Food Chemistry 150 (2014) 9–16

Contents lists available at ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem

Laccase–gum Arabic conjugate for preparation of water-soluble oligomer of catechin with enhanced antioxidant activity

Swati B. Jadhav, Rekha S. Singhal *

Food Engineering and Technology Department, Institute of Chemical Technology, Nathalal Parekh Marg, Matunga, Mumbai 400 019, India

ARTICLE INFO

Article history:
Received 11 August 2013
Received in revised form 6 October 2013
Accepted 23 October 2013
Available online 1 November 2013

Keywords:
Antioxidant
Catechin
Conjugation
Gum Arabic
Laccase
Oligomerisation

ABSTRACT

Catechin was oligomerized using free laccase and laccase-gum Arabic conjugate. The process of oligomerization was optimized with respect to solvent, ratio of solvent to buffer (0.2:10 to 1:10), pH of buffer (3–10), enzyme (575–18,400 U/mg) and substrate concentration (1–7 mM). Maximum production of oligomer was observed in methanol at ratio 0.6:10 of methanol:buffer of pH 5 using 2300 U/mg of laccase and 5 mM of catechin. The laccase-gum Arabic conjugate showed lower activity but higher stability than free laccase in methanol. Free laccase produced cross linked water-insoluble oligomer, whereas conjugated laccase produced linear water-soluble oligomer. The linear water-soluble oligomer showed higher antioxidant activity, as determined by the DPPH assay, and reducing power as compared to monomer making it suitable for biological applications. The molecular weight of the linear oligomer was found to be 13.14 kDa, which suggested it to be composed of 45 monomer units. Further characterizations of linear and cross linked oligomer were done using FTIR and differential scanning calorimetry.

1. Introduction

Catechin is a flavanoid found in green tea and wine. Flavanoids are among the best studied plant polyphenols and consist of large group of low molecular weight polyphenolic substances occurring in fruits and vegetables. They are well known for their biological and pharmacological effects including antioxidant, antimutagenic, anti-carcinogenic, antiviral, and anti-inflammatory properties (Jankun, Selman, Swiercz, & Skrzypczak-Jankun, 1997; Nakagawa et al., 1999). Applications of flavanoids became limited due to many reasons such as being restricted for few hours in the body, poor solubility in water, and their ability to act as pro-oxidant which in turn generates reactive oxygen species (Rice-Evans, Miller, & Paganga, 1996). These limitations can be overcome by increasing molecular weight of the polyphenols. High molecular weight polyphenols have been reported to exhibit enhanced biological properties with longer circulation time *in vivo* (Hagerman et al., 1998).

Many investigators have attempted to polymerize catechin as an approach to increase the molecular weight to enhance its biological properties. Amplification of antioxidant properties of catechin by polycondensation with acetaldehyde has been successfully done (Chung, Kurisawa, Kim, Uyama, & Kobayashi, 2004). Oxidative oligomerisation of epicatechin has been carried out using horseradish peroxidase (HRP) yielding water soluble oligomer exhibiting anticancer activity (Nagarajan et al., 2008). HRP catalyzes the synthesis of various polymeric flavonoids including poly-(catechin) in a mixture of buffer and 1,4-dioxane (Mejias, Reihmann, Sepulveda-Boza, & Ritter, 2002).

