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ORIGINAL ARTICLE

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Purification and characterization of polyphenol oxidase from Henry chestnuts (*Castanea henryi*)

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Abstract Polyphenol oxidase from *Castanea henryi* nuts was partially purified. Some characteristics of the enzyme were then determined to help devise measures for the prevention of undesirable enzymatic browning during storage and processing of the nuts. Preparation of acetone powder extracts of the nuts, ammonium sulfate precipitation, dialysis, and gel filtration resulted in 37-fold purification of the enzyme with a yield of 13%. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis and the excitation spectrum confirmed the enzyme preparation to be homogeneous. The approximate molecular weight of the enzyme was determined by gel filtration to be 69kD. The enzyme catalyzed the oxidation of catechol and pyrogallic acid as substrates but did not affect cresol or tyrosine. Using catechol as substrate, *p*-nitrophenol, thiourea, orcinol, and naphthol showed strong inhibition. The optimal pH and temperature for the enzyme were pH 5.0 and 40°C, respectively. The enzyme proved heat labile. When the enzyme was incubated at 70°C for 30min, the remaining activity of the enzyme was only 8%. Possible approaches to applying the results of this study to the prevention of enzymatic browning in the production of *C. henryi* nuts is also discussed.

Key words Polyphenol oxidase · Enzymatic browning · *Castanea henryi* nuts · Purification · Inhibition

Introduction

The Henry chestnut [*Castanea henryi* (Skan) Rehd. et Wils] is widely distributed in eastern and southwestern China. The tree has been planted more than ever before in these areas in recent years. The nut of *C. henryi* is one of the well-known dry fruits in southern China, and is used in traditional Chinese medicine as a tonic for the stomach and kidneys.[1,2] It is rich in protein (6.2%), lipids (2.8%), carbohydrates (64.7%), and ascorbic acid (36mg/100g) with a unique flavor and good taste.[3] However, browning of nut products during storage and processing has prevented it entering wider circulation in domestic and foreign markets.

One of the main reasons for the browning reaction of plant tissues is thought to be the result of phenolics in the plant tissue being oxidized to quinones by the polyphenol oxidase (EC 1.10.3.1) after mechanical injury or in the discordant metabolism of the tissues.[4–7] The general properties of phenol oxidases from plant sources have been reviewed, and more research on the browning reactions of plant tissues are reported.[8–11] In recent years, research into the browning of pears, litchis, bananas, and grapes has been conducted in China;[12–15] however, our knowledge of the mechanism of browning of *C. henryi* nuts is still limited. Therefore, an investigation of the properties of the enzyme is necessary if undesirable enzymatic browning during the storage and processing of *C. henryi* nuts is to be prevented.

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Materials and methods

Fresh nuts of *Castanea henryi* were collected from a plantation in the northern part of Fujian province, where most of the trees were intensively cultivated.

Sephadex G-100, catechol, protein markers of cytochrome C, ovalbumin, human gamma globulin, bovine serum albumin, phosphorylase A, and α-chymotrypsin were supplied by Sigma. β-Mercaptoethanol was obtained from Fluka, and 2,4-dihydroxyacetophenone was obtained from Tokyo Kasei. The other chemicals used in this study were of analytical reagent grade and were available in the laboratory.

Enzyme isolation and purity test

A sliced fresh sample was fully macerated in cold acetone (1:2, w/v) and kept at −15°C overnight. The sample was then agitated in a Waring blender for 20s and filtered under vacuum at room temperature to yield the acetone powder. The acetone powder was blended for 1 min in cooled 0.025 mol/l potassium phosphate buffer (pH 7.0) and the suspension was filtered through a G6 glass filter under vacuum. The filtrate was then centrifuged at 14 000*g* for 20 min at 0°C. The resultant supernatant, which contained enzyme activity, was preserved as crude enzyme.

