Original paper

Cloning of Phenylalanine Ammonia-lyase and its Role in Enzymatic Browning of Mung Bean Sprout during Cold Storage

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Received October 12, 2015 ; Accepted December 20, 2015

Mung bean sprout turns brown during cold storage. Here we cloned phenylalanine ammonia-lyase (PAL) mRNA and examined its expression during cold storage. A PAL cDNA of mung bean encoding 715 amino acids was cloned, which showed high homology to PAL genes of Fabaceae plants and had a highly conserved PAL motif including the active site (Ala-Ser-Gly). The expression of PAL mRNA increased during cold storage of mung bean sprout. The increase in expression was inhibited by the heat-shock treatment of mung bean sprout before storage. trans-Coumaroyltartronic and trans-caffeoyltartronic acids, the major substrates of polyphenol oxidase in mung bean sprout increased during cold storage. These results show that the PAL expression and biosynthesis of phenolics are essential for enzymatic browning of mung bean sprout during cold storage.

Keywords: mung bean sprout, enzymatic browning, phenylalanine ammonia-lyase, caffeoyltartronic acid, coumaroyltartronic acid

Introduction

Discoloration and browning of fresh vegetables, fruits, and minimally-processed fresh products during storage is often a limiting factor of consumer acceptance. The discoloration and browning generally occurs by oxidative polymerization of phenolics by an oxidative enzyme. Mung bean sprout is a major bean sprout in eastern Asian countries; however, it is highly perishable, with a shelf life limited by both its appearance—easily turning brown or dark in color during cold storage—and microbiological quality.

Although the discoloration of mung bean sprout is considered to be an example of enzymatic browning, there are few reports on the browning of mung bean sprout. We previously showed (Nishimura et al., 2012) that heat-shock treatment inhibited browning by repressing the induction of phenylalanine ammonia-lyase (PAL; EC 4.3.1.24) activity, one of the key biosynthetic enzymes of polyphenols or phenolics. However, the PAL gene of mung bean sprout has not yet been cloned, and whether the induction of PAL activity occurs at the level of mRNA or protein remains to be elucidated. Further, the phenolics which act as a substrate of polyphenol oxidase (PPO) of mung bean sprout and their involvement in browning are not known, although some properties of mung bean PPO (Takeuchi et al., 1992; Shin et al., 1997) and the identification of sprout phenolics (Strack, 1985) have been reported. As the polyphenol content of fresh mung bean sprout is very low (Barroga et al., 1985), it is thought that the polyphenols are synthesized during storage and are oxidized by PPO to form a brown pigment (Nishimura et al., 2012).

The aim of this study was to clone the PAL gene of mung bean sprout and demonstrate PAL induction and alterations in phenolics
in terms of browning during cold storage of mung bean sprout.

Material and Methods

Materials  Mung bean (Vigna radiata) sprout specimens were purchased from a retail shop in Tokyo between 2011–2013 and used for experiments without further storage.

Cloning of PAL  About 1 g of mung bean sprout specimens was frozen using liquid nitrogen and pulverized by a pestle, then total RNA was extracted using RNeasy Plant Mini Kits (Qiagen, Tokyo, Japan) according to the manufacturer's instructions. cDNA was obtained from the RNA extract using the PrimeScript RT Reagent Kit (Takara Bio, Ohtsu, Japan). In a preliminary experiment, more than 20 combinations of PAL sequences of such plants as Allium sativum (Tuan et al., 2010; Forward (F): 5’-TMCARG GMATCTCH GCCATMMG-3’, Reverse (R): 5’-GCCGTYNSACRTGGTNGT VA-3’), Euphorbia pulcherrima (Tao et al., 2011; F: 5’-GGAAAATTCCTTCAAG-3’, R: 5’-TACATAGAAGGG ATTACA-3’), Lactuca sativa var. longifolia (Campos et al., 2004; F: 5’-GAYCCNYTNAAYTGGG-3’, R: 5’-CCYTGRA ARITNNCNCCRTG-3’), and Trifolium spp. (Howles et al., 1994; R: 5’-AAGAATTCACRCTCYTGGTTRTGYTGTC-3’, and a sequence similar to plant PAL (Jiang et al., 2011; F: 5’-TNCCNCTNTCNACTACATHGCAGG-3’, R: 5’-AARCAYCA YCCNGNGCARRATH-3’) were used for the polymerase chain reaction (PCR). PCR products obtained were obtained from three combinations of primers (Allium-F/Allium-R, Allium-F/Trifolium-R, and plant-F/Trifolium-R). Among the combinations of these primers, only the PCR product obtained using the primer pair Allium-F/Trifolium-R could be sequenced. A new primer pair (F: 5’-TTACCC CTGTTTGGCCACTTC-3’, R: 5’-AACTTCCGCTAAAAATGTGC-3’) was designed based on the sequence data. A partial cDNA of the PAL gene (368 bp) was then obtained by PCR using this primer pair and sequenced. The 3’-terminal was determined by the rapid amplification of cDNA ends (RACE) method using PolyT primer, 5’-GGCCACGCGTCGAATTCATGGAG-3’ and a PCR primer (F: 5’-CAATCACGGCTTCTGGAGAT-3’; R: 5’-GGCCACGCGTCGACTAGTACTTTTTTT-3’). The 5’-terminal was determined by the inverse PCR method. Subsequent to phosphorylation of the 5’-terminus of the 3’-terminal primer (5’-AACTTCCGTAAAAATGTGC-3’) with polynucleotide kinase (PNK, Takara Bio), a 5’-phosphorylated cDNA was obtained using this primer and a PrimeScript RT Reagent Kit (Perfect Real Time, Takara Bio). After this cDNA was cyclized (Ultra-Turrax T18 homogenizer, Kinematica Inc., Lucerne, Switzerland) for 1 min. After the homogenate was refluxed for 30 min, the filtrate was obtained; this procedure was repeated twice more. The combined filtrate was concentrated in vacuo and adjusted to pH 2–3, and then the phenolics were extracted with ethyl acetate. After evaporation to dryness, the phenolics were dissolved in 3 mL of methanol and determined using the Folin-Denis method.

