Journal of Environmental Biology
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September 2010, 31(5) 655-660 (2010)
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Preparation and assay of C-glucosyltransferase from roots of *Pueraria lobata*

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(Received: July 25, 2008; Revised received: February 02, 2009; Re-revised received: February 28, 2009; Accepted: March 30, 2009)

Abstract: *C-glucosyltransferase (EC 2.4.1.X) is one of the key enzymes for the biosynthesis of puerarin. This paper describes the methodology in purification and assay of the enzyme for the first time in Puerarin lobata (Willd.) Ohwi. C-glucosyltransferase from roots of P. lobata was extracted and partially purified by $(NH_4)_2SO_4$ saturation. The effects of pH, temperature, and substrate concentration on the activity of the enzyme were investigated. The properties of the puerarin produced by C-glucosyltransferase were studied by thin layer chromatography (TLC) and high performance liquid chromatography (HPLC). The peak activity of C-glucosyltransferase was detected in fraction of by 80% saturation of $(NH_4)_2SO_4$ and the optimal conditions for enzymatic reaction were 35.5 μmol l^{-1} of isoliquiritigenin and 560 μmol l^{-1} of UDP-G at pH 8.1, 28°C for 1 h. Mn^{2+} at 1 mmol l^{-1} and Al^{3+} at 1 mmol l^{-1} increased the enzyme activity, while Mg^{2+} inhibited its activity. The enzyme activity in Nicotiana tabacum and P. lobata were detected under the above assay conditions. Higher activity was found in roots than in leaves and stems of P. lobata, while no enzyme activity was detected in leaves of N. tabacum. It was the first time that activity of C-glucosyltransferase, which transforms isoliquiritigenin to puerarin, was detected in P. lobata.*

Key words: *Pueraria lobata (Willd.) Ohwi, Biosynthesis of puerarin, C-glucosyltransferase, C-glucosylation*
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Introduction

Pueraria lobata (Willd.) Ohwi (Fabaceae) is one of the oldest medicinal plants in traditional Chinese medicine. The dried roots of *P. lobata*, Puerariae Radix, called Gegen in Chinese, have been used as a main ingredient in traditional prescriptions for the treatment of early symptoms of common colds and as an antipyretic, antidiarrhetic, diaphoretic, and antiemetic agent (Rong *et al*., 1998; Liu *et al*., 2002). The main active and pharmacological constituents of Puerariae Radix are isoflavones, of which daidzein, daidzin (daidzein-7-O-glucoside) and puerarin (daidzein-8-C-glucoside) are most important (Cao *et al*., 1999). Some investigations have showed that puerarin, the main active compound in *P. lobata*, is an effective antioxidant and is also effective against glutamate excitotoxicity on cultured mouse cerebral cortical neurons (Guerra *et al*., 2000). While many studies have focused on increasing puerarin production by plant cell culture (Liu and Li, 2002; Chen and Li, 2007), root hairy culture (Liu *et al*., 2002) and on pharmacological effects (Jiang 2006), the enzyme biochemistry and the regulation of the biosynthetic pathway for puerarin is little known.

In nature, secondary plant metabolites are glycosylated to O (OH- and COOH-), N, S and C atoms by glycosyltransferase (GTs) using nucleotide-activated sugars as donor substrates (Jones and Vogt, 2001; Jobron *et al*., 2003). However, only a few studies have focused on C-glucoside biosynthesis and C-glucosyltransferase in microorganisms (Hoffmeister *et al*., 2003; Fischbach *et al*., 2005; Lin *et al*., 2006). In higher plants, Kerscher and Franz (1987, 1988) reported the isolation of flavanone-*C*-glucosyltransferase from cotyledons of *Fagopyrum esculentum*. Inoue and Fujita (1974, 1977) reported that the conversion of isoliquiritigenin to puerarin in intact *P. lobata* shown by carboxyl-^{14}C labeled isoliquiritigenin chasing, was catalyzed by C-glucosyltransferase, which transfers a sugar moiety to isoliquiritigenin with the formation of a C-glycosidic linkage. Melissa Brazier-Hicks and colleagues have identified, purified, purified and characterized the enzymes responsible for C-glycoside synthesis in rice and wheat (Brazier-Hicks *et al*., 2009).