An equal volume of cold acetone was added to the supernatant, and the mixture was stirred slowly for 30 min at 4°C. After centrifugation at 16 000*g* for 20 min at 0°C, the precipitate was dissolved in 0.025 mol/l potassium phosphate buffer (pH 7.0). Ammonium sulfate crystals were then added to the solution to 80% saturation with slow stirring. The mixture was kept overnight at 4°C and then centrifuged at 16 000*g* for 20 min at 0°C. The precipitate was isolated and dissolved in a minimal volume of 0.025 mol/l potassium phosphate buffer (pH 7.0), and then dialyzed for 12 h against 0.01 mol/l Tris-HCl buffer (pH 8.0) with three solution changes. The dialyzed solution was preserved as partially purified polyphenol oxidase for further determinations.

The protein content of the enzyme preparation at each stage was estimated by the method of Lowry et al.[16] using casein as standard. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) using 7.5% polyacrylamide gel was conducted following the method of Laemmli.[17] Electrophoresis was performed with tubes measuring 10 × 0.6 cm, and run at 15 V/cm for 2.5 h at 15°C. Gels were stained with Coomassie brilliant blue. The excitation spectrum of the protein band detected to have enzyme activity was measured by a Hitachi 650-10S fluorescence spectrophotometer (Excitation wavelength 280 nm).

Assay of enzyme activity

The enzyme activity of polyphenol oxidase from *C. henryi* nuts was determined according to the method described by Benjamin and Montgomery.[18] A total mixture volume of 3 ml contained 0.1 ml of the enzyme preparation, 1.9 ml of 0.025 mol/l potassium phosphate (pH 7.0), and 1.0 ml of 0.09 mol/l catechol as substrate. The change in absorbance at 420 nm was monitored at 40°C at 30-s intervals. One unit of enzyme activity was defined as the amount of enzyme that caused an increase in OD of 0.001 per minute. The increase in absorbance was linear with time for the first 120 s.

Substrate specificity

Catechol (420 nm), pyrogallic acid (334 nm), *p*-cresol (400 nm), and L-tyrosine (472 nm) solutions at 3.0 mmol/l final concentration were tested as possible substrates for the polyphenol oxidase from *C. henryi* nuts. The assay wavelengths were selected as described by Benjamin and Montgomery.[18]

The relative activities of the enzyme in the presence of ten compounds as possible inhibitors were compared to determine the effects of inhibitors. The changes of the enzyme activity when using *p*-nitrophenol and thiourea as inhibitors were further determined at different concentrations. The kinetic constants K_m and V_{max} for *p*-nitrophenol and thiourea inhibitors were obtained from the Lineweaver-Burk double reciprocal plot, and the inhibitor constants K_i for *p*-nitrophenol and thiourea were calculated from Eqs. 1 and 2, respectively.

$$K_i = \frac{I}{\left[\left(K'_m/K_m\right) - 1\right]} \tag{1}$$

$$K_i = \frac{I}{\left[\left(K_m/K'_m\right) - 1\right]} \tag{2}$$

where I is inhibitor concentration and K'_m is the apparent K_m.

Optimal temperature and heat inactivation

Reaction mixtures containing 3.3 mmol/l catechol were incubated for 10 min at different temperatures ranging from 30°C to 60°C, after which 0.02 ml of enzyme was added to initiate the reaction. The optimal temperature for the activity of the polyphenol oxidase from *C. henryi* nuts was obtained by comparing the enzyme activities recorded at different temperatures.

The activity of enzyme incubated for intervals in the range of 10–40 min was measured over the temperature range of 40°–70°C. The enzyme activity at the optimal temperature was compared with those at the different temperatures to determine the heat inactivity of the enzyme.

Optimal pH and pH stability

The enzyme activity was determined over the pH range of 4.0–8.0. The pH value corresponding to the highest enzyme activity was taken as the optimal pH. To determine the pH stability of the polyphenol oxidase, the reaction mixtures were kept for 24 h at different pH values at 21°C. The enzyme activities were then compared with that at the optimal pH 5.0. Potassium phosphate buffer (pH 7.0) and catechol substrate were used in the determination.

