Biocatalysis and Biotransformation

ISSN: 1024-2422 (Print) 1029-2446 (Online) Journal homepage: https://www.tandfonline.com/loi/ibab20

Enzymatic synthesis of a catecholic polyphenol product with excellent antioxidant activity

Yi-Xiong Wei, Xiao-Feng Lü, Hui Cheng, Jia-Yi Luo, Yong Zou & Zhen Yang

To cite this article: Yi-Xiong Wei, Xiao-Feng Lü, Hui Cheng, Jia-Yi Luo, Yong Zou & Zhen Yang (2020): Enzymatic synthesis of a catecholic polyphenol product with excellent antioxidant activity, Biocatalysis and Biotransformation, DOI: 10.1080/10242422.2020.1756789

To link to this article: https://doi.org/10.1080/10242422.2020.1756789

BIOCATALYSIS AND BIOTRANSFORMATION
https://doi.org/10.1080/10242422.2020.1756789

Taylor & Francis
Taylor & Francis Group

RESEARCH ARTICLE

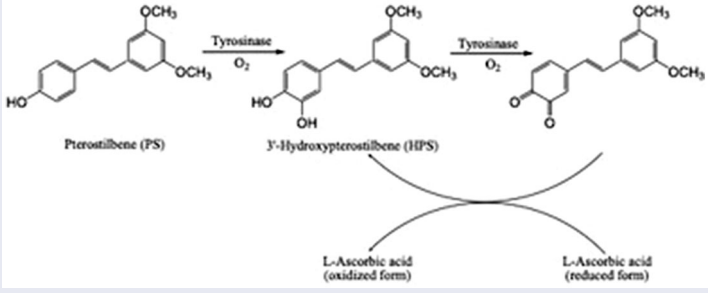
Enzymatic synthesis of a catecholic polyphenol product with excellent antioxidant activity

Yi-Xiong Wei[a]*, Xiao-Feng Lü[a]*, Hui Cheng[a], Jia-Yi Luo[b], Yong Zou[c] and Zhen Yang[b]

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

ABSTRACT

Polyphenols, especially catecholic stilbene derivatives, have attracted much attention due to the huge pharmacological effects and promising health benefits. However, their chemical synthesis via regioselective *ortho*-hydroxylation on aromatic rings is highly challenging. In this study, 3′-hydroxypterostilbene (HPS) is taken as a model product due to its strong potential as an antitumor agent. One-step enzymatic synthesis of HPS from pterostilbene (PS) was explored, with immobilised tyrosinase as catalyst. The impact of solvent, pH, temperature, oxygen and reductant concentration on the reaction was investigated, and the conversion was optimised by employing the response surface methodology (RSM). Finally, a high yield of 77.9% was obtained in 2.7 h. This study demonstrates the first successful use of a biotechnological strategy to synthesise HPS. The antioxidant activities of both PS and HPS were evaluated by using the DPPH assay, demonstrating that HPS is more potent than PS as a radical scavenger.



ARTICLE HISTORY
Received 28 August 2019
Revised 16 October 2019
Accepted 13 April 2020

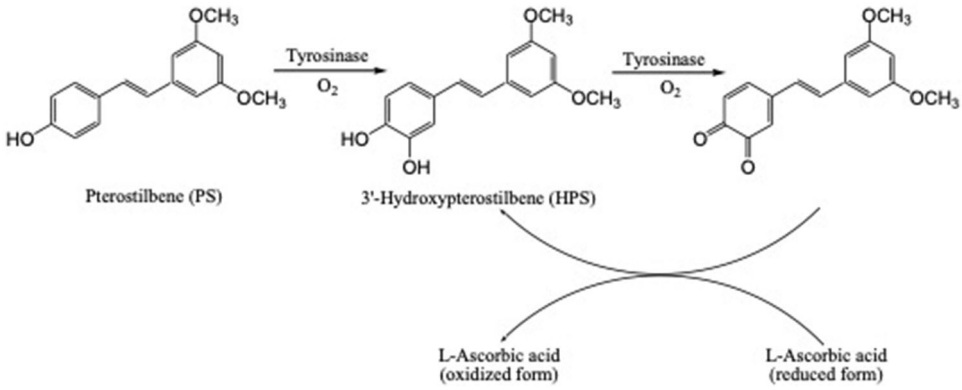
KEYWORDS
Polyphenol; 3′-hydroxypterostilbene; tyrosinase; *ortho*-hydroxylation; response surface methodology (RSM); antioxidant activity

