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Recombinant polyphenol oxidases for production of theaflavins from tea polyphenols

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ABSTRACT

Theaflavins (TFs) have attracted much attention due to their various bioactivities in black tea. This paper describes the first trial for enzymatic production of TFs by recombinant polyphenol oxidases (PPOs). PPO genes were cloned from nine species and expressed in *E. coli*. Crude enzyme assays by LC-MS revealed that eight recombinant PPOs were active for TFs production from tea polyphenols as substrates. Much higher activities were observed for crude enzymes of Md2 from *Malus domestica* (apple), Pp4 from *Pyrus pashia* (pear), and Ej2 from *Eriobotrya japonica* (loquat). When immobilized on mesoporous silica, crude Md2 was most active. The purified Md2 was immobilized and showed almost twice activity as high as its free enzyme. While the maximum activity of free enzyme was found at pH 5 and 10–30 °C, the immobilized enzyme had broader range of pH 4–6 and 10–40 °C. The activity of immobilized enzyme was relatively constant during the pH and thermal stability test. When used at 0.2 mg/ml in the beginning, the immobilized enzyme retained approximately 40% of its initial activity after 8 cycles of operation.

1. Introduction

Theaflavins (TFs) possess a benzotropolone skeleton combined by appropriate pairs of oxidized catechins, one with a dihydroxylated B ring and the other with trihydroxylated B ring [1,2]. The major TFs in black tea are theaflavin (TF1), theaflavin-3-gallate (TF2a), theaflavin-3′-gallate (TF2b) and theaflavin-3,3′-digallate (TF3) (Fig. 1). TFs contribute greatly to the colour and flavor of black tea, and have various bioactivities including antioxidant [3,4], anti-inflammatory effects [5], cancer chemoprevention [6,7], prevention of fatty liver [8,9], cardiovascular diseases [10], and inhibition of bone loss [11].

However, the concentration of TFs in black tea is quite low, only accounting for 2–20 mg/g of the dry weight of black tea [12]. It is not economical to extract TFs directly from black tea. At present, enzymatic synthesis is preferred in the preparation of TFs due to its high specificity, high yield and low side reactions [13]. Polyphenol oxidases (PPOs) have been used for enzymatic synthesis of TFs. PPOs purified from tea (*Camellia sinensis*) leaves [12] and mushrooms (*Agaricus bisporus*) [14] were successfully used to catalyze TFs synthesis. However, the process of PPO isolation and purification directly from plant tissues was complicated and inefficient. Recombinant PPOs may be a less expensive alternative. Three PPOs from *Malus domestica* [15], a tyrosinase from *Agaricus bisporus* [16] and an aurone synthase from *Coreopsis grandiflora* [17], were cloned and expressed in *E. coli* to yield substantial amount of recombinant PPO proteins, followed by characterization of catalytic activity of phenols to pigments by the activation of SDS, acidic and proteases. Although these PPOs were successfully expressed in *E. coli*, none of them has been used for TFs synthesis.

In addition, the industrial application of free enzymes is hampered by unavailable reuse and inactivation under extreme pH and high temperature. The immobilized enzyme can effectively overcome such drawbacks of free enzymes [18–20]. For example, the purified PPO from pear (*Pyrus bretschneideri*) fruit was immobilized on magnetite nanoparticles and used for synthesis of TF3 [21]. Recently, mesoporous silica has attracted much attention because of its well-ordered structure and large surface area as well as high pore volume [22–25]. These advantages may facilitate the entry of enzyme and enhance activity. Here, we describe the first trial for enzymatic production of TFs by recombinant polyphenol oxidases (free and immobilized). PPO genes

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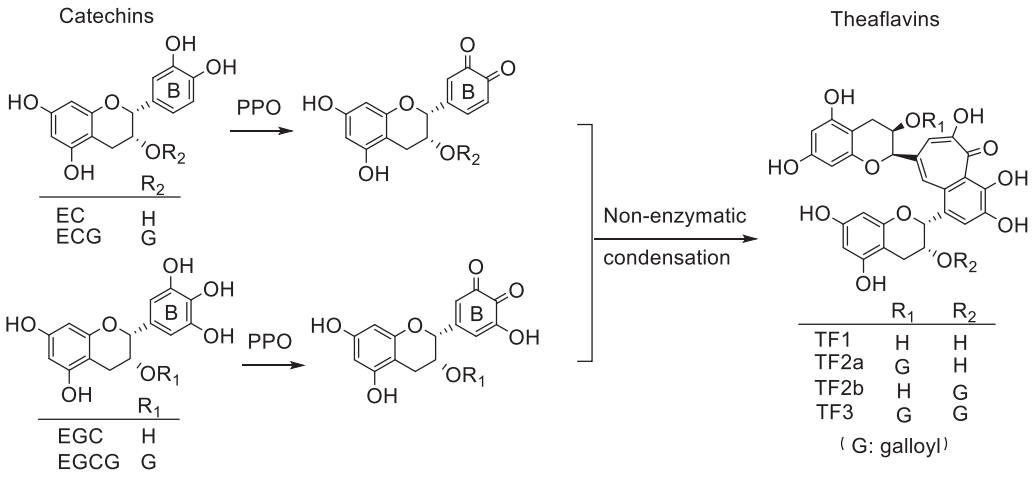