C-glucosyltransferase is one of the key enzymes in the biosynthesis pathway of puerarin. However, purification and characterization of C-glucosyltransferase from any species have not been previously reported. In this paper, a method for *C*-glucosyltransferase isolation from *P. lobata* roots and its activity assay by HPLC is described. Effects of substrate concentrations, pH values, different cations, reaction temperatures and reaction time on the activity of C-glucosyltransferase were investigated. This research will be an important basis for the identification the enzyme catalyzing the *C*-glycosylation of isoliquiritgenin to produce puerarin in *P. lobata*.

Materials and Methods

Plant material: *Pueraria lobata* (Willd.) Ohwi was collected from Shukeng Village in the mountainside in Meizhou city of Guangdong

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Province in China. In summer, fresh roots, stems and leaves of three-year-old *P. lobata* were harvested and immediately frozen in liquid N_2. *Nicotiana tabacum* was planted in pots to grow to over 0.3 m in height. The leaves were then collected and treated in the same way as for *P. lobata*.

C-glucosyltransferase preparation: Frozen vegetative organs of the plants were ground to a fine powder in liquid N_2 and homogenized at a ratio of 200 mg fresh weight to 1 ml of homogenization buffer (200 mmol l^{-1} Tris-HCl, 5 mmol l^{-1} DL-dithiothreitol (DTT) and 5 g Polyvinylpyrrolidone (PVP), pH 8.1). After centrifugation at 10,000 rpm for 30 min at 4°C, the supernatant was mixed with solid ammonium sulfate to give 50% (w/v) saturation, stored at 0°C for 1 hr and then centrifuged at 15,000 rpm for 45 min at 4°C. The new supernatant was mixed with solid ammonium sulfate to give final saturation of 80% (w/v) and centrifuged at 15,000 rpm for 45 min at 4°C. The precipitate was re-dissolved with Tris-HCl buffer (pH 8.1) containing 5 mmol l^{-1} DTT, and dialyzed against the same buffer for 12 hr at 4°C.

Determination of puerarin biosynthesis by high performance liquid chromatography (HPLC): The reaction system for the enzyme activity assay consists of 0.1 mol l^{-1} Tris-HCl buffer (pH 8.1) containing 35.5 μmol l^{-1} isoliquiritigenin, 112 μmol l^{-1} UDP-D-glucose (UDP-G) and 5 mmol l^{-1} DTT and the enzyme extract (0.5 -0.8 mg ml^{-1}) in a total volume of 220 μl. For the blank control, the isoliquiritigenin was replaced by water. Reactions were carried out at 28°C for 1 hr and terminated by addition of 10 μl of pure acetic acid. The reaction mixture was centrifuged at 1000 rpm for 5 min and the supernatant was filtered through a 0.45 μm filter membrane for HPLC analysis. The specific activity of the enzyme is expressed as ng puerarin synthesized per mg protein per min. Protein determination was carried out as described by Bradford (1976) with bovine serum albumin as a standard. Each treatment had over three replicates, and the experiments were repeated three times.

The HPLC (SPD-20A, Shimadzu) conditions were as follows: column, YMC-Pack ODS-A (4.6 mm×250 mm); eluant, MeOH: H_2O (30:70); flow rate, 1.0 ml min^{-1}; detection, 254 nm. The concentration of puerarin was calculated according to the linear regression equation $y = 49811x + 33.992$; $R^2 = 0.9991$, where y is the peak area, x is the concentration of puerarin. The relative standard deviation calculated for puerarin peak areas of 10 replicates was 1.29%. Puerarin concentrations were calculated from the standard curve. The equation was calculated using the standard puerarin (Sigma, St Louis, MO, USA). The results are presented as the means of three replicates. The experiments were repeated three times.