Introduction

Polyphenols, especially the *ortho*-hydroxylated stilbene derivatives relative to those mono-hydroxylated ones, have been of great interest due to their beneficial effects on human health as a result of their strong antioxidant properties (Scalbert et al. 2005). However, the synthesis of catecholic polyphenol products through regioselective *ortho*-hydroxylation on aromatic rings is always a big challenge to chemists. Typically, 3′-hydroxypterostilbene (HPS) has shown potential as a promising antitumor agent (Tolomeo et al. 2005; Cheng et al. 2014; Takemoto et al. 2015), but so far only chemical approaches have been used for its synthesis, which require tough reaction conditions and at least 3–4 complicated reaction steps, yet yielding a rather low yield (∼40%) (Lee et al. 2010).

In this paper, we reported the first detailed study of synthesising HPS from its original analogue, pterostilbene (PS), by means of a one-pot enzymatic process using tyrosinase (EC 1.14.18.1) immobilised as cross-linked enzyme aggregates (CLEAs) as catalyst (Scheme 1). Our previous studies have demonstrated that the immobilised enzyme is an efficient catalyst for *ortho*-hydroxylation to produce polyphenolic compounds (Xu et al. 2012; Cheng et al. 2018). This study also demonstrates the first successful use of a

CONTACT Zhen Yang zyang@szu.edu.cn College of Life Sciences and Oceanography, Shenzhen University, 1066 Xue Yuan Avenue, Shenzhen 518055, China
*These authors contributed equally to this work.
Supplemental data for this article can be accessed here.



Scheme 1. The reaction scheme for enzymatic synthesis of HPS from PS.

biotechnological strategy to synthesise HPS, and thus will shed light on the exploration of biocatalytic processes for production of catecholic polyphenol products. The antioxidant activity of HPS in comparison to PS was evaluated, sustaining that HPS is a more potent radical scavenger.

Experimental

Materials

Pterostilbene (PS) and 3′-hydroxypterostilbene (HPS) were both kindly provided by School of Pharmaceutical Sciences, Sun Yat-Sen University, China. 1,1-Diphenyl-2-picrylhydrazyl (DPPH) (96% purity) was obtained from Shanghai Macklin Biochemical Co., Ltd. (Shanghai, China). All other reagents used were of analytical grade from local manufacturers in China.

Enzymatic synthesis

Tyrosinase CLEAs were prepared as described in Xu et al. (2011). For a typical synthetic reaction, a mixture of phosphate buffer (50 mM, pH 6.0) and DMSO in a ratio of 1:1 (v/v) was used to prepare the substrate solution containing 20.0 mM PS and 40.0 mM L-ascorbic acid. The reaction was started 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 40 μL sample was taken and 20 times diluted with 50% (v/v) acetonitrile aqueous solution before being subjected to HPLC analysis as described below. All reactions were carried out at least in duplicate, subject to less than ±10% error for each data point. The yield refers to the percentage of the amount of the product formed per mole of the substrate applied.