Fig. 1. Proposed mechanisms of PPO-catalyzed formation of TFs from catechins.

from nine species were selected for heterologous expression and subsequent detection for TFs synthesis from tea polyphenols. The recombinant PPO was further immobilized on mesoporous silica to recycle, resulting in higher activity and improved stability (Fig. 2).

2. Materials and methods

Tea polyphenols (98% purity) were purchased from Shanghai Yuanye Biotechnology Co., Ltd. (Shanghai, China). Glutaraldehyde (25%, w/v), SDS, $CuSO_4$, Chitosan, ethyl acetate, methanol, 2,2′,4,4′-tetrahydroxybenzophenone (THBZ) and mesoporous silica (237.2 m^2/g in specific area, 0.842 cm^3/g in total pore volume and 14.21 nm in diameter) were purchased from Aladdin Reagent Co. Ltd. (Shanghai, China). All other chemicals and reagents were of analytical grade. Young healthy leaves of apple were obtained from local garden. All other leaves were collected in Kunming Botanical Garden. Mushrooms (*Agaricus bisporus*) and leaves of sweet potatoes were purchased from local market.

2.1. Heterologous expression and purification

The details of PPO genes were shown in Table 1. Leaves were ground in liquid nitrogen, and the genomic DNA was extracted by using the NuClean PlantGen DNA Kit (Beijing, China) according to the manufacturer's instructions. Total RNA extraction and first strand cDNA synthesis of mushroom have been performed according to standard procedures [16]. PCR amplification was performed by using DNA or 1st cDNA as templates. Specific primers for cloning and heterologous expression were listed in Tables S1 and S2. PPO genes were subcloned into pET32a (+) and transformed into *E. coli* BL21(DE3). Protein expression was induced with the addition of 0.1 mM isopropyl-β-dthiogalactopyranoside (IPTG). The cultures were grown at 16 °C shaking for 24 h with 130 rpm, and then harvested by centrifugation at 6000 rpm. The induced cells were suspended with 400 μl of citric acid-phosphate buffer (50 mM, pH 7.8) and disrupted by MSE soniprep 150 ultrasonic disintegrator (UK) on ice. Each sonication took 3 min (disruption for 15 s then pause for 15 s). The lysate was centrifuged at 4 °C in 9000 rpm for 3 min and the supernatant was collected as crude enzyme or for enzyme purification as described below. The supernatant was loaded onto a nickel chelate column (Sangon Biotech Co., Ltd., China) equilibrated with binding buffer (50 mM citric acid-phosphate, pH 7.8, 100 mM NaCl, 5 mM imidazole). The column was then washed with 3 ml of washing buffer (50 mM citric acid-phosphate, pH 7.8, 500 mM NaCl, 50 mM imidazole). The histidine-tagged protein was eluted with elution buffer (50 mM citric acid-phosphate, pH 7.8, 500 mM NaCl, 200 mM imidazole). The eluent was pooled, desalted in citric acid-phosphate buffer (50 mM, pH 7.8), and then concentrated by centrifugation (Centrifugal Filters, Amicon Ultra-15 10 K). All fractions were analyzed by SDS-PAGE.

