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Effect of additives on flavonoids, D-*chiro*-Inositol and trypsin inhibitor during the germination of tartary buckwheat seeds

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ABSTRACT

In this study, we evaluated tartary buckwheat during germination to provide an effective process that can lead to a rapid accumulation of both the total flavonoids and D-*chiro*-Inositol (DCI), as well as the elimination of trypsin inhibitor activity. At different concentrations of Al^{3+}, Cu^{2+}, and Zn^{2+}, we found significant differences of the total flavonoids and DCI accumulation, and we observed changes in phenylalanine ammonia-lyase (PAL), chalcone isomerase (CHI), and α-Galactosidase (α-Gal) activities in germinated tartary buckwheat ($p < 0.05$). Correlation analysis between these enzymes (PAL, CHI and α-Gal) activities and the total flavonoids and DCI contents revealed a significant correlation in germinated tartary buckwheat. The maximum accumulation of the total flavonoids and DCI were 1315.52 mg/100 g DW and 60.46 mg/g DW with the addition of Al^{3+}, 1315.41 and 63.59 with the addition of Cu^{2+}, and 1189.42 and 53.10 with the addition of Zn^{2+}. In these metal-treated samples, both total flavonoids and DCI were significantly higher than in the control ($p < 0.05$). Moreover, the metallic additives at the optimum concentration had no influence on total protein content, whereas a lower trypsin inhibitor activity was observed in correlation with higher protein digestibility.

1. Introduction

Tartary buckwheat grain is an important functional food material, containing proteins with high biological value and balanced amino acid composition (Bonafaccia et al., 2003; Ikeda et al., 1986), relatively high crude fiber, available zinc, copper, manganese, and dietary selenium (Stibilj et al., 2004). Tartary buckwheat also contains more flavonoids and D-*chiro*-Inositol (DCI) than common buckwheat (Fabjan et al., 2003).

Flavonoids are the most ordinary and widely distributed group of plant phenolic compounds. These secondary metabolites are derived from phenylalanine and acetate metabolism, and they perform a variety of essential functions in higher plants. Considerable attention has been given to flavonoids for their health benefits, since they possess antioxidant, antitumor, and anti-inflammatory properties (Verardo et al., 2010). Studies over the past 30 years have found that the conversion of L-phenylalanine to trans-cinnamic acid, which is catalyzed by phenylalanine ammonia-lyase (PAL), was the first step and a key step of the phenyl propanoid pathway. Similarly, chalcone isomerase (CHI) was also one of the key rate-limiting flavonoids and a biosynthetic enzyme (Kim et al., 2008).

DCI is another flavonoid, and tartary buckwheat is a vital natural source of DCI (Horbowicz et al., 1998). In its free form, DCI acts as a component of a putative mediator of insulin action with an insulin-like bioactivity. It works to increase the action of insulin and decrease blood pressure, plasma triglycerides, and glucose concentration. However, most DCI exists naturally not in its free form, but in the form of its galactosyl derivatives, named fagopyritols (chemically named α-D-galactopyranosyl-D-*chiro*-Inositols with one to three galactosyl moieties; Cid et al., 2004; Horbowicz et al., 1998). While fagopyritols failed to reduce symptoms of insulin-dependent diabetes mellitus and polycystic ovarian syndrome, these fagopyritols could be converted to free DCI during germination by the accumulation of α-Galactosidase (α-Gal) in vivo (Yang and Ren, 2008).

In spite of the presence of healthy DCI in tartary buckwheat, the grain's nutritional value is limited by the presence of antinutritional

Abbreviations: BAPNA, benzoyl-DL-arginine *p*-nitroanilide; CHI, chalcone isomerase; DCI, D-*chiro*-Inositol; α-Gal, α-Galactosidase; IU, inhibitor activity; PAL, phenylalanine ammonia-lyase; PNPG, *p*-nitrophenyl-α-D-galactopyranoside; TCA, trichloroacetic acid; TIA, trypsin-inhibitory activity; TN, nitrogen.

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factors, such as tannins, phenolic compounds, cellulose, and enzyme inhibitors (Ikeda and Kishida, 1993). Trypsin inhibitors, the dominant element of protease inhibition, are evenly distributed in buckwheat seeds; they inhibit trypsin and α-chymotrypsin activities significantly, but have inconspicuous effects on pepsin, papain, and ficin activities (Zhang et al., 2006).

Previous research has demonstrated that grain germination as a biochemical technique can effectively improve nutrients and reduce antinutrients in cereals and legumes (Randhir et al., 2004). However, there was only a limited increase in nutrient accumulation and antinutrient elimination in sprouts under normal steeping and germination conditions. In recent years, reports have shown that several nutrients, such as flavonoids, γ-aminobutyric acid, and some enzymes, increase to adapt to adverse environmental conditions, such as hypoxia, temperature stress, drought, or additive stress (Guo et al., 2012; Yang et al., 2011; Zielinska-Dawidziak and Siger, 2012). Past research of tartary buckwheat has not evaluated its germination under metallic additives stress, which may have the potential to improve the nutritional value of this grain.

In this study, the total flavonoids, DCI content accumulation, and trypsin inhibitor activity were analyzed in germinating tartary buckwheat seeds after the seeds were steeped with different additives, including $Al_2(SO_4)_3$, $CuSO_4$ and $ZnSO_4$, at different concentrations. We evaluated differential flavonoids, DCI accumulation, and antinutrient elimination of germinating tartary buckwheat seeds and sprouts which respond to different additives. This information provides a scientific basis for industrialized production of flavonoids, DCI-enriched tartary buckwheat, and high-protein tartary buckwheat.

