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# Characterization of an acidic α-galactosidase from hemp (*Cannabis sativa* L.) seeds and its application in removal of raffinose family oligosaccharides (RFOs)

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**An acidic α-galactosidase designated as hemp seed α-galactosidase (HSG) was purified from hemp (*Cannabis sativa* L.) seeds. By means of chromatographic procedures which involved chromatography on the cation-exchangers CM-cellulose and SP-Sepharose, chromatography on the anion-exchangers DEAE-cellulose and Q-Sepharose, and gel filtration on Superdex 75 using fast protein liquid chromatography, HSG was purified to electrophoretic homogeneity. Results of SDS-PAGE and gel filtration on FPLC Superdex 75 revealed that the enzyme was a monomeric protein with a molecular weight of 38 kDa. Sequences of the inner peptides of the α-galactosidase obtained by MALDI-TOF-MS showed that HSG was a novel α-galactosidase since there was a little similarity to the majority of α-galactosidases recorded in the literature. A pH of 3.0 and a temperature of 50°C were optimal for the activity of the enzyme. The activity of HSG was inhibited by the chemical modification with N-bromosuccinimide (NBS) reagent. HSG contained 16 tryptophan residues and two tryptophan residues on the surface, which were crucial to the α-galactosidase activity. The heavy metal ions $Cd^{2+}$, $Cu^{2+}$, $Hg^{2+}$ and $Zn^{2+}$ inhibited its activity. The $K_m$ and $V_{max}$ for the hydrolysis of pNPGal (4-nitrophenyl α-D-galactopyranoside) were respectively 0.008 mM and 68 μM $min^{-1}$ $mg^{-1}$. HSG also catalyzed the hydrolysis of raffinose and other natural substrates. Hence the α-galactosidase possesses a tremendous potential for food and feed industries in the elimination of indigestible oligosaccharides from leguminous products.**

**Key words**: α-galactosidases; *Cannabis sativa* L.; purification; raffinose family oligosaccharides

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**Abbreviations**: HSG, hemp seed α-galactosidase; FPLC, fast protein liquid chromatography; MALDI-TOF-MS, Matrix-Assisted Laser Desorption/ Ionization Time of Flight Mass Spectrometry; NBS, N-bromosuccinimide; pNPGal, 4-nitrophenyl α-D-galactopyranoside; RFOs, raffinose family oligosaccharides; DEPC, diethyl pyrocarbonate; DTT, dithiothreitol; EDC, carbodiimide; DIC, diacetyl; TNBS, 2, 4, 6-trinitrophenol

## INTRODUCTION

Hemp (*Cannabis sativa* L.), an annual herbaceous plant, was grown as a fiber plant and an important food plant in ancient times (Li, 1974). Hemp seeds have an enormous number of possible applications, ranging from medical therapy to use in the textile industry. Hemp seeds display an abundant content of oil, protein, carbohydrates, insoluble fiber, β-carotene, antioxidant vitamins and minerals (Callaway, 2004). Selective breeding generated cannabis plants which can be employed for specific purposes, including high-potency marijuana strains and hemp cultivars for fiber and seed production (van Bakel *et al.*, 2011). Cultivation of the marijuana strains has been banned because they contain high levels of tetrahydrocannabinol (THC), which is a psychoactive compound. Generally speaking, almost all other strains of hemp plants contain THC, albeit at a concentration much lower than that of marijuana strains. Recent research focused on the activity of compounds extracted from the high-THC strains (Appendino *et al.*, 2008) and essential oils of industrial hemp varieties (Nissen *et al.*, 2010) which display anti-microbial activity. The presence of abundant protein and other essential nutrients in hemp seeds indicates that they might be a new good source of nutrients for human beings (Galasso *et al.*, 2016).

