuna pruriens


URL cited