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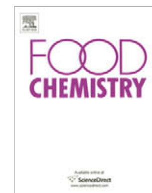

Inhibitory effects of *p*-alkylbenzoic acids on the activity of polyphenol oxidase from potato (*Solanum tuberosum*)

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ABSTRACT

In order to prevent potato from browning during preservation and processing, the effects of *p*-alkylbenzoic acids (*p*-propylbenzoic acid, *p*-butylbenzoic acid, *p*-pentylbenzoic acid, *p*-hexylbenzoic acid, *p*-heptylbenzoic acid, *p*-octylbenzoic acid) on the activity of potato polyphenol oxidase (PPO) have been studied. The PPO was extracted with PBS (pH 6.8), fractionated with ammonium sulphate, concentrated, and purified with Sephadex G-100 (1.8 × 100 cm) filtration chromatography. The active fractions were pooled and the PPO activity was determined to be 79.83 U/mg. The results showed that *p*-alkylbenzoic acids could strongly inhibit the activity of potato PPO. The values of IC_{50} of these six inhibitors were determined to be 0.213, 0.180, 0.152, 0.106, 0.075 and 0.047 mM, respectively. Kinetic analyses showed that *p*-alkylbenzoic acids used in this experiment were reversible and noncompetitive inhibitors to the enzyme. The inhibitory effects were potentiated with increasing lengths of the hydrocarbon chains, indicating that the inhibitory efficiency on the enzyme was influenced by the steric effect of the substituted groups.

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1. Introduction

Polyphenol oxidase (EC 1.14.18.1, PPO) is widely distributed in nature and has been detected in most fruits and vegetables. It is responsible for browning reactions in fruits and vegetables. Browning reactions, catalysed by PPO, occur mainly under post-harvest conditions when tissues are exposed either to stress conditions or to deterioration. PPO is a copper-containing enzyme, which catalyses both the oxygen-dependent hydroxylation of monophenols to their corresponding *o*-diphenols and the oxidation of *o*-diphenols to their cognate *o*-quinones (Mayer, 1987). Quinones are electrophilic, highly reactive molecules that can polymerise, leading to the formation of brown or black pigments (Mayer & Harel, 1991). In potato, polyphenol oxidases are also related to blackspot, an internal damage of tuber observable during their storage and resulting from impact injuries (McGarry, Hole, Drew, & Parson, 1996). This unfavourable darkening from enzymatic oxidation of phenols generally results in a loss of nutritional value and therefore becomes a major problem for the food industry (Friedman, 1996). Furthermore, PPO inhibitors should be clinically useful for the treatment of some dermatological disorders associated with melanin hyper-pigmentation (Mosher, Pathak, & Fitzpatrick, 1983) and also important in cosmetics for whitening and depigmentation after sunburn (Maeda & Fukuda, 1991). In addition, PPO is known to be involved in the moulting process of insects (Mosher et al., 1983) and adhesion of marine organisms (Marumo & Waite, 1986). Therefore, PPO inhibitors should have broad applications.

Many efforts have been addressed to the search for feasible and effective PPO inhibitors. Although many naturally-occurring PPO inhibitors have already been reported (Kubo & Kinst-Hori, 1998a, 1998b), their individual activity is either insufficient to be put into practical use or safety regulations of food additives limit their use in vivo. So, laboratory synthesis or extraction from plants (Kubo et al., 2000) has been relied upon to resolve the problem. Benzoic acid was identified as a potent mushroom tyrosinase inhibitor by Liu, Huang, and Chen (2003). It was classified as a noncompetitive inhibitor of diphenolase and monophenolase and the IC_{50} values were estimated to be 1.00 mM for diphenolase and 1.20 mM for monophenolase. Zhang, Chen, Song, and Xie (2006) also reported

Abbreviations: PPO, polyphenol oxidase; L-DOPA, L-3,4-dihydroxyphenylalanine; DMSO, dimethylsulphoxide; IC_{50}, the inhibitor concentrations leading to 50% activity lost; PBS, sodium phosphate buffer; K_m, Michaelis–Menten constant; V_m, maximum velocity.