The oligosaccharides raffinose and stachyose, also referred to as raffinose family oligosaccharides (RFOs), accumulate in the large intestine of humans and other monogastric animals deficient in α-galactosidase. This enzyme catalyzes the cleavage of the α-1,6 galactosyl linkage in these oligosaccharides. The consequent accumulation of intact oligosaccharides in the large intestine results in microbial fermentation and then flatus formation (Steggerda, 1968). Purification and characterization of α-galactosidases (α-D-galactoside galactohydrolase, EC 3.2.1.22) which are widely distributed in microorganisms, plants, and animals was reported (Ramalingam *et al.*, 2007; Wang *et al.*, 2010; Singh & Kayastha, 2012). α-galactosidases exist in a diversity of plant seeds such as sunflower seeds, white chickpea and soybean (Porter *et al.*, 1991; Kim *et al.*, 2003; Singh & Kayastha, 2012). The main function of α-galactosidases in plants is to provide the energy during the seed development stage since α-galactosidases can hydrolyze oligosaccharides (raffinose and stachyose) and polysaccharides to galactose. This hydrolytic ability is mainly employed in the sugar industry to improve sucrose crystallization by hydrolysing raffinose in beet sugar syrups (Patil *et al.*, 2010; Ferreira *et al.*, 2011). Despite its hydrolytic activity, α-galactosidase is also used for oligosaccharide synthesis by transglycosylation (Goulas *et al.*, 2009). Fabry disease is an X-linked lysosomal storage disorder caused by mutations in the α-galactosidase A gene (GLA), and the disease

relatively often results in idiopathic hypertrophic cardiomyopathy which mimics the left ventricular hypertrophy (Andreotti *et al*., 2011; Nakagawa *et al*., 2011).

Hemp seeds are widely available and rich in protein, and represent an inexpensive source of α-galactosidase. However, there are no published reports as yet. We herein report the purification and characterization of an acidic α-galactosidase (HSG) from fiber hemp (*Cannabis sativa* L.) seeds using chromatographic procedures, which entailed cation-exchangers CM-cellulose and SP-Sepharose, anion-exchangers DEAE-cellulose and Q-Sepharose, and gel filtration on Superdex 75 using fast protein liquid chromatography. The properties of HSG could lay the foundation for using this enzyme in RFOs hydrolysis.

## MATERIALS AND METHODS

**Materials**. Hemp seeds were bought in a market in Yunnan Province, China. DEAE-cellulose and CM-cellulose were products of Sigma Chemical Company, USA while SP-Sepharose and Q-Sepharose were products of GE Healthcare, USA. Superdex 75 HR 10/30 and AKTA Purifier were from GE Healthcare, USA. The substrates, 4-nitrophenyl α-D-galactopyranoside (pNPGal), guar gum, locust bean gum, melibiose, raffinose and stachyose were from Sigma. All other chemicals used were of analytical grade.

**Assay of α-galactosidase activity**. The standard assays of α-galactosidase activity were executed following the pNPGal method as previously detailed (Rezende *et al*., 2005) with some modifications. The reaction mixture, composed of 50 µl extract containing the enzyme and 50 µl 10 mM pNPGal (pH 3.6), was incubated at 50°C for 15 min, then 0.5 M sodium carbonate (400 µl) was added to stop the reaction. The amount of p-nitrophenol released was determined by measuring the absorbance at 405 nm. When other galacto-oligosaccharides (raffinose and stachyose) and polysaccharides (locust bean gum and guar gum) were used as the substrates, the amount of the formed reducing sugar was determined using 3, 5-dinitrosalicylic acid method (Miller, 1959). A glucose oxidase method kit (GOD-POD) was utilized to measure enzyme activity in the presence of melibiose. One unit of α-galactosidase activity was defined as the amount of enzyme that produced 1 µmol of p-nitrophenol from galactose/glucose per minute. The enzyme activity was expressed as specific activity (units/mg of protein). Each reaction and the corresponding controls were performed in triplicate. Data represent means ± S.D. (n=3).