The effective factors on the C-glucosyltransferase activity: For optimizing the reaction system and conditions, the C-glucosyltransferase activity was tested by changing the relative effective factors. The pH was changed from 3.0 to 10.8 in the reaction system. Isoliquiritigenin concentration was 8.9, 17.8, 35.5, 53.3 and 78.1 μmol l^{-1}; UDP-G concentration was 112, 224, 336, 448 and 556 μmol l^{-1}. The reaction conditions were also optimized, such as the temperatures ranging from 24 to 27°C and the incubation time varying from 0.5 to 2 hr. To analyze the effect different ions on the enzyme activity, an investigation was carried out by adding 1 mmol l^{-1} Al^{3+}, Mg^{2+}, Ca^{2+}, Cu^{2+}, Mn^{2+}, Zn^{2+} and EDTA in the reaction system respectively.

Enzyme activity assay by thin-layer chromatography (TLC): 1 g Silica gel G 250 was mixed with 3 ml 0.5% sodium carboxy methyl cellulose and homogenized in a mortar. TLC was performed on 10 cm×10 cm glass backed silica gel, 0.2 mm thick TLC plates. The silica gel plate was activated at 110°C for 1 hr before use. TLC was carried out with the solvents chloroform: methanol: water (5: 2: 0.1, v/v/v). The enzyme reaction mixtures were incubated at 28°C for 1 hr and then spotted onto silica gel plate with 2 μl for each spot. The plates were dried at 110°C for 3 min after the separation and scanned by gel imaging system under UV light at 250 nm.

Statistical analysis: All data on enzymatic activity were the mean values of three independent experiments. Statistical analysis was carried out using Student's t-test by DPS statistical package. Each treatment had five replicates, and the experiments were repeated three times.

Results and Discussion

C-Glucosyltransferase activity assay: The C-glucosyltransferase activity could not be detected in crude enzyme extracts before concentrated by precipitation with ammonium sulfate. Its activity was detected in the precipitate of 80 to 100% saturation of ammonium sulfate. The enzyme activity in the precipitate of 80% saturation was 122 and 37% higher than that of 90 and 100% saturation, respectively. This indicated that the highest level of enzyme activity was in the precipitate of 80% saturation of ammonium sulfate (Fig. 1).

The effects of pH on C-glucosyltransferase activity were shown in Fig. 2. These data showed significant differences in C-glucosyltransferase activity at different pH values. The enzyme activity increased dramatically with the elevation of pH value from 3.0 to 8.1, but it started to fall at even higher pH, indicating 8.1 as the optimal pH for the enzyme to catalyze the conversion of isoliquiritigenin to puerarin. The enzyme activity at pH 8.1 was 7.5 times higher than that at pH 3.0. At pH 10.8, the activity was only 1.3% of that at pH 8.1.

C-glucosyltransferase activity in response to different isoliquiritigenin concentrations was measured at optimum pH 8.1 (Fig. 3A). As the concentrations of isoliquiritigenin was going up from 8.9 to 53.5 μmol l^{-1}, the enzyme activity increased. The activity reached the highest level when isoliquiritigenin concentration was 53.5 μmol l^{-1}. But isoliquiritigenin at 78.1 μmol l^{-1} resulted in a slight decrease in C-glucosyltransferase activity.

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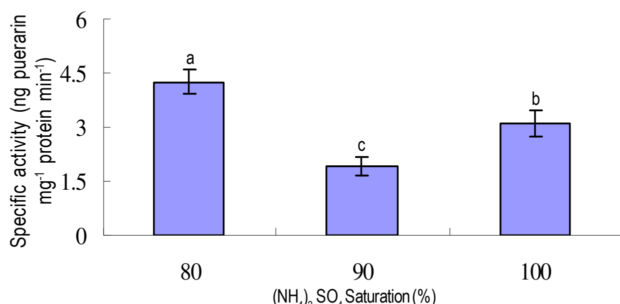


Fig. 1: The effect of $(NH_4)_2SO_4$ saturation on C-glucosyltransferase activity extracted from the root of *P. lobata*. The different letters above the bars indicated the significant differences at $p<0.05$