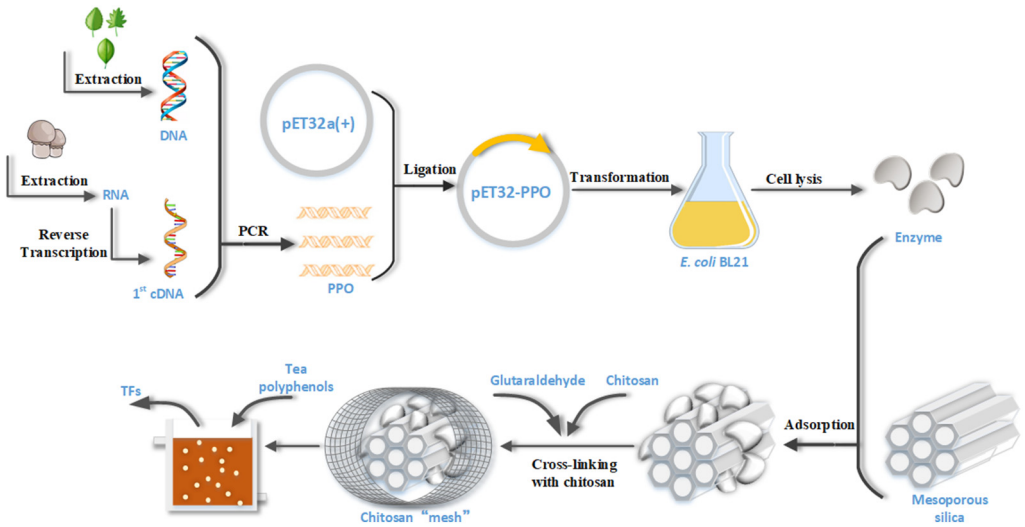

Fig. 2. Schematic illustration of TFs production by recombinant PPOs.

Table 1
PPO genes selected for cloning from plants and mushrooms.

Species	Gene name	GenBank number	Full-length ORF (bp)	Gene sequence (remove transit peptide)	M (calculated) kDa	Ref
Agaricus bisporus	*Ab3*	GQ354801.1	1731	1731	63.4	[26]
Agaricus bisporus	*Ab4*	GQ354802.1	1836	1836	67.2	[16]
Camellia sinensis	*Cs*	DQ513313.1	1800	1668	61.2	[27]
Coreopsis grandiflora	*Cg1*	KC972611.1	1809	1563	57.3	[17]
Eriobotrya japonica	*Ej2*	JX025010.1	1731	1614	59.2	[28]
Ipomoea batatas	*Ib*	AY822711.1	1767	1503	55.1	[29]
Juglans regia	*Jr1-m*	FJ769240.1	1812	1038	38.1	[30]
Juglans regia	*Jr1*	FJ769240.1	1812	1512	55.4	[30]
Juglans regia	*Jr2*	XM_018949737.1	1833	1518	55.7	–
Malus domestica	*Md2*	LT718523.1	1764	1485	54.5	[15]
Malus domestica	*Md3*	LT718524.1	1833	1521	55.8	[15]
Pyrus pashia	*Pp1*	HQ729709.2	1782	1641	60.2	[31]
Pyrus pashia	*Pp4*	GU906265.2	1764	1482	54.3	–
Vitis vinifera	*Vv*	U83274.1	1824	1515	55.6	[32]

2.2. Enzyme assays

The amount of protein was determined according to Bradford assay [33] with the standard curve shown in Fig. S2. The enzymatic reaction was carried out in 500 μl citric acid-phosphate buffer (50 mM, pH 5) containing 0.8 mg/ml tea polyphenols, 10 μM $CuSO_4$ and 1.4 mg/ml crude enzyme (the total protein of supernatant) at 30 °C for 40 min. For activation of the latent enzyme, 2 mM SDS was included in the assay mixture. The lysate of *E. coli* cells with empty pET32a(+) was used as control. Each reaction was carried out in triplicate. The reaction was stopped by adding equal volume of ethyl acetate. 5 μg of 2,2′,4,4′-tetrahydroxybenzophenone (THBZ) as internal standard was added in each reaction before extraction.

Biochemical characterization was further performed using the purified Md2 enzyme (free or immobilized). The optimum pH at pH 3/4/5/6/7 was investigated under 30 °C; the optimal temperature at 10/20/30/40/50/60 °C was evaluated under pH 5. The reaction was carried out in 500 μl citric acid-phosphate buffer (50 mM) containing 0.8 mg/ml tea polyphenols, 2 μM $CuSO_4$, 0.4 mM SDS and 0.2 mg/ml enzyme at a certain temperature for 40 min. The pH stability test was carried out in 500 μl citric acid-phosphate buffer (50 mM, pH 3/4/5/6/7) containing 0.2 mg/ml enzyme at 4 °C for 48 h, and then 0.8 mg/ml tea polyphenols, 2 μM $CuSO_4$ and 0.4 mM SDS were added for reaction at 30 °C for 40 min. The thermal stability test was carried out in 500 μl citric acid-phosphate buffer (50 mM, pH 5) containing 0.2 mg/ml enzyme at 30 °C for 0/6/12/24 h, and then 0.8 mg/ml tea polyphenols, 2 μM $CuSO_4$ and 0.4 mM SDS were added for reaction at 30 °C for 40 min. The supernatant containing 0.2 mg/ml proteins from *E. coli* cells with empty pET32a(+) was used as control, incubating at 30 °C for 40 min. Each reaction was carried out in triplicate. The reaction was stopped by adding equal volume ethyl acetate. Internal standard (THBZ) of 5 μg was added in each reaction before extraction.