**Purification of α-galactosidase from hemp seeds**. The hemp seeds (50 g) were washed and homogenized in saline (500 mL) by using a Waring blender. After overnight extraction at 4°C, the seed homogenate was centrifuged (12 000 × *g*) for 10 min. Then $(NH_4)_2SO_4$ was added to the supernatant to 80% saturation. Five hours later, the mixture was centrifuged (10 000 × *g*) for 10 min. The precipitate was dissolved in distilled water and dialyzed to eliminate $(NH_4)_2SO_4$ prior to chromatography on a DEAE-cellulose column (2.5 cm × 20 cm) which had previously been equilibrated with 10 mM Tris-HCl buffer (pH 8.6). Fractions D1, D2, D3 and D4 were eluted sequentially with 0 mM, 100 mM, 200 mM and 1 M NaCl in the starting buffer. α-galactosidase activity was found in fraction D2 which was dialyzed against distilled water and then chromatographed on a column (2.5 cm × 10 cm) of CM-cellulose equilibrated with 10 mM NaAc-HAc buffer (pH 4.0). Unadsorbed proteins were eluted with the starting buffer while adsorbed proteins were desorbed by addition of 100 mM, 300 mM and 1 M NaCl successively in the starting buffer. After the dialysis, the fraction (CM3) with activity was chromatographed on a column of Q-Sepharose (0.5 cm × 20 cm) previously equilibrated with 10 mM Tris-HCl buffer (pH 8.6). After the removal of unadsorbed proteins with the starting buffer, the column was eluted with 100 mM NaCl in the starting buffer to yield the active fraction (Q2). It was then further separated by ion exchange chromatography on an SP-Sepharose column (0.5 cm × 10 cm). After unadsorbed proteins (fraction SP1) all came off the column in 10 mM NaAc-HAc buffer (pH 4.6), the column was eluted with a linear gradient of 0–300 mM NaCl in the same buffer. The active peak (SP2) was finally chromatographed on a Superdex G-75 HR10/30 column by fast protein liquid chromatography using an AKTA Purifier (GE Healthcare, US)

**Determination of molecular mass of the isolated α-galactosidase**. SDS-PAGE of the isolated α-galactosidase was conducted in a 12% acrylamide gel at pH 8.8 using 25 mM Tris-glycine buffer containing 0.1% (w/v) SDS as reported before (Laemmli&Favre, 1973). The gels were stained in 0.1% (w/v) Coomassie blue, 30% (v/v) methanol and 10% (v/v) acetic acid in water. Electrophoretic mobility of the isolated α-galactosidase was compared to that of PageRuler Unstained Protein Ladder (Thermo Fisher) and the molecular mass was estimated from the calibration curve of electrophoretic mobility against molecular mass. The gels were then destained in the above-mentioned solution without Coomassie blue. The molecular weight was also estimated by using gel filtration on an FPLC Superdex 75 HR10/30 column (GE Healthcare).

**Analysis of the amino acid sequence of the isolated α-galactosidase**. The isolated α-galactosidase was digested with trypsin and subjected to analysis by MALDI-TOF-MS. Some high-quality peptides were subsequently analyzed by ESI-MS/MS. Amino acid sequences of the inner peptides were obtained using MALDI-TOF-MS and ESI-MS/MS.

**Biochemical properties of the isolated α-galactosidase**. Biochemical properties of HSG was determined according to the method of Zhang *et al*. (2015) with slight modifications, including the effects of pH, temperature, metal ions and chemical modification reagents on the enzyme activity.

The optimal pH for the enzyme was assayed at 50°C in the pH range from 2.0 to 8.0 in 100 mM $Na_2HPO_4$-citric acid buffer. To determine pH stability the enzyme was incubated at room temperature for 1 h in buffers of various pH values. The residual α-galactosidase activity was determined using the aforementioned enzyme assay conditions.

The activity of the isolated enzyme at the optimum pH was determined in the temperature range from 4 to 60°C. The optimal temperature for the enzyme could thus be ascertained. The temperature stability of the purified α-galactosidase was measured after incubation at different temperatures for 30 min and subsequent determination of the residual α-galactosidase activity under the standard conditions.

The influence of different concentrations (1.25, 2.5, 5, and 10 mM) of chloride salts of the metal ions ($Mg^{2+}$, $Hg^{2+}$, $Mn^{2+}$, $Pb^{2+}$, $Fe^{2+}$, $Fe^{3+}$, $Ca^{2+}$, $Cu^{2+}$, $K^{+}$, $Cd^{2+}$, $Zn^{2+}$, $Al^{3+}$) on the isolated α-galactosidase activity was exam-

**Table 1. Summary of purification procedure of hemp seeds (from 50 g hemp seeds)**

| Purification step | Yield (mg) | Total activity (U)[a] | Specific activity (U/mg)[b] | Recovery rate (%) | Purification fold[c] |
|---|---|---|---|---|---|
| Crude extract | 233.2 | 12400 | 53.1 | 100 | 1 |
| Ammonium sulfate precipitate | 76 | 9049.3 | 119.0 | 72.9 | 2.2 |
| D2 | 24.7 | 4935.0 | 199.8 | 39.7 | 3.7 |
| CM3 | 5.29 | 3051.9 | 576.9 | 24.6 | 10.8 |
| Q2 | 2.37 | 1828.5 | 771.5 | 14.7 | 14.5 |
| SP2 | 1.27 | 1131.4 | 890.8 | 9.1 | 16.7 |
| SU1 | 0.128 | 182.8 | 1428.5 | 1.4 | 26.8 |