Both laccase and peroxidase can be used for the polymerization of catechin. Laccase has shown many advantages over peroxidase in catalyzing a variety of substrates. In particular, it can use molecular oxygen instead of hydrogen peroxide for polymerization reactions. Laccase has applications in medical diagnosis, pharmaceutical industry, agriculture, preparation of anticancer drugs, cosmetics, and preparation of polymers of valuable compounds (Couto & Herrera, 2006). Laccases (benzenediol: oxygen oxidoreductase, EC 1.10.3.2) are multi-copper-containing enzymes which reduce molecular oxygen to water and simultaneously perform one electron oxidation of various substrates such as diphenols, methoxy-substituted monophenols, and aromatic and aliphatic amines (Kudanga, Nyanhongo, Guebitz, & Burton, 2011). Laccases have been reported to catalyze the oligomerisation or polymerization of many phenolic compounds which show an increased antioxidant capacity (Kurisawa, Chung, Uyama, & Kobayashi, 2003). Laccase has been used to prepare oligomers of catechin (Osman, Wong, & Fernyhough, 2007), and polymers that are insoluble in water but soluble in organic solvents such as dimethyl formamide and which show amplified antioxidant activity. The water-insoluble nature of such polymers restricts their application (Kurisawa et al., 2003). Hence, there is need to develop a controlled polymerization process to prepare water soluble polymer of catechin with enhanced antioxidant activity.

* Corresponding author. Tel.: +91 22 33612501; fax: +91 22 33611020.
E-mail address: rs.singhal@ictmumbai.edu.in (R.S. Singhal).

0308-8146/$ - see front matter © 2013 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.foodchem.2013.10.127

Enzyme–polysaccharide interactions are well known in the literature. These interactions can be covalent (Gomez, Ramirez, & Villalonga, 2000) or non-covalent (Li et al., 2010). Conjugation of polysaccharide cause structural changes to the enzyme and affects its thermal and pH stability (Jadhav & Singhal, 2012). Enzyme-polysaccharide interactions alter the enzyme activity and stability depending on the nature and structure of the enzyme and polysaccharides. The use of such enzyme-polysaccharide conjugates may affect the oligomerization ability of the enzyme which needs to be evaluated in detail.

In this work, we have conjugated laccase with gum Arabic using a previously reported method. Free and conjugated laccase were used for the oligomerization of catechin, whereas the process of oligomerization was optimized with respect to solvent, ratio of solvent to buffer, pH of buffer, substrate and enzyme concentration. Oligomers prepared under optimized conditions were evaluated for antioxidant activity and reducing power. Further characterization was done by size exclusion chromatography, FTIR and differential scanning calorimetry (DSC).

2. Materials and methods

2.1. Materials

Catechin and laccase (11,500 U/mg) from *Trametes versicolor* were purchased from Sigma Aldrich, Mumbai, India. Gum Arabic was purchased from Himedia, Mumbai, India. Sodium metaperiodate and sodium borohydride were obtained from S. D. Fine Chemicals, Mumbai. All other chemicals were of AR grade and procured from reliable sources.

2.2. Preparation of laccase-gum Arabic conjugate

Sodium metaperiodate (0.1 M) solution was prepared in 0.1 M sodium acetate buffer of pH 5.0 and used as the oxidizing solution. Gum Arabic (16 mg/ml) was oxidized in 10 ml of oxidizing solution in dark for 90 min, after which the oxidation was stopped by adding 0.3 ml of ethylene glycol, and kept for 1 h in dark. Oxidized gum Arabic solution was dialyzed against 0.1 M sodium acetate buffer of pH 5.0 at 4 °C overnight. Laccase solution (protein concentration of 1.6 mg/ml) was prepared in 20 mM sodium citrate buffer of pH 5.0 and mixed with equal volume of gum Arabic solution and kept for conjugate formation for 20 h at room temperature (~28 ± 2 °C) as reported in our previous work (Jadhav & Singhal, 2012). Sodium borohydride (20 mg) was then added to 10 ml of conjugate mixture to reduce remaining oxidized sites of gum Arabic and kept for 4 h. Finally, the prepared conjugate solution was dialyzed against 20 mM of sodium citrate buffer of pH 5 at 4 °C overnight (Ahmed, Saleh, & Abdel-Fattah, 2007; Villalonga, Gomez, Ramirez, & Villalonga, 1999). This conjugate was used for analyzing the activity and stability of laccase.