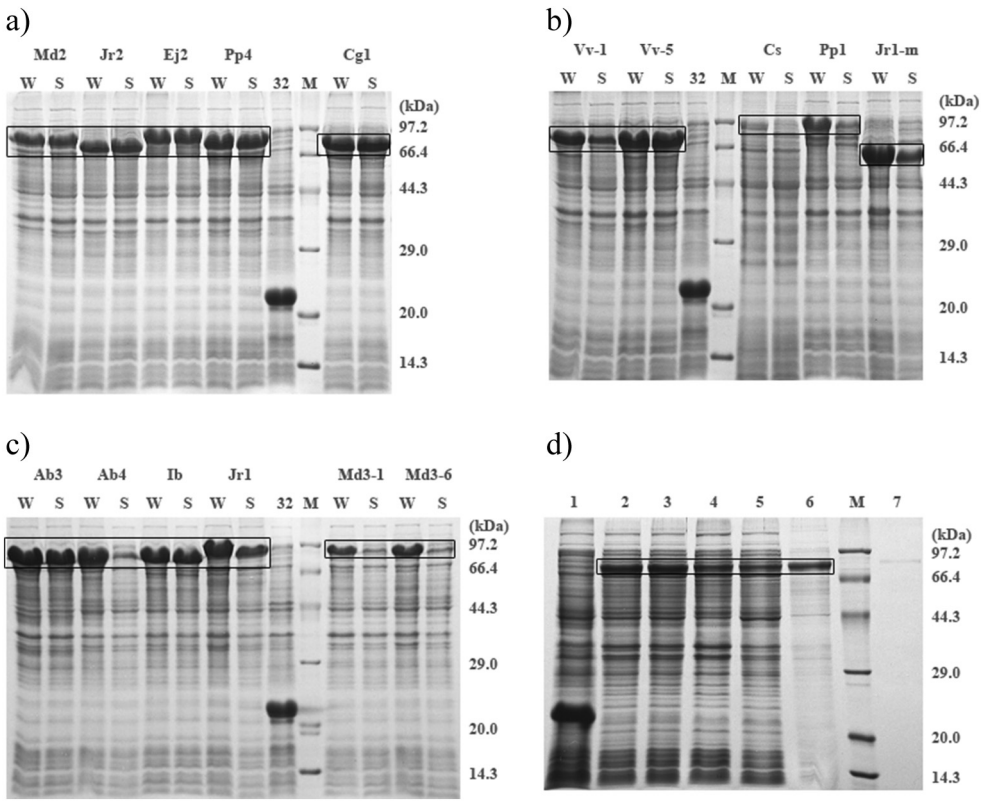

Fig. 3. SDS-PAGE analysis of His-tagged PPOs (a–c) and the purified Md2 (d). W, total lysate; S, soluble fraction; 32, *E. coli* with empty pET32a(+) as the control; M, protein size marker; 1, extracts of *E. coli* with empty pET32a(+) as the control; 2, whole-cell crude protein; 3, soluble crude protein; 4, passing through; 5, washing; 6, eluate of the purified protein; 7, stripping.

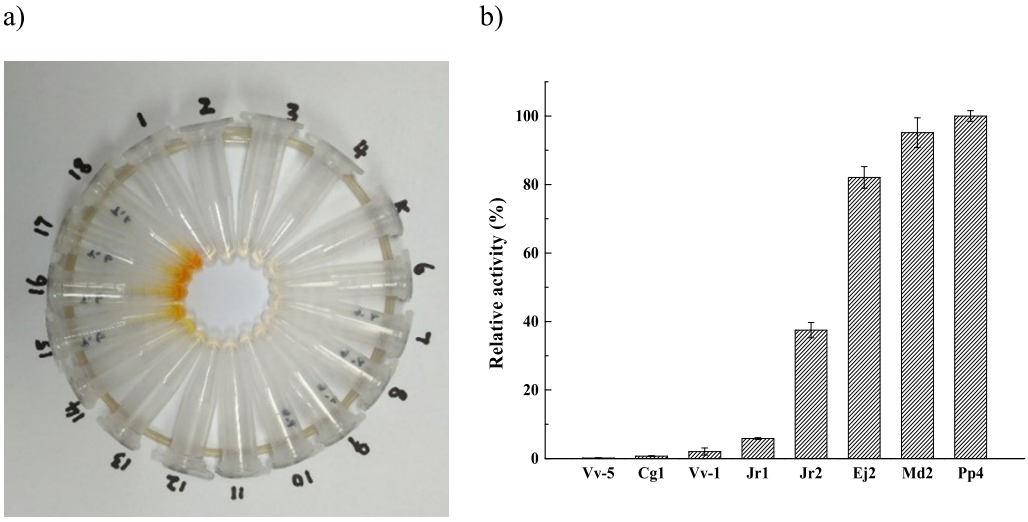

Fig. 4. a) The colour reaction of crude PPOs; b) relative activity of crude PPOs. Numbers of 1–18 denote the control (1), Ab3, Ab4, Ib (4), Md3-1, Md3-6, Cs (7), Pp1, the control, Jr1-m (10), Vv-5, Cg1, Vv-1, Jr1, Jr2, Md2 (16), Ej2, Pp4.

The extract was evaporated to dry and added 50 μl methanol for LC-MS detection. The LC-MS (Agilent 1290/6530 UPLC-Q-TOF) instrument conditions were optimized as Dual ESI: VCap 3500 V, Gas Temperature 350 °C, Drying gas 9 l/min, Nebulizer 35 psig; MS TOF: Fragmentor 135 V, Skimmer 60 V, OCT1RFVpp 750 V. The mass spectrometer was run in negative ionization mode and scanned from 50 to 1800 *m/z*. The samples were separated by an Agilent ZORBAX SB-C18 Rapid Resolution 3.5 μm pore size 4.6 mm × 150 mm column (30 °C) at a flow-rate of 0.5 ml/min. Chromatograph was conducted with elution of 30% to 37.5% B over 4.5 min, then to 75.5% B within 0.1 min and finally to 77% B over 1.4 min, where B was acetonitrile and A was 0.1% formic acid. All the LC–MS data were listed in supplementary materials. Enzyme activity was calculated by the ratio of peak areas of TFs to the corresponding internal standard, using control to remove the background TFs from tea polyphenols.

2.3. Enzyme immobilization

The immobilization was performed according to the method described by Gao et al. [22]. Mesoporous silica of 50 mg was dispersed in 25 ml citric acid-phosphate buffer (50 mM, pH 7.8) containing 0.19 mg/ml crude enzyme (or 0.22 mg/ml pure enzyme), and the mixture was shaken at 25 °C in 220 rpm for 24 h. Then 1.0 ml chitosan solution, prepared by dissolving 0.5 g of chitosan powder in 100 ml of 0.5% v/v hydrochloric acid (HCl, 38%), together with 1 ml of 1.0 wt% glutaraldehyde was added into the solution. The new mixture was stirred for another 20 min at room temperature. Finally, the mixture was centrifuged and the supernatant (unimmobilized protein) was subjected to protein quantification. The pellet containing immobilized PPOs was washed 3 times with citric acid-phosphate buffer (50 mM, pH 5). To assess the activity in relation to the amount of immobilized enzyme on support (mg/g), Md2 crude enzyme of 0.15 mg/ml, 0.22 mg/ml and 0.31 mg/ml were used, respectively.

$$\text{immobilized yield } (\%) = \frac{Wo - W}{Wo} \times 100\% \quad (1)$$

$$\text{amount of enzyme immobilized on support}(\text{mg/g}) = \frac{Wo - W}{Ws} \quad (2)$$

Wo is the amount of initial protein, *W* is the amount of unimmobilized protein in the supernatant and *Ws* is the amount of support.

2.4. Reusability and storage of immobilized enzyme

The reusability of immobilized enzyme was assessed by 8 repeated consecutive reactions. After each cycle, equal volume of ethyl acetate was added to extract the products. The biocatalysts were collected by centrifugation and washed with buffer. The next reaction was started with fresh substrate solution. The reaction was carried out in 500 μl citric acid-phosphate buffer (50 mM, pH 5) containing 0.8 mg/ml tea polyphenols, 2 μM $CuSO_4$, 0.4 mM SDS and

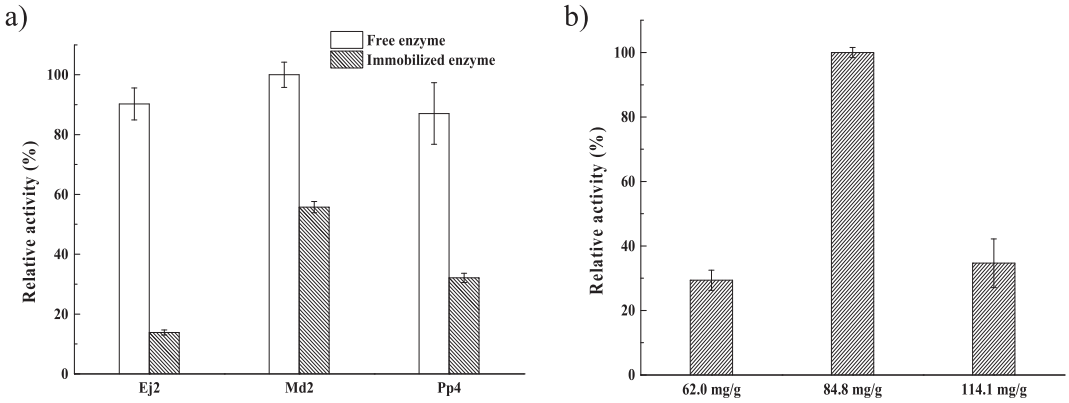

Fig. 5. a) Relative activity of free and immobilized crude enzymes of Ej2, Md2 and Pp4; b) activity in relation to the amount of crude Md2 immobilized on support.

Table 2
The immobilization yield and the amount of enzyme immobilized on support.

PPOs	Ej2	Md2	Pp4
Immobilization yield (%)[a]	86.8	82.4	88.6
Amount of enzyme immobilized on support (mg/g)[b]	84.2	79.9	85.9

[a] Percentage of the amount of protein immobilized on the support to the amount of initial protein.
[b] Ratio of the amount of protein immobilized on the support to the amount of support.

0.2 mg/ml immobilized Md2 enzyme at 30 °C for 40 min. The reaction was stopped by adding equal volume of ethyl acetate. Internal standard (THBZ) of 5 μg was added in each reaction before extraction. The free Md2 in citric acid-phosphate buffer (50 mM, pH 7.8) and immobilized Md2 in citric acid-phosphate buffer (50 mM, pH 5) were stored at 4 °C for 0/5/10/20/25 days, and then used for enzyme assays as described above. Each reaction was carried out in triplicate.

3. Results and discussion

3.1. Characterization of recombinant PPOs

PPO genes cloned from eight species of plants and one species of mushroom (Table S3, Fig. S1) were subcloned into pET32a(+) vector and transformed into *E. coli* BL21(DE3) for subsequent heterologous expression. Based on SDS-PAGE analyses, soluble recombinant proteins were observed for PPOs of Md2, Jr2, Ej2, Pp4, Cg1, Vv-5, Ab3 and Ib (Fig. 3a–c). No recombinant protein was observed for Cs, which was consistent with the previous report [34]. Ab4 with high expression level was found as inclusion bodies [26]. Vv-1, sharing 93.4% amino acid identity with Vv-5, was much lower in the expression level and solubility than those of Vv-5.

Crude PPO enzymes, including Cg1, Ej2, Jr1, Jr2, Md2, Pp4, Vv-1 and Vv-5, were active for TFs production from tea polyphenols, as demonstrated by LC-MS detection and the typical orange or orange-red colour of TFs in the reaction tubes (Fig. 4a). Based on LC–MS analyses, the most active PPOs were Ej2, Md2, and Pp4 (Fig. 4b, Table S4).

PPO can be an effective biocatalyst for production of TFs [1,35]. Previous studies have focused on native PPOs isolated directly from tea leaves [12], pear fruits [21] or mushrooms [36]. To the best of our knowledge, this is the first report on recombinant PPOs for production of TFs.

3.2. Immobilized crude enzymes

We further attempted to immobilize the recombinant PPOs for better stability and reusability to satisfy industrial applications. Due to their higher activities, crude enzymes of Ej2, Md2 and Pp4 were immobilized on mesoporous silica. Enzyme assays revealed that the activity of immobilized Md2 was almost twice as high as that of the immobilized Pp4, even four times of that of the immobilized Ej2 (Fig. 5a, Table S5). Compare to free enzymes, the immobilized enzymes lost some of their activities. This may be caused by the interference of impurity proteins or the inactivation of enzyme during the process of immobilization [19,21]. Although Md2 showed the lowest immobilization yield and

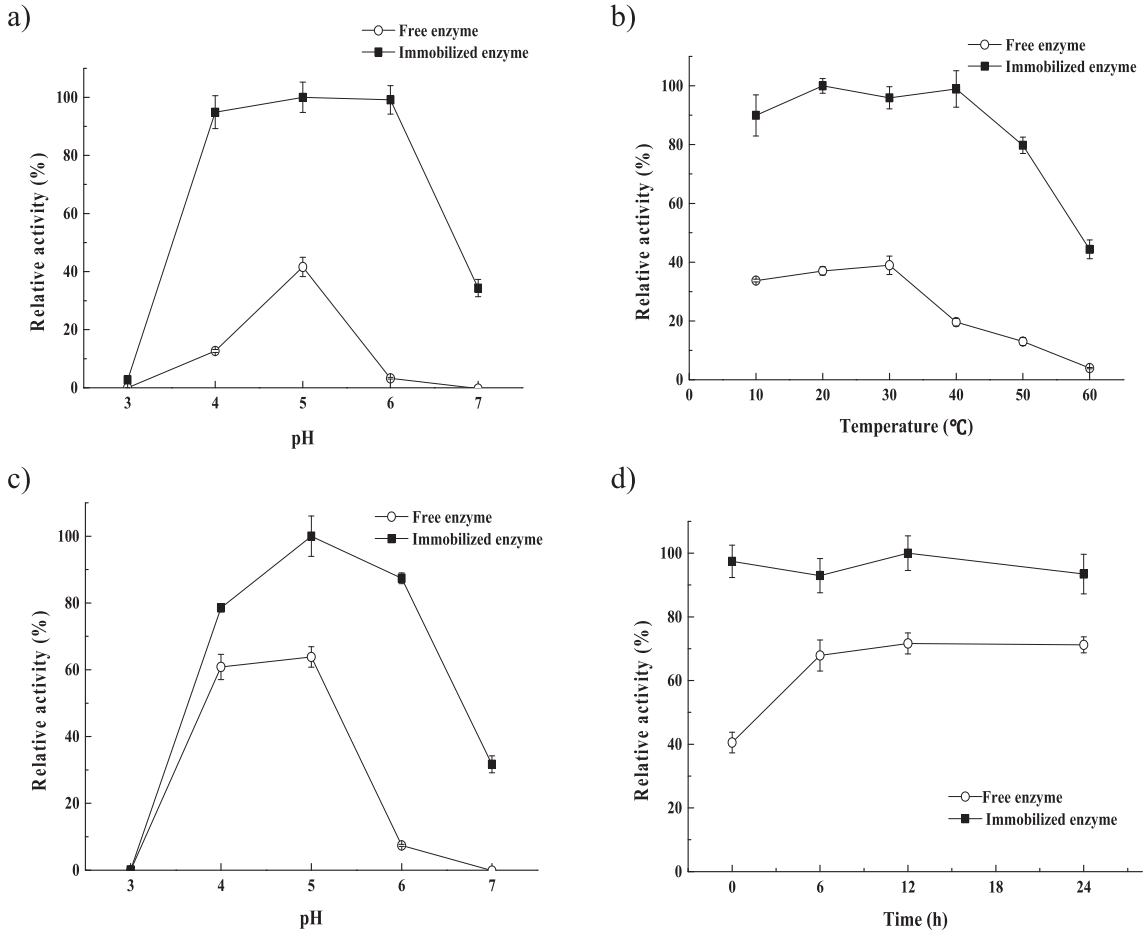

Fig. 6. Dependence of free and immobilized Md2 activity on pH (a), temperature (b), pH stability (c), and thermal stability (d).

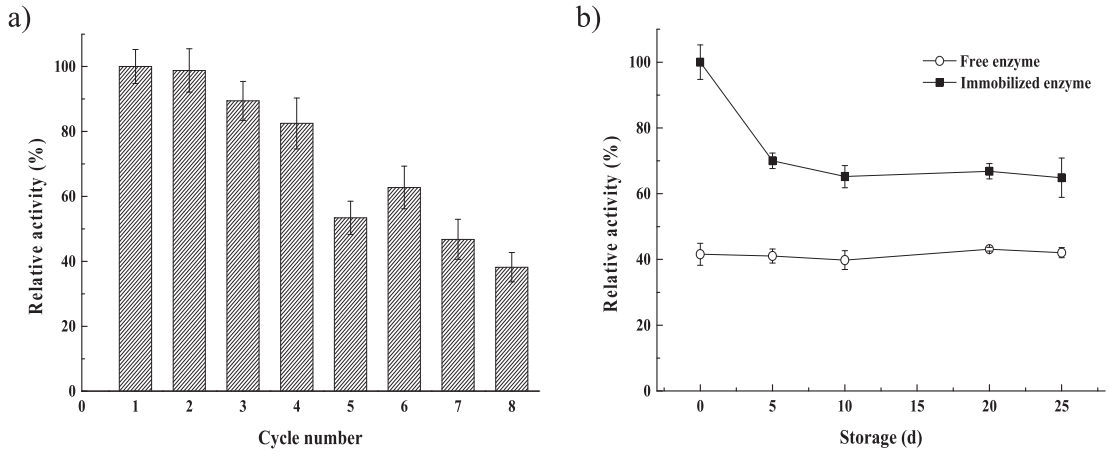

Fig. 7. a) Reusability of immobilized Md2; b) dependence of free and immobilized Md2 activity on storage.

amount on the support (Table 2), its activity was significantly higher than Ej2 and Pp4. Crude Md2 was further investigated for the effect of different amount of enzyme immobilized on support. As shown in Fig. 5b and Table S6, the optimal amount of enzyme immobilized on support was 84.8 mg/g. Too heavy immobilization led to a substantial decrease in enzyme activity. The adsorption of excess protein on the support may cause blockage of the pores so that the substrate molecules cannot enter the pores to react with the enzyme.

3.3. Characterization of the His-tag purified Md2

After purification under native condition on Ni-NTA His • Bind Superflow, the analysis of the Md2 elute on SDS-PAGE led to the detection of one major band corresponding exactly to the predicted size of the recombinant protein (Fig. 3d).

Compared to the free enzyme, the immobilized Md2 showed two-fold higher activity (Fig. 6). The improved activity of Md2 was partly due to its more complete immobilization on the support without the interference of impurity proteins. In addition, the chitosan might have an activation effect on PPO, which helped to expose active site to facilitate the contact between substrates and the active center [37]. The support itself may be another factor for the high activity due to the hydrophilic groups existed in the support combined with the hydrophilic amino residues lead to the active center exposed to the substrates [22].

The effect of pH was evaluated in the pH range of 3–7 (Fig. 6a, Table S7). The maximum activity was found at pH 5 for the free Md2, while the immobilized Md2 had wider range of pH 4–6. Free enzyme was more sensitive to pH than that of the immobilized enzyme. The maximum activity of the immobilized Md2 at 10–40 °C was higher than the free Md2 at 10–30 °C (Fig. 6b, Table S8). After staying at 4 °C for 48 h in the citric acid-phosphate buffer (pH 3/4/5/6/7), the activities of the free Md2 increased at pH 4 and pH 5 while those of the immobilized Md2 were relatively constant (Fig. 6a, c; Tables S7, S9). After staying at 30 °C for more than 6 h in the citric acid-phosphate buffer (pH 5), the free Md2 reached its maximum activity (Fig. 6d, Table S10). During the test of thermal stability, no significant difference was observed for the immobilized Md2. PPOs are typically expressed as latent pro-enzyme which can be activated by acidic solvent, SDS and proteases [38,39]. Staying for hours at pH 4 or 5 may activate the free enzyme, as shown in Fig. 6c. Although SDS was added for activation at the beginning of the reaction, the PPO activity was not fully activated and excessive SDS could inhibit the free enzyme activity. On the other side, the activity of immobilized enzyme may be completely activated during the immobilized process, leading to a higher level of pH stability and thermal stability.

3.4. Reusability and storage of immobilized Md2

Reusability is one of the advantages for the immobilized enzyme. As shown in Fig. 7a and Table S11, the immobilized Md2 (0.2 mg/ml) retained approximately 40% of its initial activity even after 8 cycles of operation. The number of recycles is closely related to the amount of immobilized enzyme used. If used at 1.0 mg/ml in the beginning, the immobilized Md2 could retain almost 100% of its initial activity after 10 cycles (data not shown). During storage of 0–25 days at 4 °C, the immobilized Md2 kept 70% of its initial activity after storage of 25 days, while the free enzyme barely changed (Fig. 7b, Table S12). The activity, stability and reusability of enzyme were greatly increased by using mesoporous silica as the support. Further work will need to optimize the immobilized PPOs for industrial use.

Abbreviations

TFs	theaflavins
TF1	theaflavin
TF2a	theaflavin-3-gallate
TF2b	theaflavin-3′-gallate
TF3	theaflavin-3,3′-digallate
PPO	polyphenol oxidase
IPTG	isopropyl-β-dthiogalactopyranoside
THBZ	2,2′,4,4′-tetrahydroxybenzophenone

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Conflict of interest

The authors declare no competing financial interest.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijbiomac.2019.04.142.

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