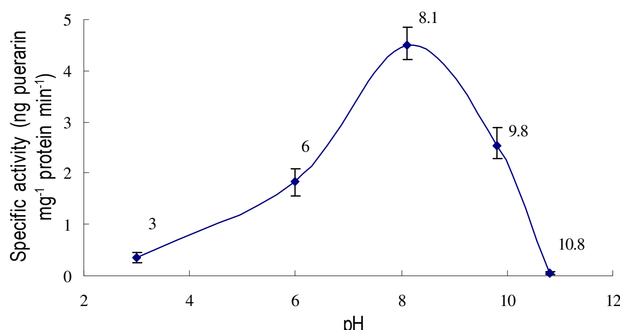
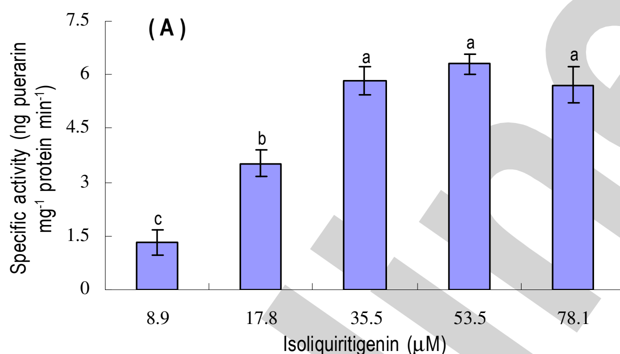


Fig. 2: The effects of pHs on C-glucosyltransferase activity extracted from the root of *P. lobata*



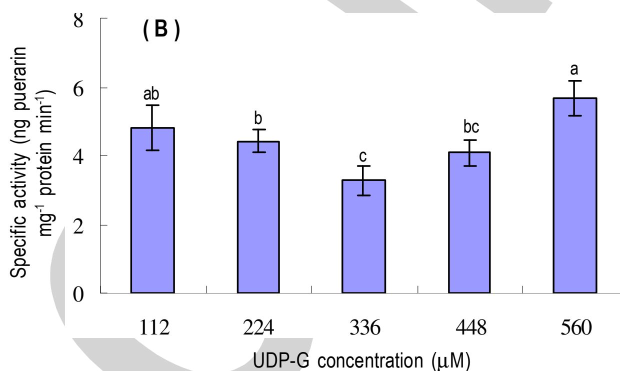


Fig. 3: The effects of isoliquiritigenin (A) and UDP-G concentrations (B) on C-glucosyltransferase activity extracted from the root of *P. lobata*. The different letters above the bars indicated the significant differences at $p<0.05$

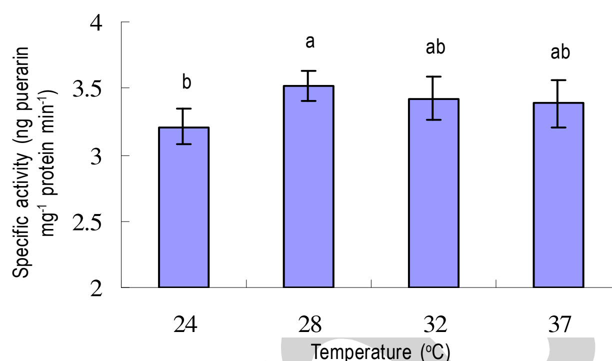


Fig. 4: The effects of temperatures on C-glucosyltransferase activity extracted from the root of *P. lobata*. The different letters above the bars indicated the significant differences at $p<0.05$

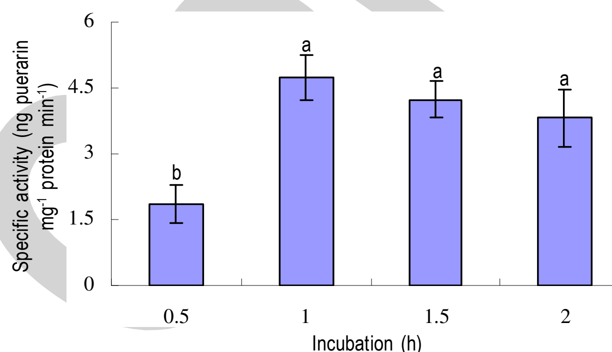


Fig. 5: The effect of incubation time on C-glucosyltransferase activity extracted from the root of *P. lobata*. The different letters above the bars indicated the significant differences at $p<0.05$

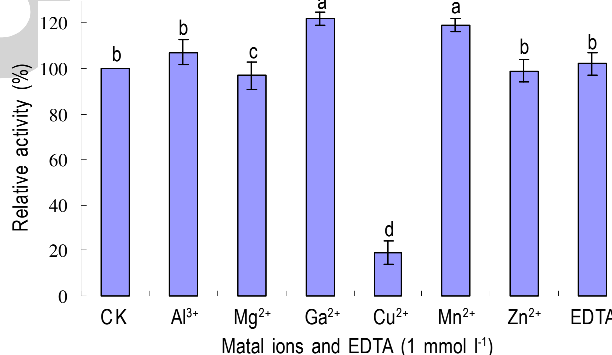


Fig. 6: The effect of inorganic ions and EDTA on C-glucosyltransferase activity extracted from the root of *P. lobata*. The different letters above the bars indicated the significant differences at $p<0.05$

Since no significant difference in C-glucosyltransferase activity was seen between the isoliquiritigenin concentrations of 35.5 and 53.5 $\mu mol\ l^{-1}$, 35.5 $\mu mol\ l^{-1}$ of isoliquiritigenin was used in the standard reaction system.

The effects of various UDP-G concentrations (112 to 560 $\mu mol\ l^{-1}$) on C-glucosyltransferase activity were shown in Fig. 3B. The enzyme activity decreased first and then increased with the changes of UDP-G concentrations from 112 to 336 $\mu mol\ l^{-1}$ and from

336 to 560 μmol l^{-1}, respectively. The lowest activity was seen at 336 μmol l^{-1} of UDP-G and the highest activity seen at the concentration of 560 μmol l^{-1}.

The activity of C-glucosyltransferase was tested at different points of temperatures: 24, 28, 32 and 37°C for 1 hr (Fig. 4). The highest and lowest activity was at 28 and 24°C, respectively.

The C-glucosyltransferase activity in related to the reaction time was showed in Fig. 5. When the reaction time was prolonged from 0.5 to 1 hr, the activity of C-glucosyltransferase enhanced from 2.1 to 4.9 ng mg^{-1} protein min^{-1}. Within 1.5-2 hr period, the enzyme activity decreased with the increase of reaction time. Based on the above results, it can be concluded that the maximal enzyme activity was at 28°C and pH 8.1 for 1 hr.

An investigation was then extended to the effects of several ions and EDTA on the activity of C-glucosyltransferase. Under the optimal reaction condition, Ca^{2+} was most effective in increasing the enzyme activity among the ions examined. The enzyme activity was increased by 22% over control with 1 mmol l^{-1} Ca^{2+}. In contrast, Cu^{2+} strongly inhibited the enzyme activity, with 80% decrease seen of enzyme activity at 1 mmol l^{-1}. Mn^{2+} slightly increased, while Mg^{2+} slightly inhibited the activity. Al^{3+}, Zn^{2+} or EDTA at 1 mmol l^{-1} did not show any significant effect (Fig. 6).

C-glucosyltransferase activity by TLC: Puerarin exhibited a strong absorption under UV light at 250 nm while isoliquiritigenin had a much weaker absorption at the same spectrum (Fig. 7A). The reaction solution with both isoliquiritigenin and UDP-G added appeared as brighter spots on the TLC plate (Fig. 7B, lanes, 1, 2 and 4). The position of the bright spots was aligned with spot position of puerarin standard (Fig. 7B, lane 7). In comparison, those with either isoliquiritigenin or UDP-G added appeared as dim spots (Fig. 7B, lanes 3 and 5) and the latter may have been caused by other UV-absorbent material rather than puerarin in the reaction. Isoliquiritigenin alone did not give any visible spot (Fig. 7B, lane 6). The results indicated that puerarin synthesized in lanes 1, 2 and 4. The above enzyme preparation method and reaction system established for *P. lobata* were tested for *N. tabacum* and the product (puerarin) formed was analyzed by HPLC. The results showed that these methods did not apply to *N. tabacum* as no puerarin product was detected in the reaction. Parallel reaction showed that puerarin was produced with the enzyme preparation from *P. lobata*. The enzyme (C-glucosyltransferase) activity was higher in root than in leaf and stem (Fig. 8).

An overwhelming number of different glycosides are found in nature, since the sugar moiety can be linked to the oxygen, carbon, nitrogen, or sulfur atoms. While the biosynthesis of O-glycosides as well as S- and N-glycosides is well understood, the study on C-glycoside biosynthesis has been much less documented

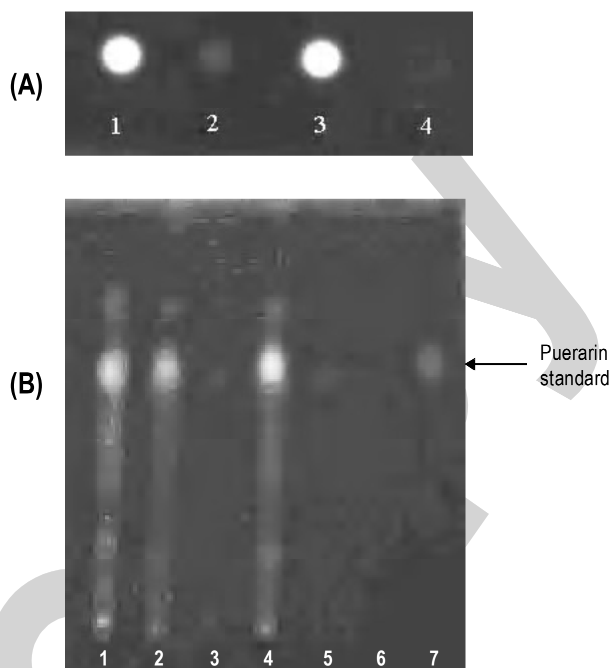


Fig. 7: Assay of C-glucosyltransferase activity. (A) standard substances (1 and 3 puerarin 2 and 4 isoliquiritigenin). (B) root extract assay (The reaction solution contained crude enzyme 0.5 mg ml^{-1}, Tris-HCl buffer 0.15 mol l^{-1}, isoliquiritigenin 0.1 mg ml^{-1}, UDP-G 0.1 mg ml^{-1}, and puerarin 0.1 mg ml^{-1}. 1: without puerarin, 2: without Tris-HCl buffer, 3: without Tris-HCl buffer and UDP-G, 4: all, 5: Crude enzyme and UDP-G, 6: isoliquiritigenin, 7: puerarin)

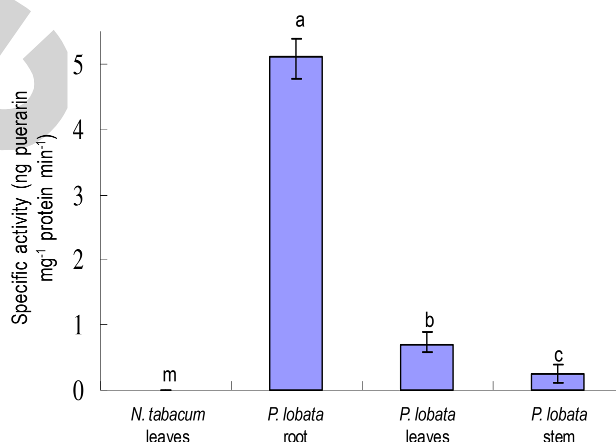


Fig. 8: The activity of C-glucosyltransferase in *N. tabacum* leaves and *P. lobata* (the different letters above the bars indicated the significant differences at $p<0.05$)

