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Characterization of germin-like protein with polyphenol oxidase activity from *Satsuma mandarine*

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ABSTRACT

Polyphenol oxidases (PPOs) catalyzing the oxygen dependent oxidation of phenols to quinones are ubiquitously distributed in plants and are assumed to be involved in plant defense against pests and pathogens. A protein with high PPO activity was identified in *Satsuma mandarine*, extracted with Tris–HCl buffer, purified by salt precipitation and column chromatography, and characterized by mass spectrometry as germin-like protein (GLP), which belongs to pathogenesis related protein (PR) family. In the present study, the structure and enzymatic properties of GLP were characterized using spectroscopy methods. Based on native PAGE analysis, the molecular weight of GLP was estimated to be 108 kDa and GLP was identified as a pentamer containing five subunits of 22 kDa. The optimum pH and temperature for PPO catalyzing activity of GLP was 6.5 and 65 °C, respectively. Kinetic constants were 0.0365 M and 0.0196 M with the substrates catechol and pyrogallol, respectively. The structural characterization of GLP provided better insights into the regions responsible for its PPO activity.

1. Introduction

Citrus is the most important evergreen fruit crop of strong economic and nutritional relevance that is widely distributed in tropical and subtropical regions of the world where moisture and soil quality are adequate [1]. Citrus contains abundant vitamin C and phenolic antioxidants, which play an important role in reducing the risk of degenerative diseases, such as cancer, diabetes, cardiovascular, and neurological disease [2]. During storage, citrus always accompanied with browning reaction which is responsible for diminished quality. The investigations regarding citrus browning reaction have been mainly focused on the typical Maillard-type browning [3,4]. Studies about enzymatic browning by polyphenol oxidases (PPOs) of citrus were rarely been reported.

PPOs are widely distributed in plants, which play an important role in browning reaction [5,6], catalyzing the oxygen-dependent oxidation of phenols to quinones. Quinone are highly reactive molecules that covalently modify and cross-link various cellular nucleophiles via a 1, 4 addition mechanism to melanin-like black or brown condensation polymers [7], causing deterioration in color, flavor, and nutrition.

PPOs extracted from different plants such as bananas [8], Bartlett pears [9], tomatoes [7], and Snake fruits [10] have been studied extensively due to their significance in the food industry. However, studies about the characteristics of citrus enzymatic browning-related proteins and their importance to the citrus storage period or defense functions are limited. In this study, a protein in the peel fraction of citrus with high PPO activity was identified as germin-like protein (GLP).

The name germin was given based on the first described member of the family, wheat germin [11]. Further studies on GLPs suggested that GLPs exist in all organs during developmental stages and participate in many processes that are significant for plant development and defense system. Researchers have suggested that GLPs are involved in three types of functions: some possess enzymatic activity, such as oxalate oxidase [12,13] and superoxide dismutase (SOD) [14–16], others seem to be structural proteins, while some others function as receptors [13]. The present study is the first report providing evidence that the plant GLPs possess PPO activity. Because both PPOs and GLPs are known to be involved in plant defense to protect against pests and pathogens [5,6,17,18], it is likely that some inherent relationship or

Abbreviations: GLP, germin-like protein; PPO, polyphenol oxidase; SOD, superoxide dismutase; PR, pathogenesis-related protein; CD, circular dichroism spectrum; PSD, particle size distribution; DSL, dynamic light scattering; Tris, tris(hydroxymethyl)aminomethane; PVPP, cross linking polyvinylpyrrolidone.

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similarities exist between these two classes of proteins. Therefore, we performed detailed biochemical analysis of the GLP extracted from the peels of *Satsuma mandarine* exhibiting PPO activity.

2. Material and methods

2.1. Material and chemicals

Fresh *S. mandarine* were harvested from identical age plants in Wenzhou. The peels were isolated from the fruits, dried under the sun, and stored at 4 °C. All chemicals and reagents used were of analytical grade, purchased from Sigma Chemical Co. (St. Louis, USA). DEAE Sepharose Fast Flow and Sephacryl S200 resin were purchased from GE Healthcare (Milan, Italy). Buffers were prepared with water purified using the Milli-Q purification system (Millipore Ibérica, Madrid, Spain).