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that salicylic acid and its derivatives could inhibit the activity of tyrosinase. However, there is little published about the inhibition mechanism of *p*-alkylbenzoic acids on PPO. In our efforts to develop the new, low-cost, easy-to-prepare, and highly active PPO inhibitors, several *p*-alkylbenzoic acids derived from benzoic acid were tested for PPO inhibitory capacity. The aim of this present experiment was, therefore, to carry out a kinetic study of the inhibition on the activity of PPO from potato by *p*-alkylbenzoic acids and to evaluate the kinetic parameters and inhibition constants characterising the system.

2. Materials and methods

2.1. Materials

Potato tubers were obtained from the Longyan Agricultural Science Research Institute. *p*-Propylbenzoic acid (**a**); *p*-butylbenzoic acid (**b**); *p*-pentylbenzoic acid (**c**); *p*-hexylbenzoic acid (**d**); *p*-heptylbenzoic acid (**e**); *p*-octylbenzoicacid (**f**) and L-3,4-dihydroxyphenylalanine (L-DOPA) were purchased from Aldrich (St. Louis, MO, USA). Dimethylsulphoxide (DMSO) was obtained from Sigma (St. Louis, MO, USA). All other reagents were of analytical grade. The water used was re-distilled and ion-free.

2.2. Enzyme extraction and purification

PPO was prepared from potato tubers according to the method of Huang, Lin, Qiu, Shi, and Chen (2006). Potato tubers (250 g) were cut into small pieces and homogenised in 250 ml of 50 mM PBS (pH 6.8) containing 0.1 M NaCl and extracted for 2 h. Then the homogenate was centrifuged at 15,000 rpm for 30 min. The supernatant, which is the crude extract, was collected and fractionated with ammonium sulphate. The final concentration of ammonium sulphate was 40% saturation. The precipitate was collected by centrifugation at 5000 rpm for 30 min, dissolved in a small amount 50 mM PBS (pH 6.8) and then dialysed against 10 mM PBS (pH 6.8) until no sulphate could be detected. The crude preparation was further chromatographed on a Sephadex G-100 column (1.8 × 100 cm, obtained from Sigma (St. Louis, MO, USA)) which was pre-equilibrated with 0.05 M PBS buffer (pH 6.8). The column was eluted with the same buffer with a flow rate of 18 ml/h and 3 ml per tube. All steps were carried out at 4 °C. Fractions with enzyme activity were pooled. One unit (U) of enzymatic activity was defined as the amount of enzyme catalysing the formation of 1 μmol of L-DOPA oxidation per minute at 30 °C.

2.3. Protein concentration

The protein concentration was measured by the Lowry method (Lowry, Rosebrough, Farr, & Randall, 1951) with bovine serum albumin as the standard.

2.4. Assay of enzyme activity

The enzyme activity assay was performed as reported by Chen, Song, Wang, and Huang (2003). In this investigation, L-DOPA was used as the substrate. The reaction media (3 ml) for the activity assay contained 0.5 mM L-DOPA in 50 mM PBS (pH 6.8). The enzyme activity was determined by following the increasing absorbance at 475 nm accompanying the oxidation of the substrate with a molar absorption coefficient of 3700 (M^{-1} cm^{-1}) (Jimenez, Chazarra, Escribano, Cabanes, & Garcia-Carmona, 2001).

The inhibition was first dissolved in DMSO and used for the experiment at 30 times dilution. The final concentration of DMSO in the test solution was 3.3%. Controls, without inhibitor but containing 3.3% DMSO, were routinely carried out (Qiu, Chen, & Wang, 2005). The extent of inhibition by the addition of the sample was the percentage necessary for 50% inhibition (IC_{50}). The inhibition type was assayed by the Lineweaver–Burk plot, and the inhibition constant was determined by the second plots of the apparent K_m/V_m or $1/V_m$ versus the concentration of the inhibitor (Shi, Chen, Wang, Song, & Qiu, 2005).

A Beckman UV-650 spectrophotometer was used for absorbance and kinetic measurements. All measurements were carried out at 30 °C.

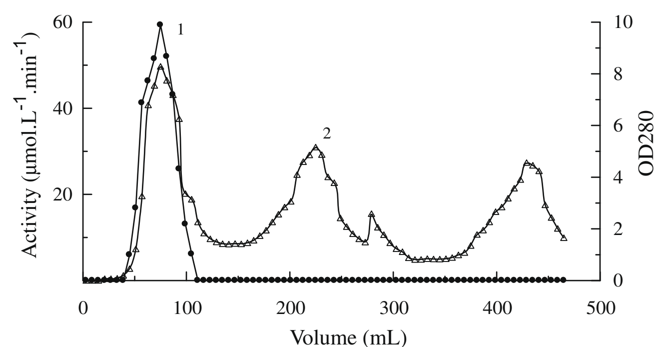

Fig. 1. Column chromatography of potato PPO on Sephadex G-100. 1 and 2 represents the protein concentration and the enzyme activity, respectively.

3. Results

3.1. Enzyme extraction and purification

The PPO from potato (*Solanum tuberosum*) was first extracted by PBS (pH 6.8), and then fractionated with ammonium sulphate. The crude preparation was concentrated and put on Sephadex G-100 column. The results of purification chromatography are shown in Fig. 1. The active fractions were pooled and the protein concentration and the specific activity were determined to be 2.27 mg/ml and 79.83 U/mg, respectively.

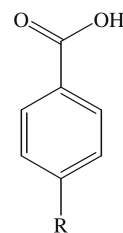

Fig. 2. Chemical structure of *p*-alkylbenzoic acids.

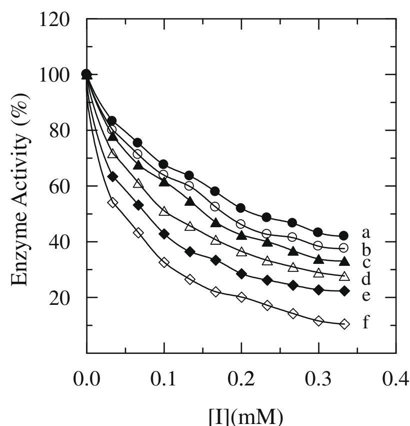

Fig. 3. The inhibition of *p*-alkylbenzoic acids on the activity of potato PPO for the catalysis of L-DOPA at 30 °C. Assay conditions: 3.0 ml reaction systems contained 0.5 mM L-DOPA, 50 mM sodium phosphate buffer, pH 6.8, and 3.3% DMSO ((**a**): *p*-propylbenzoic acid; (**b**): *p*-butylbenzoic acid; (**c**): *p*-pentylbenzoic acid; (**d**): *p*-hexylbenzoic acid; (**e**): *p*-heptylbenzoic acid; (**f**): *p*-octylbenzoicacid).

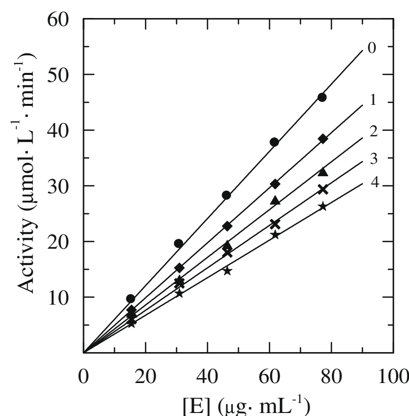

Fig. 4. The effect of concentrations of potato PPO on its activity for the oxidation of L-DOPA at different concentrations of *p*-propylbenzoic acid (**a**). Concentrations of the inhibitors for curves 0–4 were 0, 0.1, 0.2, 0.3 and 0.4 mM, respectively.

3.2. Concentration effects of p-alkylbenzoic acids on the potato PPO

Using *p*-propylbenzoic acid (**a**); *p*-butylbenzoic acid (**b**); *p*-pentylbenzoic acid (**c**); *p*-hexylbenzoic acid (**d**); *p*-heptylbenzoic acid (**e**); *p*-octylbenzoicacid (**f**) (see Fig. 2 for structures) as inhibitors, compounds (**a**)–(**f**) were tested for their effects on the activity of the potato PPO for the oxidation of L-DOPA. The inhibitory course is shown in Fig. 3. With increasing the concentrations of these *p*-alkylbenzoic acids, the activity of potato PPO markedly decreased concentration-dependently. From Fig. 3, the values of IC_{50}, the inhibitor concentration leading to 50% activity lost, of compounds (**a**)–(**f**) were estimated to be 0.213, 0.180, 0.152, 0.106, 0.075 and 0.047 mM, respectively. The data are summarised in Table 1. For *p*-substituted alkylbenzoic acids, the inhibition strength followed the order: (**a**) < (**b**) < (**c**) < (**d**) < (**e**) < (**f**). Inhibitory effects were potentiated with increasing lengths of the hydrocarbon chains.

3.3. The inhibitory mechanism of p-alkylbenzoic acids on the activity of the potato PPO

The inhibition mechanism by *p*-alkylbenzoic acids on the enzyme for the oxidation of L-DOPA was studied. The plots of the remaining enzyme activity versus the concentrations of enzyme in the presence of different concentrations of *p*-alkylbenzoic acids gave a family of straight lines, which all passed through the origin. All of these inhibitors had the same behaviour. Fig. 4 shows the relationship of enzyme activity to the enzyme concentration in the presence of different concentrations of compound (**a**). Increasing the inhibitor concentration resulted in a decrease of the slope of the line, indicating that the inhibition of *p*-alkylbenzoic acids on the enzyme was a reversible reaction course. The presence of these inhibitors did not bring down the amount of the efficient enzyme, but just resulted in the inhibition and the decrease of the L-DOPA oxidation activity of the enzyme.

3.4. Inhibition type and inhibition constants of p-alkylbenzoic acids on the activity of potato PPO

The inhibitory kinetics of potato PPO by *p*-alkylbenzoic acids was studied. Under the experimental conditions employed, the oxidation reaction of L-DOPA by potato PPO followed Michaelis–Menten kinetics. The double-reciprocal plots of the enzyme inhibited by *p*-hexylbenzoic acid are shown in Fig. 5. The results showed that the plots of $1/v$ versus $1/[S]$ gave a family of straight lines with different slopes, but they intersected one another on the *X*-axis. Accompanying the enhancement of the inhibitor concentration, the values of V_{max} descended but the values of K_m remained the same, which indicates that *p*-hexylbenzoic acid was a noncompetitive inhibitor of the enzyme. This behaviour indicated that *p*-hexylbenzoic acid could bind, not only with free enzyme, but also with the enzyme–substrate complex, and their equilibrium constants are the same. The inhibition constants for the inhibitor binding with the free enzyme (K_I) and enzyme–substrate complex (K_{IS}) were determined by the plot of the values of $1/V_{max}$ versus the inhibitor concentrations (inset in Fig. 5). Similar results were obtained with the other five compounds. They are also noncompetitive inhibitors of the enzyme, and the inhibition constants (K_I and K_{IS}) were determined by the same methods and results summarised in Table 1 for comparison.

4. Discussion

Recently, benzoic acid derivatives have been targeted for inhibition of mushroom tyrosinase, such as alkoxybenzoic acids (Chen et al., 2005), 4-vinylbenzoic acid (Song, Chen, Wang, Qiu, & Huang, 2005) and alkylbenzoic acids (Huang et al., 2006). They were noted

Table 1
Kinetics and inhibition constants of *p*-alkylbenzoic acids on potato PPO.

Compounds	IC_{50} (mM)	Inhibition	Inhibition type	Inhibition constants (mM)
a	0.213	Reversible	Noncompetitive	0.224
b	0.180	Reversible	Noncompetitive	0.199
c	0.152	Reversible	Noncompetitive	0.166
d	0.106	Reversible	Noncompetitive	0.116
e	0.075	Reversible	Noncompetitive	0.086
f	0.047	Reversible	Noncompetitive	0.059

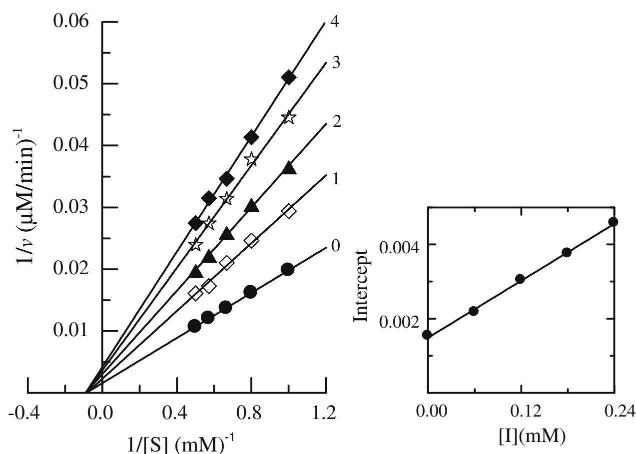

Fig. 5. Lineweaver–Burk plots for inhibition of *p*-hexylbenzoic acid (**d**) on the oxidation of L-DOPA by potato PPO. Concentration of (**d**) for curves 0–4 was 0, 0.06, 0.12, 0.18 and 0.24 mM, respectively. The inset represents the plot of $1/V_{max}$ versus the concentration of (**d**) to determine the inhibition constant.

to inhibit both diphenolase activity and monophenolase activity of mushroom tyrosinase and the *p*-alkylbenzoic acids were displayed to be of the uncompetitive type. In this study, the effects of six *p*-alkylbenzoic acids on the oxidation of L-DOPA by potato PPO have been investigated. The results show that all of the tested *p*-alkylbenzoic acids have inhibitory effects on the enzyme activity. They were displayed as reversible inhibitors, but the inhibitory types were determined to be noncompetitive. The inhibitory types of the six *p*-alkylbenzoic acids on the mushroom tyrosinase and potato PPO enzymes were different, indicating that the six inhibitors have different molecular inhibitory mechanisms on the two enzymes. The *p*-alkylbenzoic acids, not only can bind with free PPO, but also with potato PPO–substrate complexes; however, they can only bind with mushroom tyrosinase–substrate complexes. Walker and Wilson (1975) suggested the existence of two distinct sites on the enzyme: one for the binding of the substrate and another, adjacent site for binding the inhibitor. The result of the inhibitory type leads us to suppose that *p*-alkylbenzoic acids inhibitors attach to a site different from the active site and hinder the binding of substrate to the enzyme through steric hindrance or by changing the protein conformation. Since the substrate can bind with the enzyme to some extent, it may induce the enzyme conformation to change so that the hydrophobic pocket becomes bigger. We can hypothesise that the combination of the substrate with the enzyme molecule will induce a new hydrophobic pocket in the enzyme–substrate complex, and the *para*-position hydrocarbon chain can just be inserted into the pocket. Among these *p*-alkylbenzoic acids tested, *p*-octylbenzoic acid (**f**) was the most potent inhibitor, suggesting that the hydrophobic pocket accepts the eight-hydrocarbon-chain well. From the results, the combination between inhibitor (with *para*-position) hydrocarbon chain and enzyme–substrate complex is easier to engender and tighter, which means that the inhibitor could be embraced by the hydrophobic pocket. According to the results, we might design more novel and potent inhibitors of PPO for food preservatives in the future.

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