(Kerscher and Franz, 1988). Though large numbers of C-glycosides have been obtained from plant sources, little was known about the isolation and properties of C-glucosyltransferase, the enzyme for C-glycoside biosynthesis. Only a few C-glucosyltransferases involved in the biosynthesis of the natural products have been characterized so far (Liu *et al*., 2006), such as, the pathogen-associated C-glucosyltransferase IroB (Fischbach *et al*., 2005; Lin *et al*., 2006), and UrdGT2 (Baig *et al*., 2006). In plant, a few C-

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glucosyltransferases have been detected, purified and/or characterized, including a flavonoid C-glucosyltransferase from *Oryza sativa* (Ko *et al*., 2008), and 2-hydroxyflavanone-6 (or 8)-C-glucosyltransferase from *Fagopyrum esculentum* (Kerscher and Franz, 1987, 1988).

In this experiment, the C-glucosyltransferase from the root of *P. lobata* was partially purified by 50-80% saturation of ammonium sulfate, but it lost the activity after separation through Sephsdex G-25 (data not shown). The TLC method employed in the current study provides a qualitative analysis for puerarin formation, while the HPLC method provides a quantitative analysis for the production of puerarin, which serves an index of C-glucosyltransferase activity. The results demonstrated that *P. lobata* root had higher C-glucosyltransferase activity than leaf and stem. The comparison experiment looking at the product formation indicated that this reaction system worked for *P. lobata* but not for *N. tabacum*. The C-glucosyltransferase from *P. lobata* could convert isoliquiritigenin and UDP-G into puerarin *in vitro*. In this study, only UDP-G was used as sugar donor, because UDP-G is a common glucose donor for most glucosyltransferases involved in secondary metabolism. Some previous reports illustrated that glucosyltransferase exhibited a wide range of substrate specificity for sugar donors (Kerscher and Franz, 1988; Modolo *et al*., 2007). Further investigation is required to better understand the substrate specificity for the catalytic activity of C-glucosyltransferase.

It is known that the optimum pH values for a majority of glucosyltransferases fall within the range of 7.5-8.5. In addition to the pH value, the enzyme activity is also affected by reaction temperature and substrate concentrations (Hosel, 1981). The current study showed that the C-glucosyltransferase isolated from the root of *P. lobata* had an optimum pH of 8.1. The temperature tests indicated that the optimum temperature for C-glucosyltransferase was 28°C, which is consistent the results for other glucosyltransferase results (Yabuya *et al*., 2002).

Our data showed that the effect of UDP-G concentrations on C-glucosyltransferase activity was of two phases. This differs from most other (Kerscher and Franz, 1988), whose activities increased all the way along with the rising of the substrate concentrations tested. Metal ions are one of the most important factors influencing enzyme activity. This study showed that at pH 8.1, Ca^{2+} at 1 mmol l^{-1} stimulated the reaction, Cu^{2+} inhibited the reaction and the Al^{3+}, Zn^{2+} and EDTA had no significant effect. This characteristic of the enzyme was similar to that of hesperetin-7-O-glucosyltransferase (Berhow and Smolensky, 1995).

The C-glucosyltransferase in the crude extract from *P. lobata* root could convert isoliquiritigenin to puerarin. But this was not seen for the extract from *N. tabacum* leaves as measured by HPLC. This suggests the discrepancy in enzyme activities between the different plant species.

To better reveal the characteristics of C-glucosyltransferase, enzyme preparation with higher purity would be required. However, this seems to be difficult due to so many kinds of glucosyltransferases existing in minute amount (Vogt and Jones, 2000). In this study, G25 gel was tried to replace the dialysis method to desalt the crude enzyme preparation, but the result was unsatisfactory because of the significant loss of C-glucosyltransferase activity (data not shown). In order to better understand the biosynthesis of C-glucoside, and the regulation of puerarin and daidzin production in *P. lobata*, the C-glucosyltransferase would be the significant topic which is worth of further study.

Acknowledgments

The authors would like to thank Prof. J. Xu for helpful discussion on this study and Dr. J. M. Lin and Prof. H.H. Li for the revision of the manuscript. This work was supported by a grant from The Natural Science Program of Guangdong Province of China (No. 04010337).

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