[a]Total activity: α-galactosidase activity (U/mL) in each step × Volume (mL); [b]Specific activity: Total activity/Yield; [c]Purification fold: Specific activity of each step/Specific activity of the first step.

ined. The effects of chemical modification reagents comprising N-bromosuccinimide (NBS), diethyl pyrocarbonate (DEPC), dithiothreitol (DTT), carbodiimide (EDC), diacetyl (DIC) and 2, 4, 6-trinitrophenol (TNBS) on α-galactosidase activity were also investigated. The enzyme was incubated with various metal ions and chemical modification reagents at 4°C for 2 hours and then the residual α-galactosidase activity was assayed. The activity of the enzyme without any additive was taken as 100%.

Each reaction and the corresponding controls were performed in triplicate.

**Determination of tryptophan residues number in the isolated α-galactosidase.** NBS was dissolved in 10 mM NaAc-HAc buffer (pH 4.5). The number of modified tryptophan residues was determined spectrophotometrically according to the method of Spande using the following equation (Spande & Witkop, 1967):

$$n = \Delta A \times 1.31 \times M_r \times V/(W \times 5500)$$

where: ΔA is the difference in absorbance between the native and the tryptophan-modified enzyme.1.31 is an empirical factor, 5500 is the molar extinction coefficient for Trp ($M^{-1}$ $cm^{-1}$), Mr is the molecular mass of the isolated α-galactosidase, and V is the reaction volume.

For modifying the tryptophan residues in the interior of the enzyme molecule, the enzyme solution mixed with 8 M urea was boiled for 5 min. Then different quantities of NBS (4 mM) were added to modify the mixture until the absorbance at 280 nm reached the minimum value. The total number of tryptophan residues was calculated using the abovementioned formula. For determination of the tryptophan residues on the isolated α-galactosidase surface, a similar method was used except for the treatment with urea (Du *et al.*, 2013).

**Substrate specificity and kinetic studies**. For ascertaining the substrate specificity of the isolated α-galactosidase, the activity of the enzyme in the presence of different types of substrate, encompassing the synthetic substrate pNPGal, oligosaccharides (raffinose, melibiose, stachyose) and galactomannans (locust bean gum and guar gum) was determined in accordance with the methods described above.

The $K_m$ and $V_{max}$ of purified α-galactosidase were determined using substrates pNPGal (1–10 mM) and raffinose (50–300 mM) in 0.1 M $Na_2HPO_4$-citric acid buffer at 30°C with the pNPGal method or DNS method. The apparent Michaelis constant ($K_m$) and $V_{max}$ were calculated from the Lineweaver-Burk plot which was created using the initial rates obtained at various substrate concentrations and constant amount of the enzyme.

**Statistical analysis**. The data were analyzed by SPSS version 24.0 for Windows (SPSS Inc., Chicago, IL, USA). Results were represented as the mean ± standard deviation (S.D.).

## RESULTS

### Isolation of hemp seed α-galactosidase and determination of its molecular mass and inner-peptide sequence

HSG was purified from hemp seeds by employing a protocol that comprised, sequentially, precipitation with 80% saturated ammonium sulfate, and chromatographic separation on anion exchanger DEAE-cellulose, cation exchanger CM-cellulose, anion exchanger Q-Sepharose, and cation exchanger SP-Sepharose. α-galactosidase activity was found in peaks D2, CM3, Q2, and SP2 (Table 1). The purified fraction SU1 was obtained by size exclusion chromatography of SP2 on a Superdex G-75 HR 10/30 column (Fig. 1). The results of the purification of hemp seed α-galactosidase were summarized in Table 1. HSG displayed a molecular mass of 38 kDa as estimated by SDS-PAGE (Fig. 2) and gel filtration (Fig. 1). The amino acid sequences of three inner pep-