HPLC analysis

HPLC analysis of the substrates and products of the above synthetic reactions was performed on a Shimadzu LC-16 HPLC system (Kyoto, Japan) equipped with an SPD-16 UV/Vis detector and a 150 × 4.6 mm, 5 μm inertsil ODS-SP column (GL Sciences Inc., Tokyo, Japan). A mixture of solution A (0.5% acetic acid/CH_3CN (95:5, v/v)) and solution B (CH_3CN/0.5% acetic acid (95:5, v/v)) in a ratio of 1:1 (v/v) was employed as the mobile phase with a flow rate of 1.0 mL/min. The sample volume for injection was 10 μL. The absorbance at 306 nm was followed within 15 min.

Structural analysis

The product was structurally characterised by using ^1H NMR (Avance-400 NMR Spectrometer; Bruker, Billerica, MA) and FT-IR (WQF-520 FT-IR Spectrometer, Beijing Beifen-Ruili Analytical Instrument (Group) Co., Ltd., Beijing, China) spectroscopies.

RSM experimental design

A three-factor-three-level Box–Behnken design (BBD) was carried out using Design-Expert 8.0.6, a DOE software developed by Stat-Ease, Inc. (Minneapolis, MN). Samples were taken for HPLC analysis every 15 min for up to 4 h, and the maximal concentration of the product obtained was taken as the response parameter for the model. Experimental results were analysed by applying the ANOVA (analysis of variance) technique implemented in the Design-Expert software.

DPPH assay

A mixture of 485 μL anhydrous ethanol, 15 μL sample solution (PS, HPS or ascorbic acid predissolved in DMSO at a specified concentration), and 1000 μL