Estimation of molecular weight

The molecular weight (MW) of the polyphenol oxidase from *C. henryi* nuts was estimated by gel filtration chromatography on a Sephadex G-100 (column 25 mm i.d. × 500 mm) with protein markers of cytochrome C (MW 12 400), human gamma globulin (MW 16 500), α-chymotrypsin (MW 25 000), ovalbumin (MW 45 000), bovine serum albumin (MW 68 000), and phosphorylase a

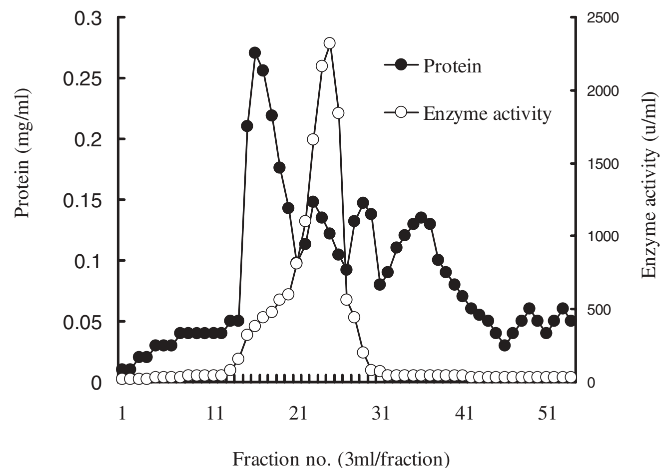

Fig. 1. Eluent profile of polyphenol oxidase on Sephadex G-100

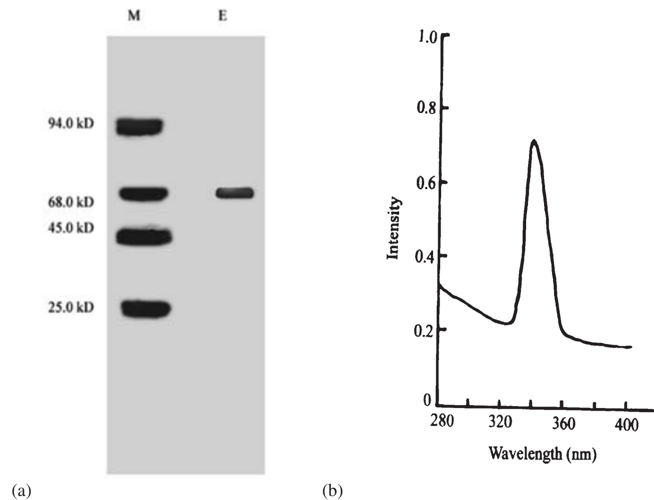

Fig. 2. **a** Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of the partially purified polyphenol oxidase from *Castanea henryi* nuts. *Lane M*, protein markers; *lane E*, enzyme preparation. Sample size 0.05 ml. **b** Excitation spectrum at 280 nm

(MW 94 000). Blue dextran 2000 (MW 2×10^6) was used to determine the void volume (V_0) eluting with 0.025 mol/l KH_2PO_4–NaCl buffer (pH 7.0) at a flow rate of 0.25 ml/min. The protein markers were chromatographed and the elution volume (V_e) was measured. The distribution coefficient (K_{av}) was given by Eq. 3.

$$K_{av} = (V_e - V_0)(V_t - V_0)^{-1} \tag{3}$$

where V_t stands for the total bed volume. The calibration curve was obtained by plotting log MW versus K_{av}. The MW for the polyphenol oxidase could be obtained from the curve by extrapolation or from the linear regression of Log MW = 5.251 − 3.872 K_{av}, ($r^2 = -0.991$).

Statistical analysis

All data obtained in this investigation were analyzed by the χ^2-test and linear regression analysis.

Results and discussion

Preparative procedure of the enzyme

Common treatment of the acetone powder was conducted as an initial preparation. The ammonium sulfate precipitation was used to remove high molecular weight proteins and sugars and to concentrate the sample. After fractionation on the Sephadex G-100 column, a peak of polyphenol oxidase activity was found. The elution profile of the polyphenol oxidase from *Castanea henryi* nuts on Sephadex G-100 is illustrated in Fig. 1. Using this procedure fourfold and 14-fold purification were achieved at the acetone powder step, and the ammonium sulfate precipitation, respectively. Table 1 shows that the purification of the peak fraction on Sephadex G-100 was about 37-fold with a yield of about 13%. The content of total protein decreased 285-fold in this process, and the total activity of the enzyme decreased by 7.7 times. Although a lot of enzyme was lost in the preparative process to obtain the acetone powder or in the buffer extraction, the initial preparation of the acetone powder effectively eliminated most of the colored matter from the sample and helped the next assay from the enzyme activity.