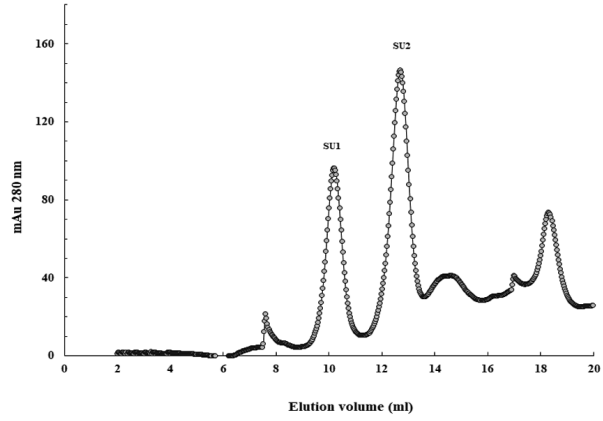


**Figure 1. Gel filtration of SP2 on a Superdex 75 HR10/30 column.**
The molecular mass of SU1 was 38 kDa.

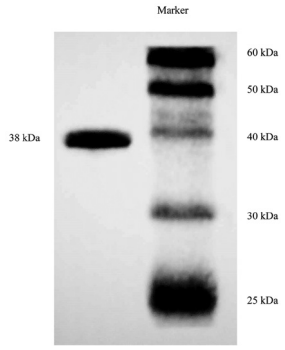


**Figure 2. SDS-PAGE results.**
**Left lane**: fraction SU1 representing purified hemp seed α-galactosidase. **Right lane**: molecular mass standard. The molecular mass of SU1 was 38 kDa.

tides as determined by ESI-MS/MS were LFGVTTL-DVVR, TQDGGTEVVEAK and DDLFNINAGIVK.

## Physiochemical properties of purified hemp seed α-galactosidase

Hemp seed α-galactosidase demonstrated optimal activity at pH 3.0 (Fig. 3A). It was stable only at pH 2-4 (Fig. 3B). There was an abrupt fall in activity (about 60% reduction) when the pH reached 5. No activity could be detected in alkaline pH. The optimum temperature for HSG activity was 50°C (Fig. 3C), but about 65% of the initial activity was lost after incubation at 50°C for 60 min (Fig. 3D). The metal ions $Cd^{2+}$, $Cu^{2+}$, $Hg^{2+}$ and $Zn^{2+}$ strongly inhibited the activity of HSG, whereas $Mg^{2+}$ and $Pb^{2+}$ ions partially inhibited the enzyme activity in this study (Table 2). The effects of six chemical modification reagents (NBS, DEPC, DTT, EDC, DIC, TNBS) on the activity of hemp seed α-galactosidase were tested. Only NBS had an obvious effect. After incubation with 0.1 mM NBS for 30 min, the enzyme lost all α-galactosidase activity (Fig. 4). When 90 µl NBS was added to the completely denatured enzyme, A280nm reached the minimum value which demonstrated that the tryptophan residues

**Table 2. Effects of metal ions on activity of hemp seed α-galactosidase (results represent mean ± S.D., n=3)**

| Metal ion concentration | Relative galactosidase activity (%) | | | |
|---|---|---|---|---|
| | 10 mM | 5 mM | 2.5 mM | 1.25 mM |
| $Fe^{2+}$ | 102±0.02 | 97±0.01 | 102±0.01 | 77±0.02 |
| $K^{+}$ | 100±0 | 89±0.01 | 83±0.04 | 84±0.01 |
| $Ca^{2+}$ | 99±0.03 | 76±0.01 | 93±0.02 | 97±0 |
| $Cd^{2+}$ | 0±0 | 4±0.01 | 5±0.01 | 11±0 |
| $Cu^{2+}$ | 0±0 | 1±0.01 | 4±0.01 | 2±0.01 |
| $Hg^{2+}$ | 0±0.01 | 2±0.01 | 0±0.01 | 4±0.01 |
| $Mg^{2+}$ | 71±0.01 | 58±0.05 | 64±0.01 | 85±0.02 |
| $Mn^{2+}$ | 111±0.09 | 108±0.01 | 76±0.01 | 69±0.01 |
| $Pb^{2+}$ | 76±0.02 | 42±0.01 | 30±0 | 43±0.01 |
| $Zn^{2+}$ | 8±0.01 | 6±0 | 26±0 | 32±0.01 |
| $Al^{3+}$ | 136±0 | 121±0.02 | 112±0 | 122±0.01 |
| $Fe^{3+}$ | 128±0.01 | 103±0.1 | 45±0.3 | 78±0.01 |