Preparation and determination of phenolics  Methanol (50 mL) was added to a sample of mung bean sprouts (about 25 g) and homogenized (Ultra-Turrax T18 homogenizer, Kinematica Inc., Lucerne, Switzerland) for 1 min. After the homogenate was refluxed for 60 min, the filtrate was obtained; this procedure was repeated twice more. The combined filtrate was concentrated and adjusted to pH 2–3, and then the phenolics were eluted with ethyl acetate. After evaporation to dryness, the phenolics were dissolved in 3 mL of methanol and determined using the Folin-Denis method.

Preparation of mung bean sprout  Mung bean sprout (about 10 g) was immersed in warm water (50°C) for 60 s with shaking, and then cooled by cold water at 4°C. Each sample was wrapped in clear food-grade plastic film (Saran Wrap, Asahi Kasei, Tokyo) and stored at 8°C for 6 days.
mixture, and left for 30 min with stirring. After concentration, phenolics were extracted with ethyl acetate at pH 2–3. The ethyl acetate fraction was concentrated and used for HPLC analysis.

Results and Discussion

Cloning of PAL mRNA from mung bean sprout  A nucleotide sequence of 2,433 bp was obtained, which included an initiation codon ATG, a termination codon TAA, and an open reading frame (2,145 bp) encoding 715 amino acids (Fig. 1). The molecular mass was calculated as about 78 kDa. The amino acid sequence contained the highly conserved PAL motif (Tuan et al., 2010) including an active site (Ala-Ser-Gly), and showed high homology to PAL genes of Fabaceae plants (Table 1). These results indicated that the cloned gene was a mung bean PAL. The Ala-Ser-Gly segment is considered to be autocatalytically converted to 4-methylideneimidazole-5-one (Fig. 2-A; Baedecker and Schulz, 2002; Ritter and Schulz, 2004; Cooke et al., 2009) to show PAL activity. As the amino acid sequence of mung bean PAL showed 84.3% similarity to that of parsley PAL (Ritter and Schulz, 2004), the structure of mung bean PAL was considered to be similar to that of parsley PAL. Based on the structure of parsley PAL, a plausible structure of mung bean PAL was depicted using Swiss-Model Workspace (Biasini et al., 2014; Arnold et al., 2006) as shown in Fig. 2-B, which was drawn as a tetramer.

PAL expression during cold storage of mung bean sprout  In a preliminary experiment, we compared 18S rRNA, actin, and tubulin as internal quantification standards. Actin was selected as an internal standard because it formed a single product, the actin Ct value was the closest to that of PAL, and the change in the delta Ct value by dilution was the least among the three standards (data not shown). Kundu et al. (2013) showed that the expression of actin in Vigna mungo, a legume, was stable under the stress of virus and salinity, although they described that use of two or more normalizers was more reliable for qPCR quantification.

PAL expression of mung bean sprout was then examined during cold storage using qPCR (Fig. 3). The expression was increased during cold storage and was the highest at day 2. As the heat-shock treatment repressed the induction of PAL activity of mung bean sprout (Nishimura et al., 2012), the effect of heat-shock treatment on the expression of PAL mRNA was then examined (Fig. 4). It was clearly shown that the induction of PAL mRNA during cold storage was repressed by the heat-shock treatment. This result supports our previous report (Nishimura et al., 2012) showing that heat-shock treatment repressed the accumulation of phenolics and browning of mung bean sprout, by inhibiting the induction of PAL activity during cold storage. Although the heat-

Table 1. Sequence similarity of the PAL gene among several Fabaceae plants.