Figure 2 shows a single band of protein that corresponded with the active band in the SDS-PAGE. There was also a single excitation peak at 340 nm in the fluorescence spectrum with the excitation wavelength set at 280 nm. These observations confirmed the enzyme preparation to be homogeneous.

Substrate specificity

Catechol, pyrogallic acid, cresol, and tyrosine were tested as possible substrates for the polyphenol oxidase from *C. henryi* nuts. As shown in Table 2, the enzyme catalyzed the oxidation of catechol and pyrogallic acid, but had no effect on cresol or tyrosine. This suggests that the enzyme lacks cresolase activity. The apparent K_m and V_{max} for catechol as substrate were 14 mmol/l and 222 U/ml, and those for pyrogallic acid were 16 mmol/l and 148 U/ml, respectively. Judging from the K_m and V_{max} values, catechol is better than pyrogallic acid as a natural substrate.

Effect of temperature on the enzyme activity

The polyphenol oxidase from *C. henryi* nuts was found to have maximum activity at 40°C. This result is very similar to reports on the polyphenol oxidase of most plant sources.[19–21] Thermostability of the polyphenol oxidase from *C. henryi* nuts is shown in Fig. 3. After separate mixtures were incubated at 40°C for 10 min and 30 min, the enzyme activity decreased by about 5% and 10%, respectively. However, the remaining activity decreased to only 8% if the

Table 1. Partial purification of polyphenol oxidase from *Castanea henryi* nuts

Purification steps	Total protein (mg)	Total activity (U, $\times 10^3$)	Specific activity (U/mg)	Purification (fold)	Yield (%)
Buffer extract of acetone powder	3875.28	321.49	82.96	1.00	100.00
Solution from acetone precipitation	867.20	288.00	332.10	4.00	89.70
Dialyzed solution of 80% $(NH_4)_2SO_4$	111.11	131.20	1180.81	14.20	40.80
Peak fraction from GPC	13.60	41.60	3058.82	36.87	12.94

GPC, gel permeation chromatography on Sephadex G-100

Table 2. Determination of substrate specificity of polyphenol oxidase from *Castanea henryi* nuts

Substrates	Wavelength (nm)	Activity ($\times 10^3$ U/mg protein)	K_m (mmol/l)	V_{max} ($\times 10^3$ U/ml)
Catechol	420	43.00	14.30	222.00
Pyrogallic acid	334	36.00	16.40	148.00
Cresol	400	–	–	–
Tyrosine	472	–	–	–

Kinetic constants K_m and V_{max} were obtained from Lineweaver-Burk plots of initial velocity data

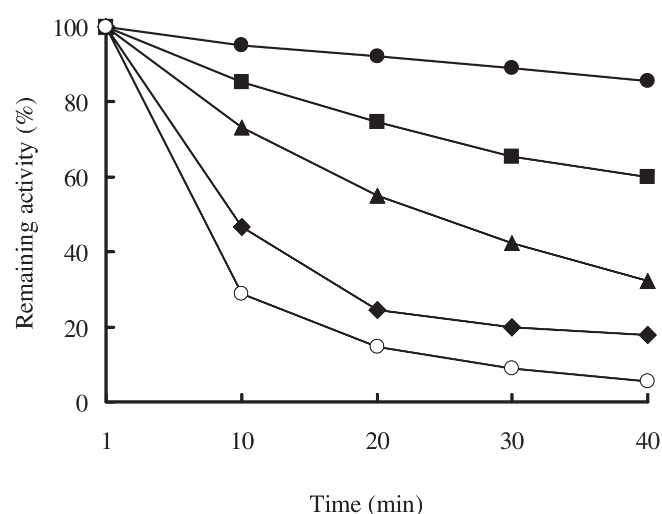

Fig. 3. Thermostability of the polyphenol oxidase from *Castanea henryi* nuts. *Filled circles*, 40°C; *squares*, 45°C; *triangles*, 50°C; *diamonds*, 60°C; *unfilled circles*, 70°C