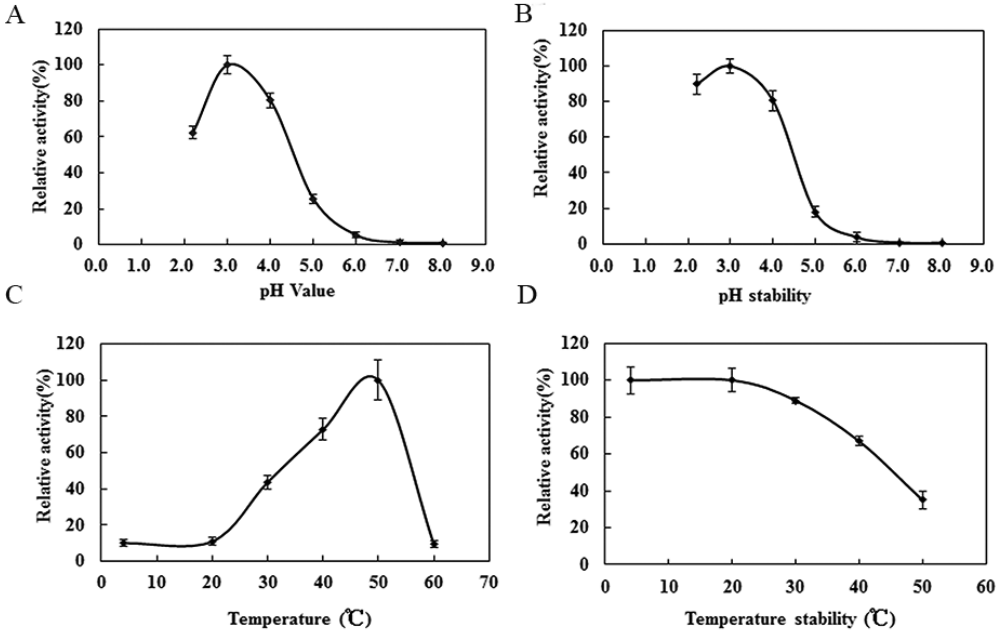


**Figure 3. Characterization of the enzymatic properties of HSG.**
(**A**) Effect of pH on α-galactosidase activity was determined at 50°C in buffers ranging from pH 2.0 to 8.0. The activity obtained at pH 3.0 was taken as 100%. (**B**) pH stability of α-galactosidase activity was determined by measuring α-galactosidase activity under standard assay conditions (pNPGal) after pre-incubation of the enzyme at room temperature for 60 min in buffers with pH ranging from 2.0 to 8.0. The activity of an enzyme sample at pH 3.0 was defined as 100%. (**C**) Effect of temperature on α-galactosidase activity was determined at 4–60°C. The activity obtained at 50°C was taken as 100%. (**D**) Thermostability of α-galactosidase activity was determined after pre-incubation of the enzyme at different temperatures ranging from 4–50°C for 60 min. The activity of enzyme sample under 4°C was defined as 100%. Data represent means ± S.D. (n=3).

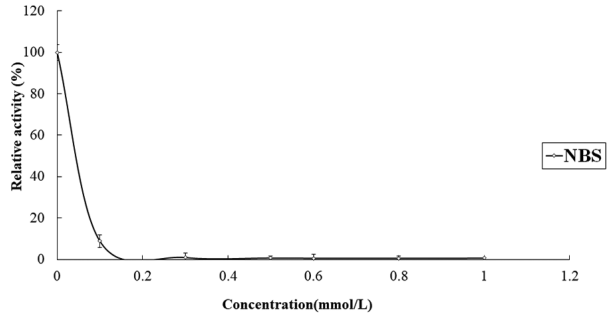


**Figure 4. Effect of chemical modification agent NBS on hemp seed α-galactosidase activity.**
The data were reproducible among the experiment replicates. Data represent means ± S.D. (n=3). NBS, N-bromo-succinamide