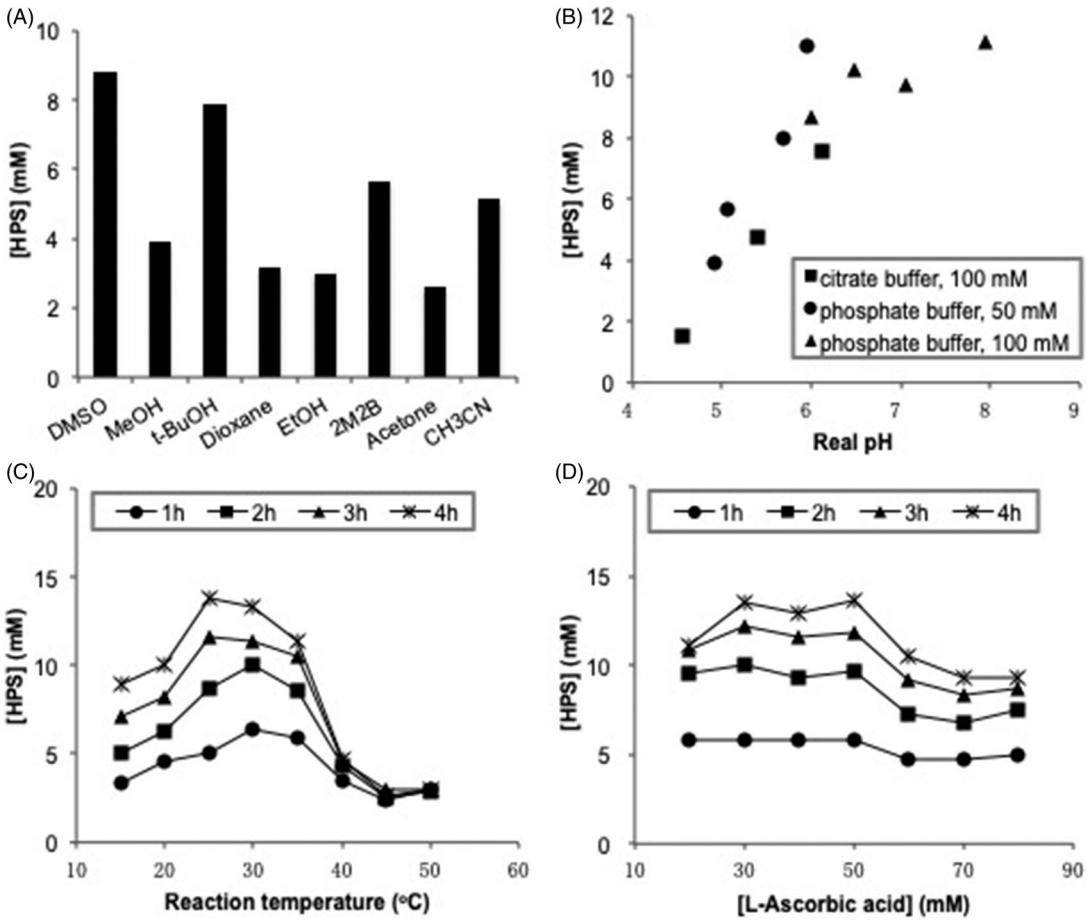


Figure 1. Effect of different reaction conditions: (A) solvent; (B) pH; (C) reaction temperature; (D) L-ascorbic acid concentration. (A) The reaction medium was a mixture of phosphate buffer (50 mM, pH 8.0) and an organic solvent in a volumetric ratio of 1:1, and the product concentration was determined after 2 h of reaction. Eight solvents were screened: dimethyl sulphoxide (DMSO), methanol (MeOH), *tert*-butanol (*t*-BuOH), dioxane, ethanol (EtOH), 2-methyl-2-butanol (2M2B), acetone, acetonitrile (CH_3CN). (B) Three buffer systems were used: citrate buffer (100 mM, pH 3–5), phosphate buffer (50 mM, pH 5–8) and phosphate buffer (100 mM, pH 5–8), and the product concentrations obtained at 2 h were plotted against the real pHs in the reaction system. (C, D) Product concentrations obtained at 1, 2, 3 and 4 h were plotted against reaction temperatures and L-ascorbic acid concentrations, respectively.

0.2 μM DPPH in anhydrous ethanol was placed in dark at room temperature for 30 min before the absorbance at 517 nm was recorded. The radical scavenging effect of the antioxidant was calculated as $(A_0 - A)/A \times 100\%$, where A is the absorbance of the sample solution and A_0 is that for the control solution (not containing the sample).

Results and discussion

The product from the enzymatic reaction was isolated as white powders and identified as HPS by HPLC (Figure S1), ^{1}H NMR (Figure S2) and IR (Figure S3). In order to optimise the synthetic process, the impact of the following reaction conditions was investigated and RSM was applied.

Effect of solvent

As the substrate PS is poorly soluble in water, addition of a hydrophilic solvent may be beneficial to enhancing the substrate solubility in the reaction system. Screening of the eight solvents reveals that dimethyl sulphoxide (DMSO) was the best to promote the synthetic reaction (Figure 1(A)) and was therefore selected in the later experiments. It is worth mentioning that the enzymatic reaction rate was not much related to either the substrate solubility in the solvent system or the nucleophilicity/hydrophilicity of the solvent.

Effect of pH

pH is well known as an important factor controlling the enzyme activity. In this study, three buffer systems were employed to cover a broad pH range: citrate

buffer (100 mM, pH 3–5), phosphate buffer (50 mM, pH 5–8) and phosphate buffer (100 mM, pH 5–8). As shown in Figure 1(B), the formation of the product increased with an increase in pH, levelling off within the range of pH 6–8. Comparatively, phosphate buffer seemed to be slightly more favourable than citrate buffer in promoting the product formation. It has to be noted that the real pH in the reaction system was more or less shifted due to the addition of L-ascorbic acid. In the later experiments, phosphate buffer (50 mM, pH 8.0) was normally used to generate a pH 6.0 reaction medium.

Effect of temperature

Temperature is also an important factor for an enzymatic reaction. As shown in Figure 1(C), the optimal temperature for the tyrosinase CLEAs-catalysed synthetic reaction was 30 °C for the first two hours but was 25 °C for the later two hours, both lower than the optimal reaction temperature for the free enzyme (50 °C) (Yang et al. 2007).