Table 3. The pH stability of polyphenol oxidase from *Castanea henryi* nuts

pH	Activity ($\times 10^3$ U/mg protein)	Relative activities[a] (%)
4.0	1.44	52.94
4.2	1.71	62.58
4.4	1.92	70.59
4.6	2.35	86.40
4.8	2.53	93.01
5.0	2.72	100.00
5.2	2.46	90.44
5.4	2.24	82.35
5.6	1.85	68.01
5.8	1.72	63.24
6.0	1.64	60.29
6.2	1.58	58.09

[a] Enzyme mixtures were kept at 21°C for 24h at the nominated pH before activities were determined

enzyme was incubated at 70°C for 30min. Therefore, this enzyme proved heat labile, and this property may be used to control enzymatic browning during processing and storage.

Effect of pH on the enzyme activity

The effect of pH on enzyme activity was determined in a series of experiments with pH increments of 0.2 over a pH range of 4.0–8.0. At pH 4.0 and pH 8.0, about 70% and 1% of the maximal activity were detected, respectively. The optimum pH for the enzyme was determined to be pH 5.0. The optimum pH for the polyphenol oxidase of higher plants varies with plant species. Values for the polyphenol oxidases from burdock, apple, mango, strawberry, and cling peaches, and other plant sources are reported to be roughly neutral (pH 7.0).[22–27] As shown in Table 3, the relative activity of the enzyme is high over the narrow range of pH 4.5–5.5, indicating the pH range of enzyme stability. Significant loss of enzyme activity occurs when the enzyme is outside this pH range.

Effect of inhibitors on the enzyme activity

Ten compounds in different concentrations were examined as inhibitors to enzyme activity. Table 4 shows the effects of these compounds as inhibitors in tests using catechol as substrate (4mmol/l final concentration). The results showed that all these compounds were effective in inhibiting enzyme activity to some extent. Among them, *p*-nitrophenol, thiourea, orcinol, and naphthol showed strong inhibition, with relative activities of about 50%–70% with concentrations of 1.0–5.0mmol/l.

Figure 4 demonstrates the effect of *p*-nitrophenol as an inhibitor (5.0mmol/l final concentration) using catechol as substrate. The Lineweaver-Burk double reciprocal plot showed that this inhibitor changed the K_m value but not the

Table 4. Inhibitor effects of selected compounds on the activity of polyphenol oxidase from *Castanea henryi* nuts

Compounds	Concentrations (mmol/l)	Relative activities[a] (%)
Blank	–	100.00
p-Nitrophenol	1.0	51.3
	5.0	37.6
Thiourea	1.0	59.4
	5.0	49.7
EDTA	1.0	82.6
	5.0	66.5
Orcinol	1.0	64.5
	5.0	32.3
α-Naphthol	1.0	74.2
	5.0	56.5
2,4-Dinitrophenol	1.0	88.4
	5.0	45.2
p-Hydroxybenzoic acid	1.0	90.3
	5.0	64.5
2,4-Dihydroxyacetophenone	1.0	90.3
	5.0	48.4
Phenylalanine	1.0	83.9
	5.0	54.9
β-Mercaptoethanol	1.0	81.0
	5.0	38.7

EDTA, ethylenediaminetetraacetic acid
[a] Relative activities of the enzyme were based on data of the blank

V_{max} value. This indicates that *p*-nitrophenol showed competitive inhibition to the enzyme. The inhibitor constant K_i for this case was calculated to be 7.5 mmol/l.

The effect of thiourea (5.0 mmol/l final concentration) as inhibitor using catechol as substrate is illustrated in Fig. 5, which shows the decrease of both K_m and V_{max}. This implies the inhibition type of thiourea to the enzyme activity was uncompetitive, and the inhibitor constant K_i was calculated to be 3.8 mmol/l.