2.2. Protein extraction

Citrus peels (200 g) were homogenized with 400 ml of ice-cold 0.5 M Tris–HCl buffer (pH 7.0) containing 5% cross linking polyvinylpyrrolidone (PVPP). The homogenate was kept at 4 °C for 12 h and centrifuged at 5000*g* for 15 min using refrigerated centrifuge (Eppendorf centrifuge 5804, Germany). The supernatant containing the crude protein was collected and fractionated with solid ammonium sulfate at 25% saturation to remove impurities and 80% saturation to precipitate the proteins with PPO activity. The precipitate was resuspended in 0.5 M Tris–HCl buffer (pH 7.0) and dialyzed against the same buffer for 30 h with more than five changes of the buffer during dialysis.

2.3. Protein purification

The protein fraction after dialysis was concentrated using an ultrafilter (Millipore Co., Bedford, MA, USA) and loaded onto a DEAE Sepharose Fast Flow column (2.6 × 30 cm) pre-equilibrated with 0.05 M Tris–HCl buffer (pH 7.0). Protein was eluted with a linear gradient of the buffer containing 0–1.0 M NaCl at a flow rate of 1.2 ml/min. 6.0 ml per fractions were collected and assayed for the PPO activity. The fractions containing PPO activity were pooled and concentrated using a stirred ultrafiltration cup – Amicon® 8050 (Millipore Co., USA) and applied to a Sephacryl S-200 column (1.6 × 90 cm) pre-equilibrated with 0.05 M Tris–HCl buffer (pH 7.0). Protein was eluted with the same buffer at a flow rate of 0.4 ml/min. Fractions with the highest PPO activity were pooled and concentrated and stored at 4 °C.

2.4. Protein content

Protein concentration was measured according to the Bradford method using BSA as a standard [19].

2.5. Assay of PPO activity

The PPO activity was assayed by monitoring the change in absorbance at 420 nm with a UV1800 spectrophotometer (Shimadzu, Kyoto, Japan). The standard reaction mixture contained 0.5 ml of the protein solution (0.2 mol/l) and 0.1 M catechol dissolved in 2.5 ml of 0.1 M phosphate buffer (pH 7.0). One unit of the enzyme activity was defined as the amount of the enzyme that increases 0.001 of absorbance per minute per milliliter of protein solution. The PPO activity was assayed in triplicate.

2.6. Electrophoresis assay

The purity of the protein was determined by native PAGE according to the method of Davis (1964) [20] with minor modifications. The gels were stained with 50 ml of 0.1 M catechol and Coomassie brilliant blue R-250 for activity and molecule weight determination, respectively. For molecular weight and subunit determination, the purified protein with PPO activity was subjected to blue native PAGE and SDS–PAGE [21].

The isoelectric point (IEP) of purified protein was measured by isoelectric focusing electrophoresis (IEF). Protein samples of 40 μg were subjected to IEF between P*I* 3 and 10. The voltage of isoelectric focusing was 30 V for 10 h, 500 V for 30 min, 2000 V for 30 min, 5000 V for 30 min, 8000 V for 1 h, and 10,000 V for 4 h. The gel was stained by Coomassie brilliant blue R-250.

2.7. ESI-QUAD-TOF-MS measurement

The identification analysis of protein with PPO activity was performed using an ESI-QUAD-TOF-MS instrument. The protein band was excised from the gel, digested with trypsin, loaded into an ionization chamber, and finally tested as different peptide sequence.

2.8. Optimal pH and pH stability

To determine the optimal pH for maximal PPO activity, 0.3 ml of 0.02 M catechol was combined with 2.6 ml of appropriate buffers (50 mM sodium citrate buffer for pH 3.5–5.5, 50 mM sodium phosphate buffer for pH 5.0–7.5 and 50 mM Tris–HCl buffer for pH 7.0–9.0) [10], and 0.1 ml protein. The pH stability of the enzyme was determined by incubating the protein in the above-mentioned buffers at 4 °C. After 24 h the protein was dialyzed to neutral pH and the residual activity of the enzyme was analyzed.