Effect of L-ascorbic acid

In this synthetic reaction, L-ascorbic acid plays the role of a reductant, converting the subsequently produced *o*-quinone back to *o*-diphenol (Scheme 1). The impact of its concentration (20–80 mM) on the synthetic reaction is presented in Figure 1(D).

L-Ascorbic acid should be applied with an appropriate amount. At a low concentration (20 mM), this reducing agent may be so insufficiently supplied that the product HPS was further oxidised, leading to a lower production yield; this was reflected by a gradual reduction in the sum of PS and HPS concentrations. The sum of these two concentrations remained fairly constant at 20 mM (the initial substrate concentration) when the reaction was carried out in the presence of 30–80 mM L-ascorbic acid, implicating that the amount of the reductant applied was sufficient to ensure no further oxidation of the product. But the decreasing yield obtained when more than 50 mM L-ascorbic acid was present may be due to the inhibitory effect of L-ascorbic acid on the enzyme (Golan-Goldhirsh and Whitaker 1984).

Effect of oxygen

As a co-substrate for the tyrosinase-catalysed oxidation reaction, oxygen is expected to play a critical role in the conversion from PS to HPS. Synthetic reactions with and without frequent sampling are compared in Figure 2. Simply by opening and closing the cap of the test tube, frequent sampling is a good way of replenishing oxygen for the reaction. The two

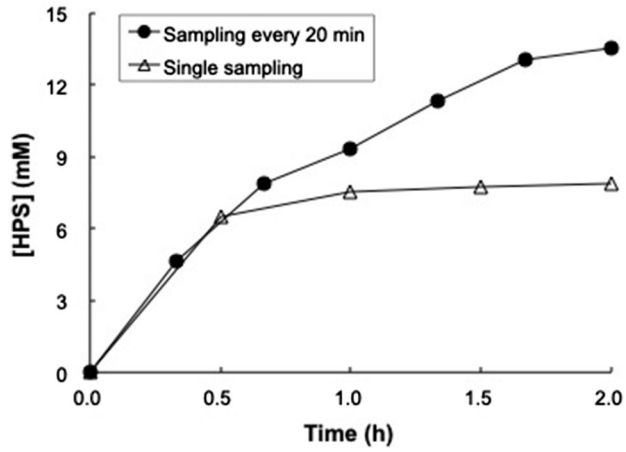


Figure 2. Effect of oxygen. Reactions were carried out at 30 °C by adding 40 mg CLEAs into the reaction system containing 20 mM PS, 30 mM L-ascorbic acid and 50% (v/v) DMSO in phosphate buffer (100 mM, pH 7.0). Frequent sampling: the test tube was sampled every 20 min, after which the test tube was capped and placed back to the incubator for continuous reaction. Single sampling: each test tube was sampled only once at a specified time and then discarded.

Table 1. Variables and levels used for the Box–Behnken design.

		Level		
Variable	Symbol	−1	0	+1
Enzyme dosage (mg)	A	20	30	40
L-Ascorbic acid concentration (mM)	B	30	40	50
Reaction temperature (°C)	C	25	30	35

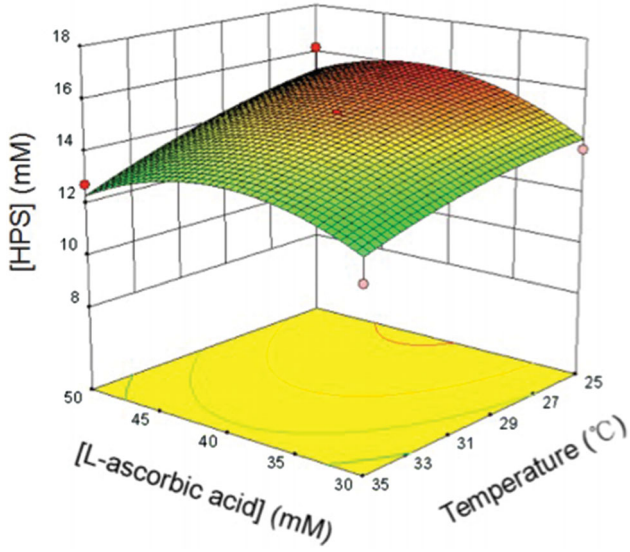


Figure 3. Response surface plot showing the mutual effects of L-ascorbic acid concentration and reaction temperature on the maximal product concentration.