Molecular weight of the enzyme

The protein band with enzyme activity coincided approximately with the known protein marker of 68 kD on the SDS-PAGE. However, the distribution coefficient K_{av} in the gel filtration on Sephadex G-100 was determined to be 0.106. According to the linear regression equation, the MW of the polyphenol oxidase from *C. henryi* nuts was estimated to be 69 kD. The MW of polyphenol oxidase from most plant sources is generally thought to be 116–128 kD, although the MW of the enzyme from different plant sources varies greatly.[7,28–30] Most reports on the estimation of the MW of polyphenol oxidase used partially purified enzyme preparations with methods such as gel filtration and SDS-PAGE, and thus showed a range of values.

In conclusion, the polyphenol oxidase from *C. henryi* nuts was found to be similar to the enzymes of most other higher plant sources, particularly with respect to substrate specificity, effects of inhibitors, optimal temperature, and optimal pH. Therefore, the properties of the enzyme suggest that it is possible to prevent enzymatic browning in the production of *C. henryi* nuts by avoiding mechanical damage and exposure to the air, using a suitable pH value, adding appropriate inhibitors, and minimizing treatment time at high temperature.

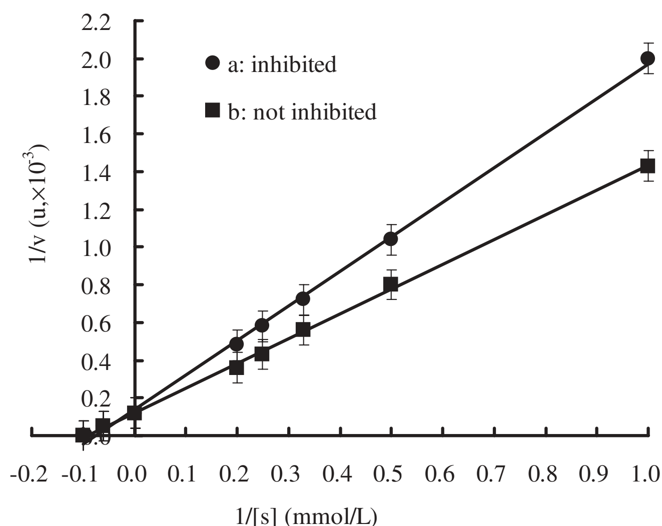

Fig. 4. Competitive inhibition of polyphenol oxidase from *Castanea henryi* nuts by *p*-nitrophenol (at 5 mmol/l using catechol as substrate)

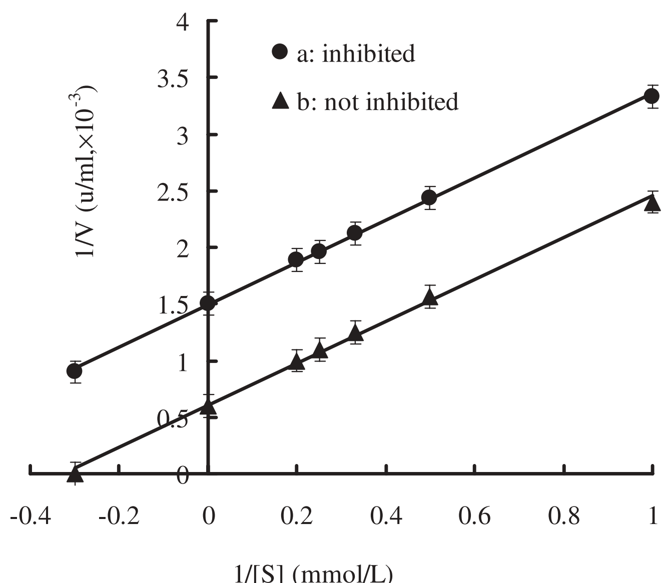

Fig. 5. Uncompetitive inhibition of polyphenol oxidase from *Castanea henryi* nuts by thiourea (at 5 mmol/l using catechol as substrate)

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