2.9. Optimal temperature and temperature stability

The optimal temperature for maximal activity was ascertained by adding the protein solution to the preheated reaction buffer at various temperatures of 25 °C, 35 °C, 45 °C, 55 °C, 65 °C, 75 °C, and 85 °C, then the mixture solution was subjected to spectrophotometric measurements at 420 nm rapidly. The thermal stability of the PPO activity was measured by incubating the tubes containing 0.1 ml protein solution and 2.6 ml 50 mM sodium phosphate buffer (pH 7.0) at various temperatures of 25 °C, 35 °C, 45 °C, 55 °C, 65 °C, 75 °C, and 85 °C in water bath for 5, 15, 30, and 45 min and rapidly cooling to room temperature. Then, 0.3 ml of 0.2 M catechol was added and the residual activity of the samples was measured.

2.10. Substrate specificity

Michaelis–Menten constant (K_m) was determined using catechol, pyrogallol, resorcinol, gallic acid, and chlorogenic acid as substrates dissolved in 100 mM phosphate buffer, pH 7.0 at various concentrations of 0.01, 0.02, 0.03, 0.04, and 0.05 M [22]. The K_m and V_{max} values were calculated from the reciprocal lines of Lineweaver–Burk plot. The kinetic data were drawn as 1/specific activity ($1/V$) versus 1/substrate concentration (1/[S]) by using $1/V$ as the vertical axis and 1/[S] as the abscissa. The vertical axis value is $1/V_{max}$ while abscissa value is $1/K_m$. Optimal protein substrate was obtained based on the constant K_m.

2.11. Circular dichroism (CD) spectral measurement

Protein solution of 1.0 mg/ml dissolved in 50 mM Tris–HCl buffer (pH 6.8) was subjected to CD spectral measurements using protein-free buffer as control. Spectral units were converted into

changes in molar extinction coefficient($\Delta\varepsilon$, $M^{-1}\,cm^{-1}$). The secondary structure composition of the protein with PPO activity was estimated from the CD spectra [23].

2.12. Fluorescence spectral measurement

Fluorescence spectral measurements were performed using an F-4600 Fluorescence spectrophotometer (Hitachi, Japan). Protein solution of 0.6 mg/ml prepared with 50 mM Tris–HCl (pH 6.8) were measured at 350 nm emission wavelength to get the maximum excitation wavelength, and then scanned at the maximum excitation wavelength to record the emission spectra. The operating conditions of excitation spectrum: λ_{ex}: 280 nm and λ_{em}: 300–400 nm; Both E_m slit and E_x slit were set as 5 nm; scan speed was set as 200 nm/min [24].

2.13. Particle size distribution measurement

The protein solution of 0.3 mg/ml at room temperature in 50 mM Tris–HCl buffers (pH 6.8) was used for particle size measurement by Zetasizer Nano-ZS device (Malvern Instruments, Malvern, Worcestershire, U.K.). The size measurement results containing protein particle size and the distribution were reported as the mean and standard deviation from at least five readings. All measurements were carried out in triplicate.

3. Results and discussion

3.1. Protein purification

The protein content and the relative enzyme activities of peak fractions were examined. Fractions 20–30 tubes eluted with 0.2 M NaCl on DEAE Sepharose fast flow column showed maximum enzymatic activity. These fractions were pooled for further purification on Sephacryl S200 column. As shown in Fig. 1(A (B)), the peak with the highest protein content and maximum PPO activity was eluted and collected for further studies.

3.2. Purity and molecular weight

The purity of the protein with PPO activity after final purification was analyzed by native PAGE. As shown in Fig. 1(B), only one band could be seen when the gel was stained with Coomassie brilliant blue R-250, indicating the homogeneity of the protein. When the gel was stained by pyrogallol for activity assay, strong activity was observed at the same protein band confirming that the purified protein was the exact one with PPO activity.

According to the results of native PAGE stained with Coomassie brilliant blue R-250, the molecular weight of the purified protein with the PPO activity was estimated to be 108 kDa. The electrophoretic pattern of the protein on SDS–PAGE revealed that the purified protein of 108 kDa contained five subunits of 22 kDa, indicating that the protein to be a homo multimeric protein and may be a pentamer. Considering the huge molecule of GLP, the active center of PPO may be located either in the cavity correctly folded by these subunits or in the hydrophobic area of each subunit to form a functional district or active center to possess the PPO activity.

3.3. LC–ESI–MS/MS identification

The band on SDS–PAGE gel was analyzed by ESI-QUAD-TOF-MS. The results identified the protein as GLP in *S. mandarine* with a highest matching score of 529 points ($p < 0.05$). The protein number is (gi|52782810) with its molecular weight of 24 kDa.