synthetic reactions exhibited the same reaction rate during the initial 30 min, but then the rate of the reaction under frequent sampling significantly exceeded the one obtained with single sampling while the later being gradually levelling off, very much due to the complete consumption of the oxygen in the single-sampled test tube. Two hours of reaction yielded 13.5 mM and 7.9 mM HPS, respectively. This clearly demonstrates that sufficient supply of oxygen is important to the enzymatic reaction.

Optimisation by RSM

Based on the above single-factor results, response surface methodology (RSM) with a three-factor-three-level Box-Behnken design (BBD) was employed for optimisation of the enzymatic synthesis of HPS (Table 1). One of the 3D response surfaces with contour plots is depicted in Figure 3. A maximal product concentration (16.6 mM) was predicted by the model with a set of reaction conditions suggested: 40 mg CLEAs (enzyme dosage), 48.3 mM (L-ascorbic acid concentration), and 25 °C (reaction temperature). Three tests were done under these conditions, and an average product concentration of 15.4 mM was obtained (translating to a maximal yield of 77.9%), which is reasonably close to the predicted value. Obviously, the RSM-mediated optimisation has effectively improved the production yield, which is also obviously much higher than the one

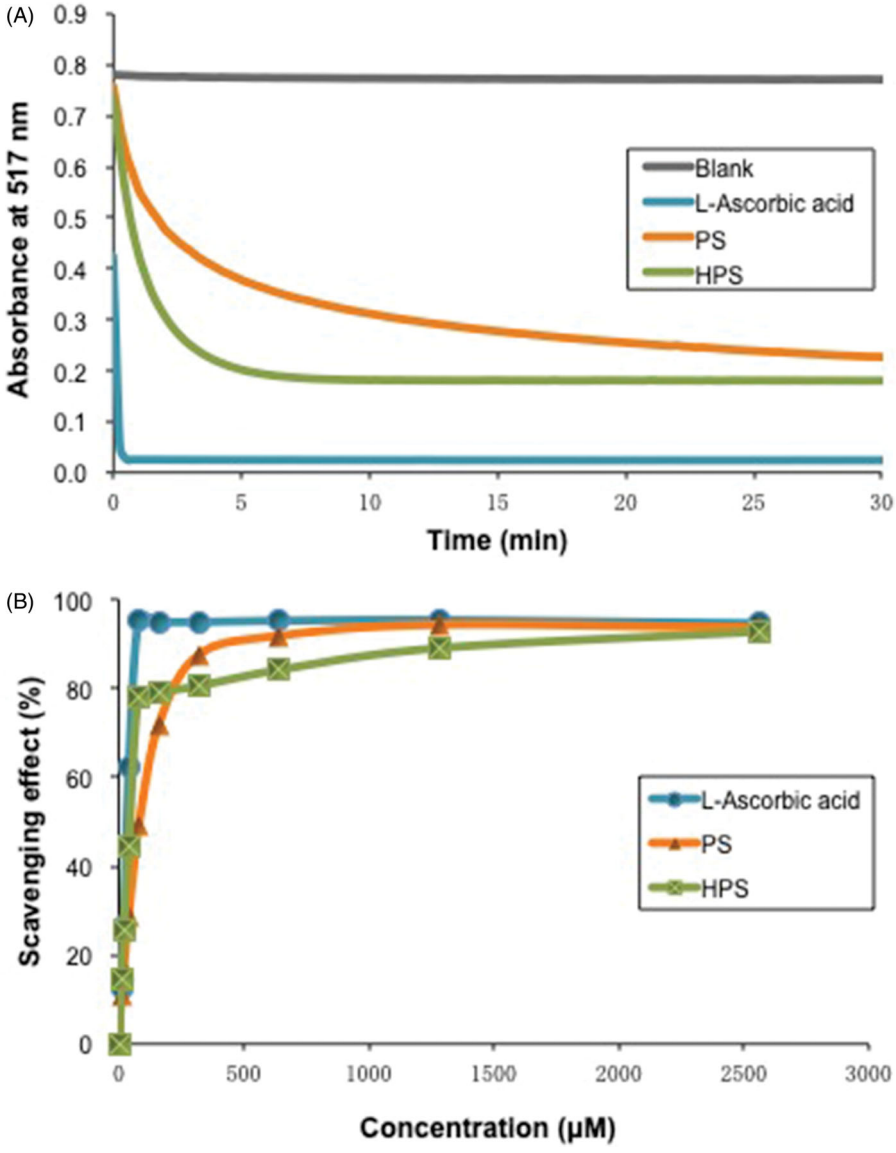


Figure 4. DPPH radical scavenging assay. (A) Variation of the absorbance at 517 nm with reaction time, in the absence of any antioxidant (blank) or in the presence of 160 μM L-ascorbic acid, PS and HPS; (B) the scavenging effects of L-ascorbic acid, PS and HPS presented at different concentrations of these antioxidants after reaction for 30 min.

normally obtained by chemical synthesis (~40%) (Lee et al. 2010).

Antioxidant activity

The antioxidant activities of PS and HPS were compared by evaluating their free radical scavenging ability using the DPPH assay (Yoshiki et al. 2001; Mishra et al. 2012). The basis of this method is the antioxidant-induced scavenging of the DPPH radical, which is reflected by a decrease in the absorbance at 517 nm. The kinetic data (Figure 4(A)) suggest that while the DPPH radical was highly stable in the absence of any antioxidant during the reaction period (30 min), addition of PS and HPS resulted in a significant drop in the absorbance, indicating that they both have a strong radical scavenging activity. While the PS-induced reduction in the DPPH level was a gradual one, HPS triggered a much more abrupt decrease in the absorbance, suggesting that HPS caused a much faster radical scavenging process than PS.

The strong radical scavenging ability of both PS and HPS was confirmed when experiments were performed with different amounts of the scavengers for a fixed period of 30 min, in comparison to the reference standard L-ascorbic acid (Figure 4(B)). Like the reaction for ascorbic acid, those for both PS and HPS were also biphasic: the scavenging effect increased sharply within the initial low concentration range (0–320 μM for PS and 0–80 μM for HPS and ascorbic acid) before becoming levelling off at a higher concentration. At low concentrations ($\leq$160 μM) HPS exhibited a higher scavenging effect than PS, presenting a similarly abrupt increase as L-ascorbic acid. The IC_{50} value (half maximal inhibitory concentration) for HPS was determined to be 46 μM, slightly higher than the one for ascorbic acid (33 μM) but smaller than the one for PS (84 μM). Lee et al. (2010) have also reported that HPS was stronger than PS in showing activity against ABTS radical.

Therefore, our experiments have demonstrated that similar to L-ascorbic acid, HPS is a more potent radical scavenger than PS in terms of both capacity and rate of scavenging. This is in support of the findings from Szekeres et al. (2010) that an increased number of hydroxyl groups on the aromatic ring structure normally indicates a better antitumor and free radical scavenging capacity, with *ortho*-hydroxylated stilbenoid derivatives more effective than other hydroxyl-substituted ones.

Conclusions

One-pot enzymatic synthesis of *ortho*-hydroxystilbenes was successfully demonstrated. A high yield of 77.9% was obtained within 2.7 h after optimisation with RSM. As compared to the generally used chemical strategy, this enzymatic process has shown promising benefits such as gentle reaction conditions, simple operations, and high production yield. This is in good agreement with our recent demonstration (Cheng et al. 2018). Comparing to its original analogue PS, the product HPS is more powerful in free radical scavenging and hence has a higher antioxidant activity.

Disclosure statement

The authors report no conflict of interest.

Funding

This work was supported by the National Natural Science Foundation of China under Grant No. 21276159 (to Z.Y.); the Key Project of Guangdong Natural Science Foundation under Grant No. 2016A030311033 (to Y.Z.); and the Science and Technology Program of Guangzhou City under Grant No. 201505041557046 (to Y.Z.).

References

Cheng H, Zou Y, Luo X, Song X-H, Yang Z. 2018. Enzymatic synthesis of catechol-functionalized polyphenols with excellent selectivity and productivity. Process Biochem. 70: 90–97.

Cheng T-C, Lai C-S, Chung M-C, Kalyanam N, Majeed M, Ho C-T, Ho Y-S, Pan M-H. 2014. Potent anti-cancer effect of 3′-hydroxypterostilbene in human colon xenograft tumors. PLoS One. 9(11):e111814.

Golan-Goldhirsh A, Whitaker JR. 1984. Effect of ascorbic acid, sodium bisulfite, and thiol compounds on mushroom polyphenol oxidase. J Agric Food Chem. 32(5):1003–1009.

Lee HS, Lee BW, Kim MR, Jun J-G. 2010. Syntheses of resveratrol and its hydroxylated derivatives as radical scavenger and tyrosinase inhibitor. Bull Korean Chem Soc. 31(4): 971–975.

Mishra K, Ojha H, Chaudhury NK. 2012. Estimation of antiradical properties of antioxidants using DPPH• assay: a critical review and results. Food Chem. 130(4):1036–1043.

Scalbert A, Manach C, Morand C, Rémésy C, Jiménez L. 2005. Dietary polyphenols and the prevention of diseases. Crit Rev Food Sci Nutr. 45(4):287–306.

Szekeres T, Fritzer-Szekeres M, Saiko P, Jäger W. 2010. Resveratrol and resveratrol analogues—structure–activity relationship. Pharm Res. 27(6):1042–1048.

Takemoto JK, Remsberg CM, Davies NM. 2015. Pharmacologic activities of 3′-hydroxypterostilbene: cytotoxic, anti-oxidant, anti-adipogenic, anti-inflammatory,

histone deacetylase and sirtuin 1 inhibitory activity. J Pharm Pharm Sci. 18(4):713–727.

Tolomeo M, Grimaudo S, Cristina AD, Roberti M, Pizzirani D, Meli M, Dusonchet L, Gebbia N, Abbadessa V, Crosta L, et al. 2005. Pterostilbene and 3′-hydroxypterostilbene are effective apoptosis-inducing agents in MDR and BCR-ABL-expressing leukemia cells. Int J Biochem Cell Biol. 37(8): 1709–1726.

Xu D-Y, Chen J-Y, Yang Z. 2012. Use of cross-linked tyrosinase aggregates as catalyst for synthesis of L-DOPA. Biochem Eng J. 63:88–94.

Xu D-Y, Yang Y, Yang Z. 2011. Activity and stability of cross-linked tyrosinase aggregates in aqueous and nonaqueous media. J Biotechnol. 152(1–2):30–36.

Yang Z, Deng J, Chen L. 2007. Effect of ionic and non-ionic surfactants on the activity and stability of mushroom tyrosinase. J Mol Catal B Enzym. 47(1–2):79–85.

Yoshiki Y, Kahara T, Okubo K, Sakabe T, Yamasaki T. 2001. Superoxide- and 1,1-diphenyl-2-picrylhydrazyl radical-scavenging activities of soyasaponin β g related to gallic acid. Biosci Biotechnol Biochem. 65(10):2162–2165.