<table>
<thead>
<tr>
<th>Fabaceae plants</th>
<th>Similarity (%)</th>
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<tbody>
<tr>
<td></td>
<td>Nucleotide</td>
</tr>
<tr>
<td>Mung bean (Vigna radiata)</td>
<td>100 (2,433 bp)</td>
</tr>
<tr>
<td>Kidney bean (Phaseolus vulgaris)</td>
<td>94</td>
</tr>
<tr>
<td>Wild soy bean (Glycine soja)</td>
<td>90</td>
</tr>
<tr>
<td>Soy bean (Glycine max)</td>
<td>89</td>
</tr>
<tr>
<td>Locust tree (Robinia pseudoacacia)</td>
<td>88</td>
</tr>
<tr>
<td>Spiderwort (Trifolium pratense)</td>
<td>85</td>
</tr>
<tr>
<td>Bird’s foot trefoil (Lotus japonicus)</td>
<td>85</td>
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</table>
shock treatment of cut lettuce also repressed the accumulation of phenolics and browning during storage (Loaiza-Velarde et al., 1997; Murata et al., 2004), the treatment did not repress PAL expression but was speculated to inhibit the translation of PAL (Campos-Vargas et al., 2005; Tanaka et al., 2011). Although the reason and mechanism are unclear, heat-shock treatment seems to have influenced differentially the expression of PAL between cut lettuce and mung bean sprout. Salman et al. (2008) reported heat-shock reduced the level of PAL mRNA in sliced endive, similar to the case of mung bean sprout.

**Change in phenolics of mung bean during cold storage**  The amount of phenolics was increased from 10.6 ± 0.6 mg/100 g at day 0 to 25.8 ± 1.1 mg/100 g at day 7 during storage at 8°C, results that coincide with the data of Nishimura et al. (2012). Next, each phenolic was examined using HPLC. Figure 5 shows a typical HPLC profile of phenolics of mung bean sprout. Peaks A and B showed the absorption maxima at 295 and 325 nm, respectively, and peaks C and D showed the absorption maximum at 310 nm. From these UV spectra and in reference to the literature (Strack et al., 1985), peaks A, B, C, and D on the chromatogram were identified as cis-caffeoyltartronic, trans-caffeoyltartronic, cis-coumaroyltartronic, and trans-coumaroyltartronic acids,
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Fig. 5. A typical HPLC profile of mung bean sprout. A, cis-caffeoyltartronic acid; B, trans-caffeoyltartronic acid; C, cis-coumaroyltartronic acid; D, trans-coumaroyltartronic acid.

respectively. To ascertain this, trans-coumaroyltartronic and trans-caffeoyltartronic acids were prepared from trans-coumaric and trans-cafeic acids, respectively. Their spectra and retention times on the DAD-HPLC corresponded with those of mung bean phenolics.

The amount of each phenolic was next examined during cold storage (Fig 6). trans-Coumaroyltartronic acid, the major phenolic, and trans-caffeoyltartronic acid were increased during cold storage. Although the amount of trans-caffeoyltartronic acid was less than trans-coumaroyltartronic acid, the former appeared to also definitely contribute to browning, since, as an o-diphenol, this compound is a better substrate for PPO than mono-phenols such as trans-coumaroyltartronic acid. In fact, about 80% of trans-caffeoyltartronic and 60% of cis-caffeoyltartronic acids were decreased during incubation of a homogenate of mung bean sprout, while about 40% of trans-coumaroyltartronic and 20% of cis-coumaroyltartronic acids were decreased (data not shown). trans-Coumaroyltartronic and trans-caffeoyltartronic acids were decreased at day 6. This decrease might indicate that these phenolics were consumed during enzymatic browning. These results suggest that trans-caffeoyltartronic acid was the best substrate for mung bean PPO and that trans-caffeoyltartronic and trans-coumaroyltartronic acids mainly contributed to the browning. It remains unclear why all the phenolics did not increase in spite of the increase in PAL gene expression. The biosynthesis of each phenolic might be regulated differentially by different PAL isozymes or down-stream of PAL.

In general, PPO is located in plastids or chlorophylls (Murata et al., 1997), while its substrates, phenolics, are in vacuoles (Yamaki, 1994). Disruption of this compartmentation leads to the interaction between phenolics and oxidative enzymes and then to enzymatic browning. Further examination of the compartmentation is necessary to understand the browning of mung bean sprout.

In conclusion, PAL mRNA of mung bean sprout was cloned. Its expression was increased during cold storage, and heat-shock treatment repressed the expression. With the induction of PAL expression, PPO substrates contributing to browning were increased during cold storage. These results reveal that PAL expression and biosynthesis of phenolics are essential for enzymatic browning of mung bean sprout during cold storage.

References