2.3. Laccase activity assay

The assay mixture for test consisted of 0.05 M sodium citrate buffer of pH 5.0 (110 μl), enzyme solution (25 μl, appropriately diluted) and the reaction was started immediately by addition of 0.216 mM syringaldazine in absolute methanol (15 μl). The oxidation of syringaldazine was followed for 2 min by measuring the absorbance at 530 nm. The assay mixture for the blank consisted of 0.05 M sodium citrate buffer of pH 5.0 (110 μl), distilled water (25 μl) and syringaldazine 0.216 mM in absolute methanol (15 μl). Extinction coefficient (ε) of the oxidation reaction was 65 mM^{-1} cm^{-1} (Elsayed, Hassan, Elshafei, Haroun, & Othaman, 2012). One unit of enzyme activity was defined as the micromole of substrate oxidized per min per ml of enzyme solution at room temperature and pH 5.0. The protein in the sample was analysed using Folin–Lowry method (Lowry, Rosebrough, Farr, & Randall, 1951) and it was calculated using bovine serum albumin as a standard in the range of 0.2–1.0 mg/ml.

2.4. Selection of solvent for enzymatic oligomerization of catechin using free laccase and laccase-gum Arabic conjugate

Catechin monomer substrate (5 mM) was mixed with free and conjugated laccase (2300 U/mg) in a reaction mixture. The reaction mixture contained organic solvent (acetonitrile, methanol, ethylene glycol and dimethyl sulphoxide) and sodium citrate buffer of pH 5 (20 mM) in a ratio of 0.6:10. The reaction was carried out for 30 min at room temperature. The reaction mixtures were then centrifuged at 8000 rpm for 10 min. Free laccase produced water insoluble oligomer which precipitated out from the solution whereas conjugated laccase produced water soluble oligomer. The water insoluble oligomer precipitate was dissolved in 1 N NaOH and diluted appropriately with distilled water. Similarly, the water soluble oligomer was also diluted appropriately. The absorbance of both oligomers was measured at 418 nm to determine the amount of compounds formed.

2.5. Activity and stability of free and conjugated laccase in various concentration of methanol

Laccase-gum Arabic conjugate was prepared as described in section 2.2. Free and conjugated laccase were checked for activity in methanol (10–80%). The activity assay was performed in methanol as described in Section 2.3. The activity of laccase in buffer was considered as the initial activity. The percent activity in methanol was calculated using the activity in the buffer as reference. The stability of the free and conjugated laccase in methanol was analysed. Both forms of laccase (20 μg/ml) were incubated in methanol (20–100%) for 30 min at room temperature. The residual activity was determined using the procedure described in Section 2.3. The activity of laccase in buffer without incubation in methanol was considered as the reference activity to calculate the percent remaining activity.

2.6. Optimization of process for the oligomerization of catechin using free and conjugated laccase

Oligomerization of catechin was carried out using free and conjugated laccase as described in Section 2.4. The process of oligomerization was optimized for the ratio of methanol to buffer (0.2:10–1:10), pH of the buffer used (3–10), enzyme (575–18,400 U/mg) and concentration of catechin (1–7 mM). The prepared oligomers by free and conjugated laccase were analysed by measuring absorbance at 418 nm.

2.7. Determination of antioxidant activity of monomer and oligomer of catechin by DPPH radical scavenging ability

Monomer and water soluble oligomer prepared by conjugated laccase were evaluated for their ability to scavenge 2,2-diphenyl-1- picrylhydrazyl (DPPH) stable free radicals using method of Yamaguchi, Takamura, Matoba, and Terao, (1998). Different concentrations of compounds (7.6–45.6 μM) were prepared in methanol. The reaction mixture consisted of sample solutions (25 μl) and 0.3 mM DPPH solution (175 μl), left to stand at room temperature for 30 min in the dark. The absorbance was measured at 517 nm. The control was distilled water. The inhibition percentage (%) of the radical scavenging activity was calculated according to the following equation: