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Enzymatic synthesis of catechol-functionalized polyphenols with excellent selectivity and productivity

Hui Cheng[a], Yong Zou[b], Xiang Luo[b], Xian-Heng Song[b], Zhen Yang[c,*]

[a] College of Life Sciences and Oceanography, Shenzhen Key Laboratory of Marine Bioresources and Ecology, Shenzhen University, Shenzhen, 518060 Guangdong, China
[b] School of Pharmaceutical Sciences, Sun Yat-Sen University, Guangzhou, 510006, China
[c] College of Life Sciences and Oceanography, Shenzhen Key Laboratory of Microbial Genetic Engineering, Shenzhen University, Shenzhen, 518060 Guangdong, China

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ABSTRACT

Polyphenol products have become more and more attractive due to their strong anti-oxidant properties and a great variety of promising pharmacological activities and beneficial effects on human health. In this study, mushroom tyrosinase immobilized as cross-linked enzyme aggregates (CLEAs) was used as the catalyst for *ortho*-hydroxylation reactions to produce 3,4-dihydroxyphenylacetic acid, piceatannol and 3′-hydroxypterostilbene from 4-hydroxyphenylacetic acid, resveratrol and pterostilbene, respectively, with excellent selectivity and productivity. This is the first report of synthesizing these three polyphenolic compounds with tyrosinase CLEAs as catalyst, and the first study of biocatalytic production of 3′-hydroxypterostilbene. Introducing a deep eutectic solvent (DES) into the tyrosinase CLEA preparation exhibited a positive effect in terms of enhancing the catalytic activity of the immobilized enzyme and also promoting the synthesis of the polyphenol products.

1. Introduction

Polyphenols are an ample family of phenolic compounds such as phenolic acids, stilbenes, flavonoids and lignans, which are characterized by the presence of several hydroxyl groups attached on aromatic rings [1,2]. Nowadays, polyphenols have attracted more and more attentions mainly due to the recognition of their antioxidant properties and, as a result, their probable role in the prevention of various diseases associated with oxidative stress, such as cancer and cardiovascular and neurodegenerative diseases [3]. The structure-activity relationship of stilbene analogues has revealed that an increased number of hydroxyl groups on the aromatic ring structure signifies better antitumor and free radical scavenging capacities; and in terms of the position of hydroxylation, *ortho*-hydroxystilbenes are more effective than other hydroxyl-substituted stilbenoid derivatives [4]. Piceatannol (Pic), 3′-hydroxypterostilbene (HPS) and 3,4-dihydroxyphenylacetic acid (DHPAA) are three of the typical examples (Scheme 1).

Piceatannol is an *ortho*-hydroxylated derivative of resveratrol (Res), a well-known naturally occurring stilbene endowed with a plethora of health-promoting effects such as anti-inflammation, −cancer, −diabetes, −obesity and −aging activities [5]. Although having been found to display a similarly broad spectrum of biological functions as resveratrol, piceatannol has shown to exhibit much higher antitumor and antioxidant properties [4]. In addition, this compound also possesses some other pharmacological activities as reviewed in [6]. All these beneficial properties have encouraged the use of piceatannol, more potent as complementary to its congener resveratrol, in health and functional foods as well as in pharmaceutical and cosmetic products.

3′-Hydroxypterostilbene is another naturally occurring *o*-dihydroxyl stilbenoid compound. The original molecule it derives from is pterostilbene (PS), which has received tremendous attention recently due to its much greater bioavailability and better biological activity, relative to its original analogue, resveratrol [5]. Although there is a paucity of published data regarding the biological activities of 3′-hydroxypterostilbene, a few studies have revealed that this compound possesses similar pharmacological activities such as being anti-oxidant, anti-inflammatory, and anti-adipogenic [7], and that it is more potent than pterostilbene against the growth of human cancer cells [8] and remarkably more effective in inducing apoptosis of leukemia cells than not only pterostilbene but also piceatannol and resveratrol [9], all suggesting that it may be a promising antitumor agent.

Although less studied, 3,4-dihydroxyphenylacetic acid is also a strong anti-oxidant agent [10]. Limited research has shown that it has anti-proliferative activity in prostate and colon cancer cells [11], and can protect against cholesterol-induced dysfunction of pancreatic β-cells [12]. In addition, this compound can also be used as a precursor for the synthesis of hydroxytyrosol and hydroxystilbenes, both of which are also natural polyphenols with a broad spectrum of beneficial effects

* Corresponding author.
E-mail address: zyang@szu.edu.cn (Z. Yang).

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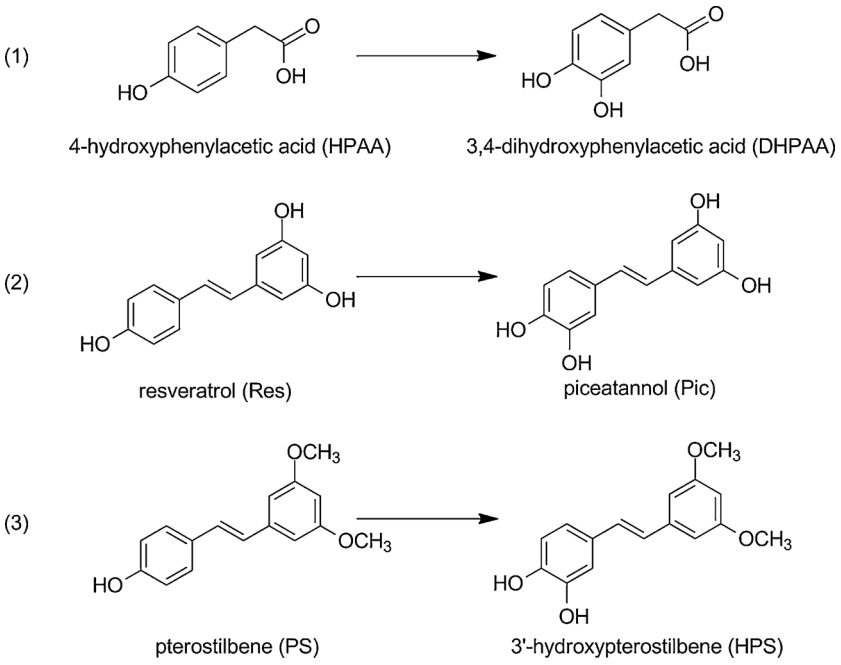


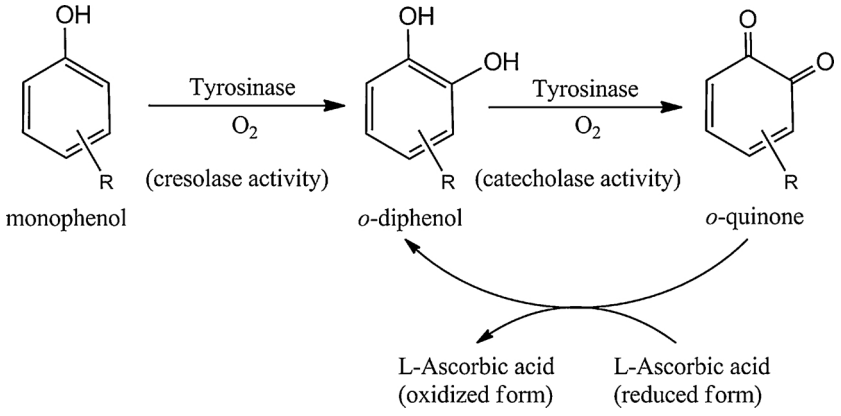
Scheme 1. The three synthetic reactions involved in this study.

on human health [13,14].

In spite of the more significant health benefits they can offer relative to their congeners, these *ortho*-hydroxylated polyphenol products are usually present in nature at much lower abundance [6]. Therefore, it is highly imperative to work out some synthetic strategies in order to supply sufficient amounts of these polyphenolic compounds for exploration and application of their pharmacological functions. Regioselective *ortho*-hydroxylation on benzene rings has always been challenging for chemical processes but can be easily achieved by means of biocatalysts. Several microbial P450 monooxygenases (CYP) and non-P450 ones (HpaBC) have been identified to be catalysts for *ortho*-hydroxylation of phenolic acids [15], stilbenoids [16–18] and flavonoids [19], however with low catalytic activity and synthetic efficiency. Tyrosinase (EC 1.14.18.1), on the other hand, is a promising candidate. The principle behind this is the strict regioselectivity of this enzyme: It is a copper-containing oxidoreductase responsible for the catalysis of the *ortho*-hydroxylation of monophenols to *o*-diphenols and their subsequent oxidation to *o*-quinones; in the presence of a reducing agent such as L-ascorbic acid the quinone product can be recycled back to the *o*-diphenol, leaving it as the sole product [20] (Scheme 2). The research conducted in our laboratory has verified that this enzyme, when immobilized in the form of cross-linked enzyme aggregates (CLEAs), is an efficient catalyst for *ortho*-hydroxylation of tyrosine with excellent productivity to synthesize L-DOPA, a drug for treatment of Parkinson's disease [21].

As a novel immobilization method more advantageous than conventional carrier-bound strategies, CLEA preparation consists of protein precipitation followed by cross-linking with each other, combining purification and immobilization into a single operation to provide easily and inexpensively prepared, yet highly stable and recyclable catalysts with remarkable catalytic efficiency. Due to these attractive features, this new immobilization method has been successfully applied to a variety of enzymes with widespread applications [22]. Our previous studies have indicated that immobilization of tyrosinase via CLEA formation can effectively improve the stability of the enzyme in aqueous solution against various deactivating conditions such as pH, temperature, denaturants, inhibitors, and organic solvents [23], and that the tyrosinase CLEAs exhibit a high catalytic power for efficient removal of phenolic compounds from wastewater [24].

The major goal of this study was to assess the feasibility of utilizing tyrosinase CLEAs as catalyst for *ortho*-hydroxylation to produce polyphenolic compounds, and DHPAA, Pic and HPS were taken as the 3 model products for this demonstration (Scheme 1). Although it is well known that biocatalytic synthesis is much more advantageous than



Scheme 2. Reaction scheme for tyrosinase-catalyzed *ortho*-hydroxylation to produce *o*-diphenols.

chemical processes, so far very few studies have been presented regarding enzymatic synthesis of polyphenolic compounds. Our study is the first one to demonstrate the use of tyrosinase CLEAs as a new biocatalyst for the synthesis of the above 3 polyphenol products, while no reports in literature have been given yet about biocatalytic production of 3′-hydroxypterostilbene.

Meanwhile, stimulated by our recent findings that deep eutectic solvents (DESs) can viably activate and stabilize enzymes when added as additives in the reaction system [25,26], we surmised that introducing a DES into CLEAs might trigger the immobilized enzyme more active or more stable. Here a DES is a new type of 'green' nonaqueous solvent that can be easily prepared by mixing an ammonium salt (such as choline chloride) with a hydrogen-bond donor (HBD, such as urea) at a specified molar ratio (for a review see [27]). It has currently attracted widespread academic and industrial interests with a broad range of applications. Therefore this study was also attempted to testify whether incorporating DES into tyrosinase CLEAs during their preparation would exert positive effects on the catalytic performance of the immobilized enzyme and on the synthesis of the polyphenol products.

2. Materials and methods

2.1. Materials

Fresh mushrooms were obtained from a local supermarket in Shenzhen, China. 3,4-Dihydroxyl-phenylalanine (L-DOPA) was purchased from Sigma–Aldrich China Inc. 4-Hydroxyphenylacetic acid (HPAA), 3,4-dihydroxyphenylacetic acid (DHPAA), resveratrol (Res) and piceatannol (Pic) were of all HPLC grade from Tokyo Chemical Industry Co., Ltd. Pterostilbene (PS) and 3′-hydroxypterostilbene (HPS) were both kindly provided by School of Pharmaceutical Sciences, Sun Yat-Sen University, China. Choline acetate (ChAc, 99%) was purchased from ShangHai Cheng Jie Chemical Co. Ltd. Choline chloride (ChCl) and all other reagents used were of analytical grade from local manufacturers in China.

2.2. DES preparation

A DES was prepared by mixing an ammonium salt (ChCl or ChAc) and a hydrogen-bond donor (urea, glycerol, acetamide, or ethylene glycol, respectively abbreviated as U, G, A or EG) at a molar ratio of 1:2, 1:1 or 2:1, following the procedures described in [25,26]. The concentration of a DES in aqueous solution refers to the molar concentration of the cholinium salt involved in the DES. It is worth noting that the term of DES is sometimes misunderstood. Strictly speaking, mixing the two components at a certain molar ratio may result in a room temperature liquid but does not necessarily make the system DEEP EUTECTIC, while only the composition leading to the LOWEST melting or freezing point is called DEEP eutectic. Here in this paper, following the general use in other literature, the term DES is still used referring to those ammonium salt-hydrogen bond donor mixtures which are liquid under ambient temperature.

2.3. Preparation of tyrosinase CLEAs with and without DES incorporation

Tyrosinase CLEAs were prepared from fresh mushrooms by precipitating the enzyme with ammonium sulfate and subsequent cross-linking with glutaraldehyde, following the procedures described in [23]. Typically, the enzyme solution (40 mL) was mixed with 10 mL of sodium phosphate buffer (50 mM, pH 6.0, in the presence or absence of 0.5 M of a DES), to which ammonium sulfate (to reach a final 50% saturation) and 1.0 mL of 25 wt% glutaraldehyde were successively added.

2.4. Activity assays for tyrosinase CLEAs

The activity of tyrosinase CLEAs in aqueous solution was determined spectrophotometrically by following the oxidation of L-DOPA to dopachrome [23,28]. Typically, 7.5 mg CLEAs were added to 20.0 mL Na phosphate buffer (50 mM, pH 6.0) containing 0.8 mM L-DOPA in a capped test tube to start the reaction, which was carried out in a shaking incubator at 25 °C with agitation of 200 rpm. All tests throughout this study were triplicated with standard deviations not greater than 5%.

2.5. Reaction to produce 3,4-dihydroxyphenylacetic acid (DHPAA) from 4-hydroxyphenylacetic acid (HPAA)

A typical reaction was started by addition of 15.0 mg of tyrosinase CLEAs into 20.0 mL of Na phosphate buffer (50 mM, pH 6.0) containing 10.0 mM HPAA and 15.0 mM L-ascorbic acid in a 50 mL conical flask, which was placed in a shaking incubator with agitation of 200 rpm at 25 °C. Periodically, a 0.15 mL sample was taken which was then four times diluted with the buffer before subjected to HPLC analysis as described below.

2.6. Reaction to produce piceatannol (Pic) from resveratrol (Res)

The reaction was normally carried out in 2.0 mL phosphate buffer (50 mM, pH 6.0) containing 20.0 mM Res, 40.0 mM L-ascorbic acid and 25% (v/v) DMSO (for pre-dissolving Res) in a shaking incubator with agitation of 220 rpm at 30 °C. 10.0 mg of tyrosinase CLEAs was added to start the reaction, and every one hour a 0.1 mL sample was taken, which was then 20 times diluted with 50% (v/v) acetonitrile aqueous solution before being subjected to HPLC analysis as described below.

2.7. Reaction to produce 3′-hydroxypterostilbene (HPS) from pterostilbene (PS)

A substrate solution containing 20.0 mM PS, 40.0 mM L-ascorbic acid and 50% (v/v) DMSO (for pre-dissolving PS) was prepared in phosphate buffer (50 mM, pH 6.0). The reaction was conducted by adding 10.0 mg CLEAs to 2.0 mL of the substrate solution, which was placed in a capped test tube in a shaking incubator with agitation of 220 rpm at 25 °C. At intervals, a 20 μL sample was taken and 20 times diluted with 50% (v/v) acetonitrile aqueous solution before being subjected to HPLC analysis as described below.

2.8. HPLC analysis

HPLC analysis of the substrates and products of the above three synthetic reactions was performed on a Shimadzu LC-20AT HPLC system equipped with an SPD-20A UV/Vis detector and a 150 × 4.6 mm, 5 μm inertsil ODS-SP column (GL Sciences Inc. Japan). The detailed conditions used for HPLC analysis of the three reactions are listed in Table S1.

3. Results and discussion

3.1. Confirmation of the products produced from the three enzymatic ortho-hydroxylation reactions

The three synthetic reactions were monitored by HPLC analysis. As shown in Fig. 1, samples removed from the reaction mixtures presented not only the peaks for the substrates (HPAA, Res and PS) but also new ones that match the authentic products (DHPAA, Pic and HPS) respectively, and these product peaks kept rising as the reactions went on, thus confirming the formation of the products. No peaks for L-ascorbic acid were observed in Fig. 1(B and C) simply because this reducing agent does not have absorbance at the detection wavelengths of 320 nm

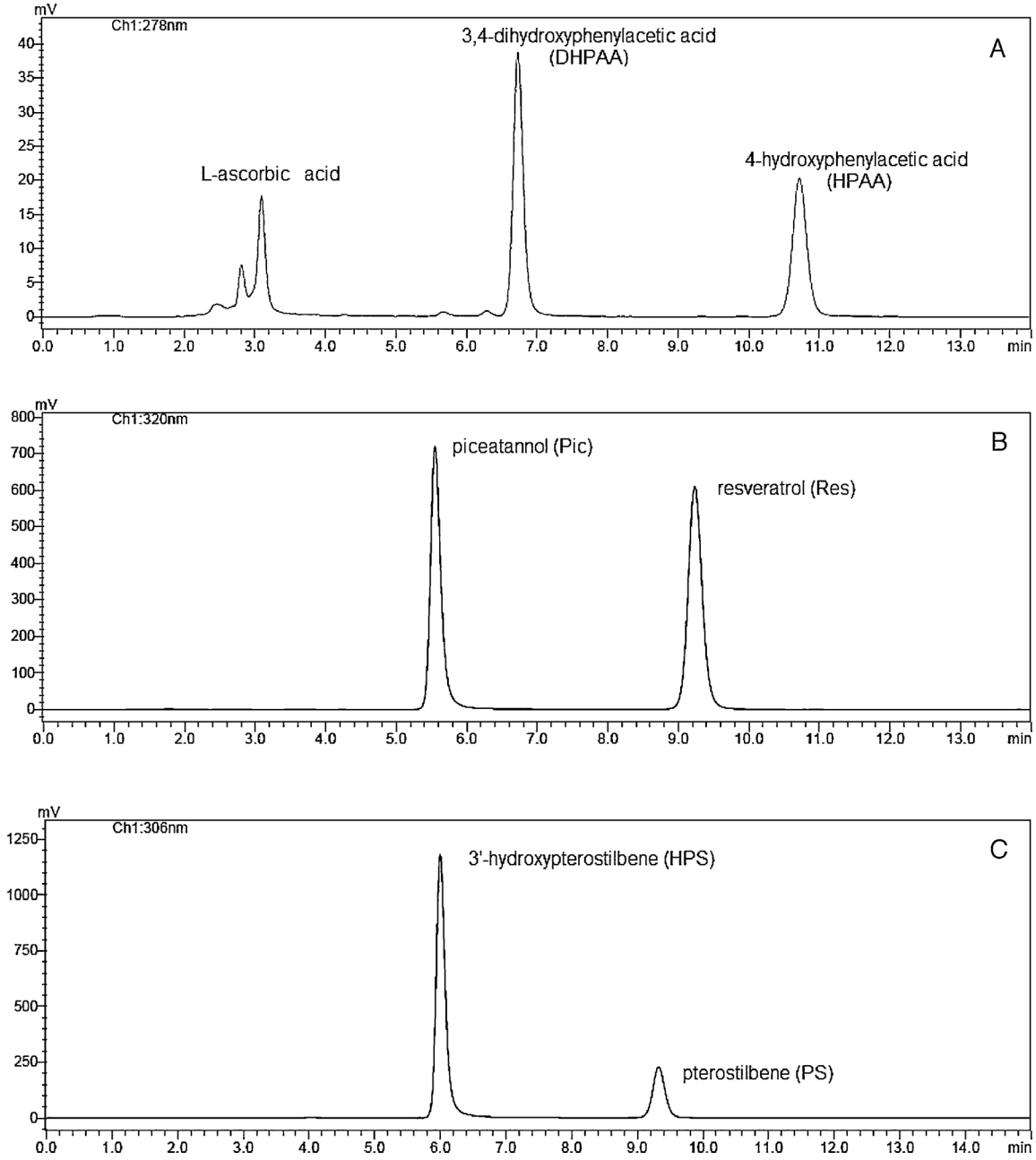


Fig. 1. HPLC chromatograms of samples from the three synthetic reactions: (A) HPAA → DHPAA, (B) Res → Pic, and (C) PS → HPS. Reactions were carried out for 2 h following the procedures stated in Sections 2.5–2.7, respectively, and the samples were taken for HPLC analysis as described in Section 2.8.

and 306 nm, which were used for HPLC detection of Res and PS, respectively. The ^{1}H NMR spectral data for the three products were supplied in the Supplementary Data file.

3.2. Synthesis of 3,4-dihydroxyphenylacetic acid (DHPAA)

The time-dependent formation of the product DHPAA and consumption of the substrate HPAA were shown in Fig. 2A, with tyrosinase CLEAs as the catalyst. Within the initial 1 h, the concentration of DHPAA notably increased while that of HPAA decreased. The reaction became leveling off after two hours of reaction, reaching a conversion of 40.6% and a yield of 99.7%. Here conversion and yield are respectively described as percentages of how much of a reactant has reacted and how much of a desired product is formed relative to the amount of the reactant that is consumed. A high yield of 99.7% obtained in this synthetic reaction is indicative of an almost quantitative conversion of HPAA to DHPAA. The fairly constant sum of HPAA and DHPAA during the whole reaction period (Fig. 2A) also confirms that the reaction is highly selective, with DHPAA as the only product. The same phenomenon was observed when the reaction was catalyzed by the free enzyme.

Effects of individual factors, namely catalyst dosage, pH, reaction temperature, L-ascorbic acid and substrate concentrations, on the hydroxylation reaction have been investigated (Fig. S1). The product formation was gradually enhanced along with an increase in the amount of the enzyme added (Fig. S1A), while the initial reaction rate presented a proportional relationship with the amount of the enzyme applied (data not shown). Within the pH range of 4–8 the concentration of the product produced was maintained fairly unchanged (Fig. S1B), while an increase in the reaction temperature from 20 to 45 °C did not show an improving impact on the conversion, with 25 °C yielding a slightly better result (Fig. S1C). Regarding the influence of L-ascorbic

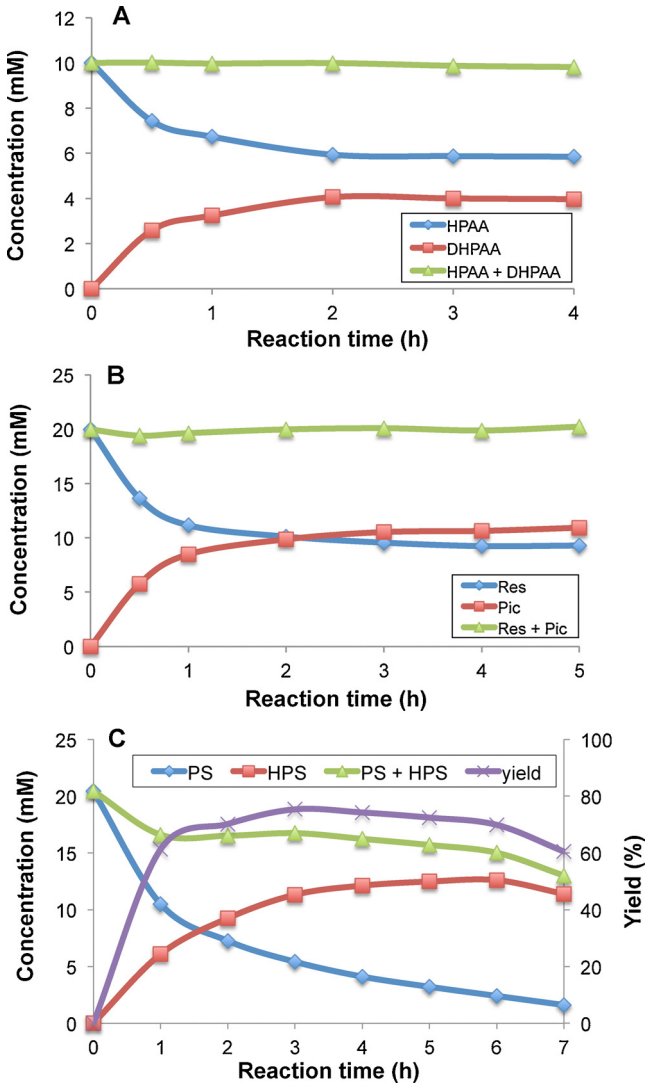


Fig. 2. Time courses of the three synthetic reactions: (A) HPAA → DHPAA, (B) Res → Pic, and (C) PS → HPS. Reactions were carried out as stated in Sections 2.5–2.7, respectively. Periodically samples were taken for HPLC analysis as described in Section 2.8.

acid (Fig. S1D), when its concentration was increased from 10 to 20 mM, there was an abrupt enhancement in the DHPAA formation, which was reasonable because L-ascorbic acid plays the role of a reductant, converting the subsequently produced *o*-quinone back to *o*-diphenol. However, when the concentration of L-ascorbic acid was sufficiently high (20–50 mM), a further increase in it did not lead to a significant change in the product formation. Although inhibitory effect of L-ascorbic acid on tyrosinase has been reported [29], it was not observed here. Additionally, an increase in the substrate concentration resulted in a higher product concentration, but accompanied by a decrease in the conversion (Fig. S1E); this is common as has been stated in [30,31]. All the above impacts were observed regardless of whether the enzyme was applied in the immobilized form of CLEAs or as free in aqueous solution, suggesting that the immobilization did not alter the catalytic properties of the enzyme.

So far there are only 7 papers published regarding enzymatic conversion of HPAA to DHPAA: Four by free enzymes (i.e., HpaBC of *E. coli* [15,32,33] and mushroom tyrosinase [34]), and three by immobilized mushroom tyrosinase [35–37]. The four papers with free enzymes have only confirmed the enzyme's ability to catalyze the conversion of HPAA to DHPAA but without quantifying their results in the synthetic

Table 1
A comparison of our results with literature data for the conversion of HPAA to DHPAA catalyzed by different forms of mushroom tyrosinase.

Ref.	Enzyme form	Initial [HPAA] (mM)	[L-ascorbic acid] (mM)	Reaction time (h)	Productivity (mM/h)
[35]	immobilized on crosslinked hexacinnamate of D-sorbitol	1	> 5.5	5	0.19
[36]	free enzyme	10	15	24	0.32
	immobilized on epoxy-acrylic resin	10	15	24	0.23
	LbL-coated[a]	10	15	24	0.29
[37]	mushroom powders entrapped in alginate gels	5	5	0.87	1.50
		5	5	2	1.05[b]
		5	10	2.7	0.67
		5	10	2.5	1.04[b]
This study	free enzyme	10	15	2	2.45
	CLEA	10	15	2	2.03

[a] The immobilized enzyme was further coated by the Layer-by-Layer (LbL) method [36].
[b] The results were obtained by *in situ* adsorption with Al_2O_3 [37].

reaction. The results reported in the three papers with the use of immobilized mushroom tyrosinase as catalyst were listed in Table 1, in comparison with the ones obtained in this study. The reactions carried out by Marín-Zamora et al. [35] have utilized not only a much higher input of L-ascorbic acid relative to the substrate concentration (> 5.5 equiv), but also two additional reagents: hydroxylamine (as an external reductant) and borate (as a trapping agent of the *o*-diphenol product) [38], all being favorable to the production of DHPAA. The *in situ* adsorption strategy developed by Kampmann et al. [37] was also to improve the product formation. Although without the aid of all these beneficial strategies, our tyrosinase CLEAs presented excellent results in terms of giving an almost quantitative yield (99.7%) and a supreme productivity (2.0 mM/h), as compared to those reported in the three papers (Table 1).

3.3. Synthesis of piceatannol

The time course of tyrosinase CLEA-catalyzed conversion of resveratrol to piceatannol was displayed in Fig. 2B. Similar as in Fig. 2A, this reaction became leveling off after 2 h, the total concentration of resveratrol and piceatannol remained fairly constant at about 20 mM during the whole reaction course, and a final conversion and yield of 53.5% and 100%, respectively, were obtained. These results imply that this reaction is also highly selective, quantitatively converting resveratrol to piceatannol. The impacts on this reaction of the enzyme amount, reaction temperature and concentration of L-ascorbic acid applied (Fig. S2) were also similar as for the reaction of DHPAA formation (Fig. S1).

Potter and coworkers [39] were the first to report the biological synthesis of piceatannol from resveratrol by using the cytochrome P450 enzyme CYP1B1. Later on, a few other enzymes and whole cells (Table 2) were discovered which are capable of catalyzing this conversion. Our study is the first to report the use of mushroom tyrosinase and their cross-linked aggregates as catalysts for this synthetic reaction. As compared to the results obtained by using other biocatalysts, tyrosinase CLEAs offer an excellent candidate in terms of promoting a complete conversion (with a yield of 100%) and a much higher productivity (2.19 mM/h).

Table 2
Synthetic results obtained in our study as compared to those reported in literature for the enzymatic conversion of resveratrol (Res) to piceatannol (Pic).

Ref.	Enzyme	Catalyst form	Initial [Res] (mM)	Final [Pic] (mM)	Productivity (mM/h)
[16]	cytochrome P450 BM3 (CYP102A1) from *Bacillus megaterium*	free enzyme	0.1		0.05
[17]	HpaBC monooxygenase from *Pseudomonas aeruginosa*	whole cells of transformed *E. coli* strain	30	8.8	0.73
[18]	HpaBC monooxygenase	whole cells of transformed *E. coli* strain	Initially 1.5 g/L, then fed continuously	4.9	0.27
[40]	tyrosinase from *Streptomyces avermitilis*	whole cells	0.1	0.078	0.02
[41]	tyrosinase (MelC2) from *Streptomyces avermitilis* MA4680	cell extract	0.5	0.11	0.65
This study	mushroom tyrosinase	CLEA	20	10.9	2.19

3.4. Synthesis of 3′-hydroxypterostilbene

Tyrosinase CLEAs can also be used to catalyze the *ortho*-hydroxylation of pterostilbene (PS) to produce 3′-hydroxypterostilbene (HPS) (Fig. 2C). As the reaction went on, HPS was built up while PS was consumed. A maximum yield of 75.4% was obtained after 3 h of reaction, together with an excellent productivity of 3.78 mM/h.

Actually, this is the first report of synthesizing HPS with a biocatalyst. The above preliminary data have shown very promising results, although it has to be stated that under the current reaction conditions this reaction is less selective than the above two, taking into account the relatively lower yields (< 100%) and also the total concentrations of PS and HPS which were always lower than 20 mM, the initial input of the substrate. More investigations on this biocatalytic synthesis leading to its optimization are being undertaken in our laboratory.

3.5. Tyrosinase CLEAs incorporated with DES

The idea that DES, when introduced into CLEAs, may render the immobilized enzyme more active or more stable has prompted us to prepare 8 DESs composed of two cholinium salts (ChAc and ChCl) and 4 HBDs (U, A, G, EG) at the same molar ratio of 1:1, and to introduce each of these DESs into tyrosinase CLEAs for their activity and stability assays. The screening results of these 8 different types of DES-CLEAs were shown in Fig. 3A. The DES incorporated in the tyrosinase CLEAs did show some positive effects on both the activity and stability of the immobilized enzyme. For instance, the ChCl/U (1:1)-CLEAs presented an activity that was 2.2-fold as high as the one obtained by the DES-free CLEAs; and at 50 °C the half-life of the ChAc/U (1:1)-CLEAs was 180 min while that for the DES-free CLEAs was 165 min. In comparison with those ChAc-based ones, the ChCl-based DESs seemed to trigger the tyrosinase CLEAs relatively more active and more stable (Fig. 3A); and the two DESs mentioned above that yielded DES-CLEAs with good activity and stability were both composed of urea as the HBD. And a later trial on the ChCl/U DES with different salt/HBD molar ratios suggested that the molar ratio of 1:2 was slightly better than that of 1:1 or 2:1 for the DES in promoting the activity of the DES-CLEAs in the order of: ChCl/U (1:2)-CLEA > ChCl/U (1:1)-CLEA > ChCl/U (2:1)-CLEA > DES-free CLEA.

The above findings were similar to our previous observations when investigating the impacts of adding DES as additive on the catalytic performance of two different enzymes: *Penicillium expansum* lipase (PEL) [25] and horseradish peroxidase (HRP) [26]. Both activity and stability of the two enzymes were found to be dependent on the choice of the salt anion and the HBD involved in the DES, and the molar ratio of the two. Based on the fluorescence and CD studies on HRP, we have concluded that relative to the ChAc-based DESs, the ChCl-based ones trigger the enzyme more active because of a looser tertiary structure and a higher α-helix content in its secondary structure. The DES-induced changes in both structure and catalytic properties of the enzyme were mainly caused by the salt anion (in the order of ChCl > ChAc)

