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Purification and characterization of chalcone isomerase from fresh-cut Chinese water-chestnut

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ABSTRACT

Chalcone isomerase (CHI) from fresh-cut Chinese water-chestnut was isolated and purified by precipitation with ammonium sulfate (35%–80%), sequential chromatography using DEAE-cellulose (DE-52) and Sephadex™ G-100. The purified CHI was obtained with a 12.35-fold purity, a yield of 6.86% and a specific activity of 125 U/mg·proteins. The molecular weight was estimated to approximately 14.4 kDa with sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The spectral and HPLC analysis demonstrated that the end enzymatic product was naringenin. Further analysis based on enzymatic characterization indicated the optimal temperature was 45 °C, as well as pH was 7.5. The CHI activity was significantly stimulated by Ca^{2+} and Cu^{2+}, but inhibited by Zn^{2+}, Fe^{2+} and Na^{+}. A significant active effect of DTT, Triton X-100 and PVP on the enzyme activity was observed, while a negative correlation was found between the CHI activity and the chemical compounds of β-mercaptoethanol, SDS and Tween 80. In addition, EDTA, ascorbic acid, sodium ascorbate and citric acid presented no significant ($p > 0.05$) influence on CHI activity. Finally, the K_m (2.91 mM) and V_{max} (45.25 U/mL) for naringenin chalcone were obtained from the Lineweaver-Burk plot. Therefore, the properties of CHI could contribute to controlling the yellowing of fresh-cut CWC.

1. Introduction

The Chinese water-chestnut (CWC, *Eleocharis luberose*) is a hydrophytic vegetable widely consumed in China, due to its peculiar taste, health benefits and high nutrient value (Pan, Li, & Yuan, 2015; Peng & Jiang, 2003). Already, peeled CWC (also named fresh-cut CWC) are also commercially available (Pradas-Baena, Moreno-Rojas, & Luque de Castro, 2015, chap. 1). However, the fresh-cut CWC has a reduced commercial and shelf life compared to the corresponding unpeeled one, as the peeled CWC always turns yellow with the extension of storage time (Oms-Oliu et al., 2010; You et al., 2012). The phenomenon that the fresh-cut CWC always turns yellow is different from enzymatic browning and the yellowing pigments in the yellowing of fresh-cut CWC were mainly flavonoids (Pan & Chen, 2007, 2008). Naringenin and eriodictyol have been isolated, purified and identified as the primary flavonoids that found in the yellowing components in substrate from the yellowing fresh-cut CWC (Pan et al., 2015).

Flavonoids including naringenin and eriodictyol are synthesized via the phenylpropnaoid pathway, and most of the enzymes involved in the flavanoids biosynthetic pathway have been characterized (Cheng et al., 2011; Forkmann, 1991; Grace & Logan, 2000; Tohge, Watanabe, Hoefgen, & Fernie, 2013; Winkel-Shirley, 2001). Chalcone isomerase (CHI) is one of the critical enzymes that mainly catalyzes the isomerization of chalcone naringenin (2′, 4, 4′, 6′-terahydroxychalcone) into naringenin (5, 7, 4'-trihydroxyflavanone) that is one of the essential yellowing pigments in the yellow fresh-cut CWC (Pan et al., 2015). Moreover, CHI also presents stereoisomerism and strong pH-dependence. Jez and Noel (2002) proved that the catalytic cyclization activity of CHI for chalcone naringenin, 4, 2′, 4'-trihydroxychalcone and 2′, 4′-dihydroxychalcone reached more than 90% but not for 4, 2′-dihydroxychalcone at pH 7.5. In contrast, the catalytic cyclization activity of CHI for chalcone naringenin and 4, 2′, 4′-trihydroxychalcone was only about 50% and even lower for 2′, 4′-dihydroxychalcone and 4, 2′-dihydroxychalcone at pH 6.0.

In previous study, some researchers have reported that the present of CHI was not only restricted to the plant kingdom (Cheng et al., 2011; Dixon, Dey, & Whitehead, 1982; Jez & Noel, 2002; Jez, Bowman, Dixon, & Noel, 2000; Kang et al., 2014; Zhou et al.,

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2014), but also was found in microorganisms (Dixon et al., 1982; Gensheimer, 2004; Herles, Braune, & Blaut, 2004). In addition, the structure and reaction mechanism of CHI have also been investigated (Jez & Noel, 2002; Jez et al., 2000), whereas limited or even elusive information is available relating to CHI from the fresh-cut CWC and the relationship between CHI and the yellowing CWC.

In present research, the isolation and purification of CHI from fresh-cut CWC was principally studied and the end enzymatic product was analyzed. In addition, the optimal activity temperature and pH values, the thermal and pH stability, the effects of metal ions and chemical reagents and the corresponding kinetic parameters were also determined. This study would be conducive to clarify the mechanism of fresh-cut CWC yellowing and understand the relationship between the yellowing of fresh-cut CWC and CHI, and then to control the yellowing of fresh-cut CWCs.

2. Materials and methods

2.1. Materials and chemicals

The CWCs were obtained from a local market at Haikou, Hainan province, China. They were firstly pre-cooled for 24 h at 4–6 °C after back to the laboratory. The fresh CWCs with uniform size and without physical damage or visual defects were selected and immediately refrigerated at −80 °C after peeling and cutting.