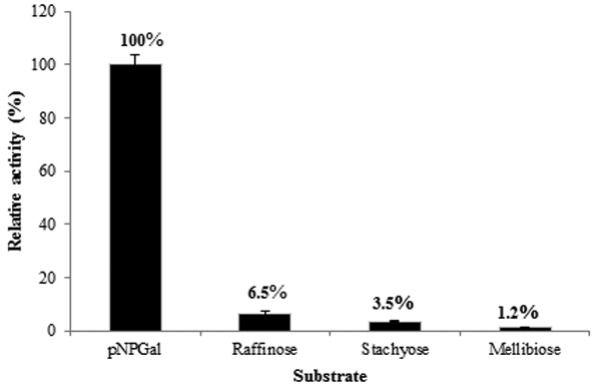


**Figure 5. Hydrolysis of natural and synthetic substrates by HSG.**
Data represent means ± S.D. (n=3).

were modified completely. The total number of tryptophan residues was calculated by the formula. HSG contained 16 tryptophan residues. The different concentrations of NBS modifying the enzyme solution can be used to calculate the number of tryptophan residues on the HSG surface. Our analysis revealed that there are two tryptophan residues on the HSG surface. Hemp seed α-galactosidase exhibited the highest specificity toward pNPGal. As compared to pNPGal (100%), the enzyme manifested lower activity toward raffinose (6.3%), stachyose (3.5%) and melibiose (1.2%) (Fig. 5). The $K_m$ and $V_{max}$ values for hemp seed α-galactosidase were 0.008mM and 68 μM $min^{-1}$ $mg^{-1}$ for the hydrolysis of pNPGal and 0.16 mM and 4.3 μmol $min^{-1}$ $mg^{-1}$ for the hydrolysis of raffinose, respectively.

## DISCUSSION

HSG was purified from hemp seeds by a series of purification steps. The specific activity of α-galactosidase toward pNPGal after 26.8-fold purification was 1428.5 U/mg with a final yield of 1.4% which exceeded those of α-galactosidases from *Bispora* sp. MEY-1 (Wang *et al.*, 2010), *Penicillium purpurogenum* (Shibuya *et al.*, 1998) and white chickpea (Singh&Kayastha, 2012).

It was a monomeric protein with a molecular mass of 38 kDa that resembled the α-galactosidases from germinating lentil (*Lens culinaris*) seeds (40 kDa) (Celem *et al.*, 2009) and *Pichia pastoris* (41 kDa) (Gao *et al.*, 2003). Nevertheless, some α-galactosidases have a substantially larger molecular mass, such as α-galactosidases from *Aspergillus terreus* (108 kDa) (Shankar *et al.*, 2009) and *Pleurotus florida* (99 kDa) (Ramalingam *et al.*, 2007).

The amino acid sequences of three inner peptides were determined by ESI-MS/MS. The sequences were LFGVTTLDVVR, TQDGGTEVVEAK and DDLFNINAGIVK which exhibited some homology with α-galactosidases from other species, with the highest extent of homology (64%) with α-galactosidase from *Treponema azotonutricium* (not shown). There is a little identity with other α-galactosidases, suggesting that hemp seed α-galactosidase is a new enzyme.

It is crucial to understand the properties of this enzyme, as it can be used in production. Using pNPGal as the substrate, the activity of HSG reached its maximum at pH 3.0 and the enzyme was stable only at acidic conditions. This was typical for α-galactosidases which usually have optima at acidic pH values. Many fungal α-galactosidases such as those from *Rhizopus* sp. demonstrated an optimum pH of 4.8 (Cao *et al.*, 2009). α-galactosidase of the fungus *Thermomyces lanuginosus* displayed a pH optimum of 5–5.5 (Rezessy-Szabo *et al.*, 2007). So did the α-galactosidase from the white-rot fungus *Pleurotus florida* (Ramalingam *et al.*, 2007).

HSG exhibited maximum activity at 50°C, about 65% of the initial activity was lost after incubation at 50°C for 60 min, which was analogous to the α-galactosidases from *Penicillium* sp. F63 CGMCC1669 and *Rhizopus* sp. F78 ACCC 30795 which showed low temperature optima at 40 and 50°C, respectively, as well as low thermostability (Mi *et al.*, 2007; Cao *et al.*, 2009).