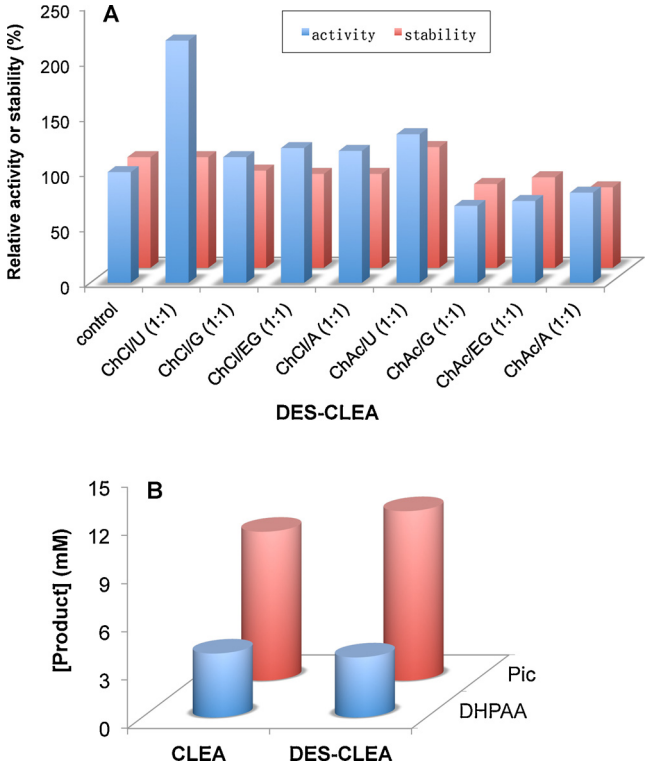


Fig. 3. Tests on DES-CLEAs: (A) A comparison of activity and stability of tyrosinase CLEAs incorporated with 8 different types of DES. Reactions were carried out as described in Section 2.4, and the relative activity and stability (%) refer to the percentage of the initial reaction rates and half-lives, respectively, both obtained at 50 °C by the DES-CLEAs relative to the ones obtained by the DES-free CLEAs (as control). (B) Production of DHPAA and Pic catalyzed by tyrosinase CLEAs, with and without the incorporation of the DES ChCl/U (1:2), after reactions for 2 h and 4 h following the procedures described in Sections 2.5 and 2.6, respectively.

rather than by the HBD. The salt effect was significantly weakened when the salt was incorporated into the DES, simply because of the presence of the H-bonding of the HBD to the salt anion. This can also be used to account for the observation that DESs formed by U or A as the HBD affected the enzyme more than those formed by G and EG, for the former two HBDs are weaker hydrogen-bond donors as compared to the polyols G and EG. On the other hand, the impacts of salt anion and HBD of the DES on the activity and stability of PEL were somewhat opposite, and this has been well explained by the interactions in the DES H-bonding network and their influences on the lipase mechanism [25]. Therefore, the impacts of DES may be different to different enzymes, depending very much on their protein structures and catalytic mechanisms. DES inserted into tyrosinase CLEAs affected the immobilized

enzyme in the same way as for the free HRP, presumably due to the fact that like the metalloenzyme HRP with the iron-containing heme group as the redox cofactor, tyrosinase is also an oxidoreductase, which contains copper instead of iron at the active site. In addition, incorporating DES into the CLEAs might be beneficial in improving the mass transfer within the catalyst, thus favoring their activity.

It is worth mentioning that in this study, the DES was introduced into the tyrosinase CLEAs by adding in the aqueous solution containing the enzyme. It is generally believed that due to the rupture of the H-bonds, DES may be totally dissociated into separate components when dissolved in water [42], but our previous studies with DES added into aqueous enzymatic reaction systems have suggested that it is the DES rather than its dissociated components that affect the enzyme activity and stability [25,26]. A few other studies [43] have also demonstrated that the eutectic network of some DES is well maintained upon serious dilution with water. It is uncertain yet whether the activation/stabilization effect induced by the incorporation of DES into tyrosinase CLEAs, as observed above, was caused by the presence of the intact DES complex or its dissociated components.

Inspired by the positive effects of DES on DES-CLEAs as presented above, we have attempted to employ ChCl/U (1:2)-CLEAs as catalyst for the two synthetic reactions, i.e., HPAA → DHPAA, and Res → Pic (Fig. 3B). For the first reaction, the DES-CLEAs yielded similar amounts of the product as the DES-free CLEAs; while for the second one, 13.8% more of the product, piceatannol, was produced by the DES-CLEAs, relative to that obtained by the DES-free ones. These preliminary results have manifested the feasibility of applying DES-CLEAs as catalyst for synthesis of polyphenol products. Further studies with regard to the use of this new type of biocatalyst are being conducted in our laboratory.

4. Conclusions

Above we have demonstrated the feasibility of enzymatic synthesis of catechol-functionalized polyphenol products such as 3,4-dihydroxyphenylacetic acid, piceatannol and 3′-hydroxypterostilbene, by using tyrosinase CLEAs as catalyst, with high selectivity and excellent productivity. Incorporating a DES into the tyrosinase CLEA preparation can render the immobilized enzyme more active and more stable, thus granting a promising benefit to the biocatalytic synthesis of polyphenolic compounds.

Declarations of interest

None.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.procbio.2018.03.028.

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