DEAE-cellulose (DE-52) was purchased from Shanghai Yuanye Bio-Technology Co., Ltd (Shanghai, China). Sephadex™ G-100, Tris (hydroxymethyl) aminomethane (Tris), DL-dithiothreitol (DTT), and polyvinyl pyrrolidone (PVP) were purchased from Solarbio Science & Technology Co., Ltd (Beijing, China). HPLC-grade methanol and acetonitrile was purchased from Tedia company, Inc. (Ohio, USA). β-Mercaptoethanol (β-ME) was purchased from Amresco Inc. (USA). All the other chemicals in this paper were of analytical or biochemical grade and were obtained from China.

2.2. Extraction of the crude enzyme

The crude CHI was extracted using the method described previously by Lister and Lancaster (1996) with some modifications. The frozen CWC sample (500 g) was blended in 1000 mL of ice-cold sodium phosphate buffer (50 mM, pH 7.5) containing 5% w/v PVP, 50 mM sodium ascorbate, 10 mM DTT and 0.1% v/v Triton X-100 and then homogenized with a Waring Blender (800S, USA). The homogenate was filtered with eight layers of degreasing gauze. The filtrate was then centrifuged at 12, 000 *g* for 20 min. The supernatant was collected as the crude CHI. All the extracting steps were conducted at 4 °C.

2.3. Purification procedures

Solid ammonium sulfate was added to the crude CHI until 35% of saturation was reached. The solution was then rested at 4 °C for 4 h and then centrifuged at 12, 000 *g* for 20 min to collect supernatant. Subsequently more solid ammonium sulfate was added to the supernatant reaching a final saturation of 80%. The solution was stored overnight at 4 °C and next centrifuged at 12, 000 *g* for 30 min to achieve precipitated fraction, which was dissolved in a minimum volume of Tris-HCl buffer (50 mM, pH 7.5), and then dialyzed overnight against the same buffer to remove salts. The solution was then concentrated by freeze-drying.

The dialyzed sample (4 mL) was deposited on a DEAE-cellulose column (2.5 cm × 20 cm) packed with DE-52 and previously equilibrated with Tris-HCl buffer (20 mM, pH 7.5). Unbound proteins were washed using the same buffer at a flow rate of 1 mL/min. The bound proteins were eluted with a linear increasing gradient of NaCl prepared in same buffer (ranging from 0 to 0.3 M) at same flow rate. The proteins were monitored at 280 nm using an on-line UV detector (HD-3, Nanjing University, China) and the peak fractions were collected to assay the CHI activity. Fractions that revealed the highest CHI activity were pooled, and the pooled fraction was dialyzed against the same buffer overnight and concentrated by freeze-drying. The concentrate was subsequently dissolved in a minimum volume of Tris-HCl buffer (50 mM, pH 7.5), loaded onto a column (1.0 cm × 50 cm) of Sephadex G-100 gel, previously equilibrated with the same buffer, and similarly eluted using the same buffer at a flow rate of 0.4 mL/min. The proteins were monitored at 280 nm using an on-line UV detector and the peak fractions were collected to assay the CHI activity. Fractions that exhibited highest enzyme activity were pooled and dialyzed against Tris-HCl buffer (50 mM, pH 7.5) then concentrated by freeze-drying to finally achieve purified CHI. One part of the purified CHI was used for sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) to determine the purity and the relative molecular mass using marker (14–80 kDa) as standard proteins, another part was used for the characterization of the enzymatic properties.

2.4. SDS-PAGE electrophoresis

The purified CHI was subjected to SDS-PAGE using 12% cross-linked gel (15 mA) and 5% stacking gel (10 mA) according to the method described by Laemmli (1970). The samples were denaturated in boiling water for 5 min and then cooled to room temperature. 10 μL of the samples and the molecular mass markers (*ProteinRuler*® I, 12–80 kDa) was used for the electrophoretic analysis. After electrophoresis, the gel was fixed using a mixture of methanol/acetic acid/water (5:1:4) for 30 min at room temperature, then stained with Coomassie brilliant blue R-250 (0.5 g) dissolved in 500 mL of methanol/acetic acid/water (3:1:6) solvent for 40 min at room temperature, followed by de-staining in the same solvent without Coomassie brilliant blue R-250 at room temperature. The washing step was repeated five times.

2.5. Assay for enzyme and protein concentrations determination

The activity of CHI was assayed according to the procedures described by Lister and Lancaster (1996) and Zhao (2014) with some modifications. The reaction mixtures containing the enzyme (200 μL), 2 mL of Tris-HCl (50 mM, pH 7.5) containing 7.5 mg/mL bovine albumin and 50 mM $NaHSO_3$ were thoroughly mixed. The reaction was initiated by adding naringenin chalcone dissolved in 2-ethoxy-ethanol (50 μL of a solution at 1 mg/mL), and then incubated at 45 °C for 30 min. One unit of CHI activity was defined as the amount of enzyme that caused the decrease of the absorption value at 381 nm of 0.01 in 1 h under the experimental conditions.

The method of Coomassie brilliant blue G-250 described by Bradford (1976) was used to estimate the protein content of CHI with bovine serum albumin as the standard protein.

2.6. Analysis of the reaction products

The reaction mixture (5 mL) as prepared above in the section **"**Assay for enzyme**"** was extracted with ethyl acetate (5 mL) three times (Pan et al., 2015) and the products were identified by spectral analysis of the wavelength ranging from 600 nm to 200 nm (Kuhn, Forkmann & Seyffert, 1978) and high performance liquid chromatography (HPLC, Waters e2695, USA) with a Betasil C_{18} column (4.6 mm × 250 mm, 5 μm). The chromatographic conditions were the same as previously described with the following modifications.