2. Materials and methods

2.1. Materials and reagents

Dried tartary buckwheat seeds (Heifeng 1, obtained from Inner Mongolia Province, China in 2011) were stored at −20 °C before the experiments. We used analytical-grade reagents, including rutin, D-*chiro*-Inositol, L-phenylalanine, bull serum albumin, tetrahydroxychalcone, *p*-nitrophenyl-α-D-galactopyranoside (PNPG), trypsin, benzoyl-DL-arginine *p*-nitroanilide (BAPNA), and pepsin (Sigma Chemical Co., Shanghai, China).

2.2. Material treatments

Tartary buckwheat seeds were sterilized using 5% (v:v) of sodium hypochlorite for 30 min, washed, and steeped in distilled water or different metallic salt solutions at 25 ± 1 °C for 8 h. Before germination, soaked seeds were spread thinly on a pallet (30 cm in width × 40 cm in length × 5 cm in height) with two layers of sterilized moist gauze, and then covered by one layer of moist gauze. Distilled water was applied every 8 h for seed germination. After germination, the fresh seed tissues were collected carefully and washed with distilled water to measure PAL, CHI, and α-Gal activity. For other assays in this study, both non-germinated and germinated seeds were dried at 50 °C for 8 h, ground with a laboratory mill, and passed through a 40-mesh screen sieve. Three replications were tested for each treatment, and the experiments were repeated three times.

2.3. Determination of total flavonoids

The total flavonoid content was performed according to the procedure of Stockova et al. (2009), with a slight modification. Approximately 200 mg of the sample was immersed in 30 mL of methanol/water (7:3, *v*/*v*) solution and kept in a stoppered 100-mL conical flask with shaking at 65 °C for 6 h; it was then centrifuged at 4000 rpm for 30 min. The supernatant was diluted with 70% methanol solution to 50 mL. Then 1 mL of the extract sample was transferred to a 10-mL volumetric flask, and 2 mL of 0.1 M aluminum chloride hexahydrate solution and 3 mL of 1 M potassium acetate solution were added. The mixture was diluted with 70% methanol solution to 10 mL, mixed well, and kept in the dark for 30 min. The treated extract sample was mixed using a vortex mixer, and the absorbance was measured at 420 nm. A standard curve was plotted using rutin as a standard (0.0500 mg/mL rutin in methanol), producing the linear regression equation ($R^2 = 0.9997$):

$$\text{Absorbance} = 29.935\ \text{rutin(mg/mL)} - 0.001$$

Total flavonoid content was expressed as rutin equivalents.

2.4. Determination of DCI

The DCI content was performed according to the procedure of Yang and Ren (2008), with a slight modification. All samples were dried in an oven at 40 °C until the weight was constant. The extraction was performed by mixing 3 g of sample with 30 mL of ethanol/water (1:1, *v*/*v*) solution in a stoppered 100-mL conical flask by shaking at 25 °C for 2 h. Next, the homogenate was centrifuged at 8000 rpm for 20 min at 20 °C. One milliliter of the supernatant was put in a vial and dried in an oven at 40 °C until the solution was completely dried. The sample was re-dissolved in1mL of methanol, filtered through a 0.45-μm Millex-HX syringe filter (13 mm) (Bedford, MA), and stored in a HPLC auto-sampler vial for immediate HPLC analysis (Yang and Ren, 2008).

HPLC analysis was conducted on a Waters-600 high-performance liquid chromatography system coupled with RID (Waters, USA). A NH_2 5 μm column (4.6 × 250 mm, Waters, USA) was used. The chromatographic separation was performed using acetonitrile/distilled water (4:1, *v*/*v*), and the flow rate was set at 1 mL/min. All analyses were performed in triplicate in three independent samples.

2.5. Assay on PAL activity

Fresh seed tissues (1.0 g) were ground with mortar and pestle at 0 °C in 10 mL of 0.05 mol/L sodium borate buffer (pH 8.8) containing 5 mmol/Lβ-mercaptoethanol, 3% PVP, and a little silicon dioxide. Homogenates were centrifuged at 10,000× *g* for 20 min at 4 °C (Centrifuge 5804R, Eppendorf, Germany). The supernatants were stored at −4 °C and used as a source of crude enzyme.

The PAL activity in crude enzyme extracts was assayed using a modified version of McCallum and Walker's (1990) method. The assay mixture consisted of 2 mL 0.05 mol/L sodium borate buffer (pH 8.8) and 0.2 mL of crude enzyme, and the reaction was initiated by the addition of 1 mL 0.02 mol/L L-phenylalanine. The reaction was conducted at 30 °C for 1 h. Cinnamic acid yield was estimated by measuring the increase of absorbance at 290 nm (A290) of the supernatant. Increase of 0.1 of A290 per hour is defined as one unit of PAL enzymatic activity. Triplicate assays were performed for each extract.

2.6. Assay on CHI activity

Fresh seed tissues (1.0 g) were ground with mortar and pestle at 0 °C in 10 mL of 0.05 mol/L sodium phosphate buffer (pH 7.8) containing 0.05 mol/L ascorbic acid, 0.018 mol/L β-mercaptoethanol, and a little silicon dioxide. Homogenates were centrifuged at 10,000× g for 20 min at 4 °C (Centrifuge 5804R, Eppendorf, Germany). The supernatants were stored at −4 °C and used as a source of crude enzyme.