The metal ions $Cd^{2+}$, $Cu^{2+}$, $Hg^{2+}$ and $Zn^{2+}$ strongly inhibited the activity of HSG, whereas $Mg^{2+}$ and $Pb^{2+}$ ions partially inhibited the enzyme activity in this study. Shankar reported that $Ag^{+}$, $Hg^{2+}$ and $Cu^{2+}$ ions completely eliminated the activity of a thermostable α-galactosidase from *Aspergillus terreus* (GR) strain (Shankar *et al.*, 2009). α-galactosidase from *Cucurbita pepo* leaves was partially inhibited by $Ca^{2+}$, $Mg^{2+}$ and $Mn^{2+}$ ions, more so by $Ni^{2+}$, $Zn^{2+}$ and $Co^{2+}$ ions, and severely inhibited by $Cu^{2+}$, $Ag^{+}$ and $Hg^{2+}$ ions (Gaudreault&Webb, 1983). Thus, α-galactosidases from different sources are similar in their inhibition by $Hg^{2+}$ and $Cu^{2+}$ ions.

Among the six chemical modification reagents, only NBS had an obvious effect on hemp seed α-galactosidase. NBS is a reagent modifying the tryptophan in the protein. Incubation of the enzyme with 0.1 mM NBS for 30 min resulted in total loss of α-galactosidase activity, signifying that tryptophan is an amino acid essential to the enzyme activity. The results were reminiscent of similar data on α-galactosidase from germinating coffee beans (Shen & Jin, 2011).

HSG contained 16 tryptophan residues including and two tryptophan residues on its surface. NBS-modification resulted in the formation of oxidized surface tryptophan residues that did not relocate to the hydrophobic interior. These results suggest that native surface tryptophan residues play a pivotal role in the activity of HSG. Thus, one can assume that these surface tryptophan residues reside in the active site. The other 14 tryptophan residues may be located on the interior of the hemp seed α-galactosidase molecule. There are several studies on the detection of tryptophan residues on enzymes by NBS modification. Teng reported that hyaluronidase contained 11 tryptophan residues, only one of which was essential for the activity of the enzyme (Teng *et al.*, 2006). There are 17 tryptophan residues on inulinase and 2 of them play a pivotal role in the enzymatic activity (Liu *et al.*, 2007).

HSG showed the highest specificity for pNPGal. Generally, α-galactosidase exhibited higher activity with the synthetic substrate (pNPGal) compared to natural substrates (melibiose, raffinose and stachyose) (Ferreira *et al*., 2011), HSG did not act on the polysaccharides, locust bean gum and guar gum. According to their substrate specificities, α-galactosidases can be divided into two groups (Dey *et al*., 1993). The first group contains α-galactosidases active only on oligosaccharides with a low degree of polymerization, for example melibiose, raffinose and stachyose. The second group of α-galactosidases consists of enzymes active on polymeric substrates and short oligosaccharides (Comfort *et al*., 2007). The inability of HSG to act on polymeric substrates such as locust bean gum and guar gum was possibly due to the large, multimeric structure of α-galactosidase which restricts the accessibility of polymeric substrates to the enzyme active site.

The Michaelis constant for pNPGal determined in the kinetic experiments was considerably lower than the corresponding value for raffinose; however, it is evident that synthetic substrates are hydrolyzed more efficiently than natural galacto-oligosaccharides by most α-galactosidases.

## CONCLUSION

In this study, an acidic α-galactosidase from hemp (*Cannabis sativa* L.) seeds was purified and characterized for the first time. The molecular mass of the hemp seed α-galactosidase as determined by SDS-PAGE and gel-filtration was 38 kDa. The isolated α-galactosidase demonstrated pronounced activity and stability at acidic pH values, which makes it valuable for applications at these pH values. The α-galactosidase contained 16 tryptophan residues with two of them located on the surface and playing a key role in the activity of the enzyme. The purified enzyme could efficiently hydrolyze natural substrates such as raffinose. The results suggest that hemp seed α-galactosidase has tremendous potential in the beet sugar and food and feed industries for the elimination of indigestible oligosaccharides from legumes.

### Declaration of interest

We declare that we have no conflict of interest.

### Author Contributions

H. Wang designed the study. G. Tian and Y. Zhao prepared materials and reagents. W. Zhang and F. Du performed the experiment. T. B. Ng wrote the manuscript. All authors reviewed the manuscript.

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