As shown in Fig. 2(A), some peptides of GLP in *S. mandarine* (SGLP) match with those in *Citrus sinensis* (gi|52782810) and *Citrus limon* (gi|393008685). The peptide matching GLP in *C. sinensis* (gi|52782810) is TDPGHLQDVXVAINDPKXGVFVNRK, while in *C. limon* (gi|393008685), the two peptides, TDPGHLVDVCVAINDP KDGVFVNGKFCKDP and IDYAPYGQRPPHIHPR, match that of GLP. From the matching point of view, GLP in *S. mandarine* (SGLP) and *C. sinensis* (gi|52782810) share a higher degree of identity, suggesting that *C. L.* may share similar response to plant defense elicitors.

GLPs are cell wall glycoproteins with robust quaternary structures presented in all angiosperm families that show unusual resistance to biotic as well as abiotic stress-related processes, such as detergent treatment, heat denaturation and degradation by

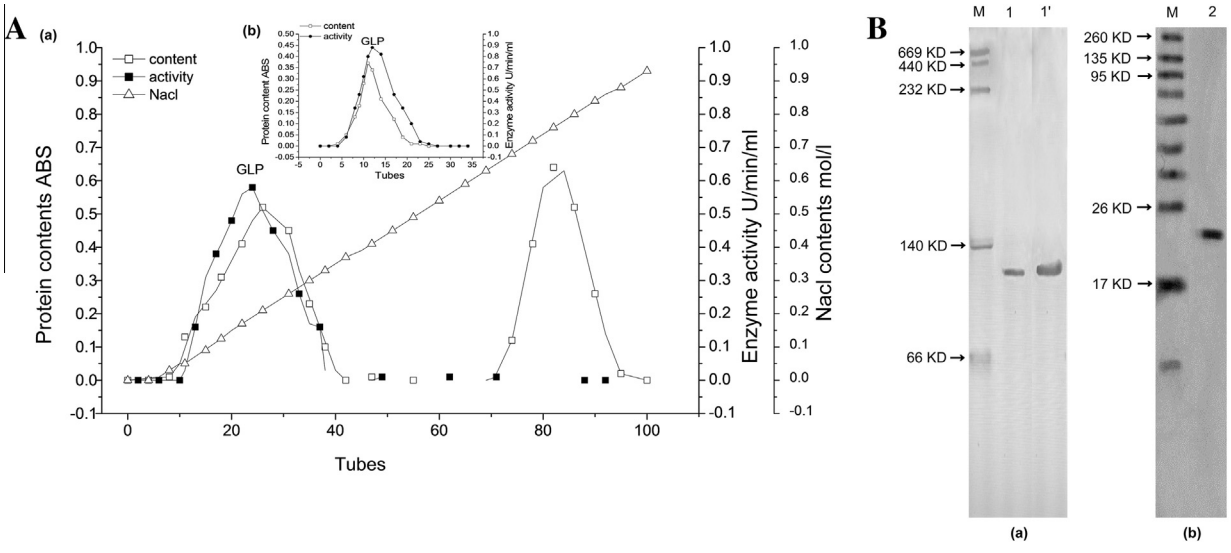

Fig. 1. (A (A)) Elution profiles of proteins from DEAE Sepharose fast flow column. (-□-) represents the protein content, which was measured by the absorbance at 280 nm; (-■-) represents the PPO activity (U/ml/min) of each tubes measured by the activity assay; (-△-) represent the content of NaCl, which elutes proteins from the column and separate the proteins by using different concentration of the eluent. (A (B)) Elution profiles of PPO from a Sephacryl S200 column. (-□-) represents the protein content, which was measured by the absorbance at 280 nm; (-■-) represents the PPO activity (U/min/ml) of each tubes measured by the activity assay. (B) Electrophoresis analyses of GLP. M: Standard protein markers and their molecular masses; Lane 1: Native-PAGE of purified protein with PPO actively stained with Coomassie brilliant blue R-250; Lane 1′: Native-PAGE of purified protein with PPO actively stained with pyrogallol; Lane 2: SDS–PAGE of purified protein stained with Coomassie brilliant blue R-250.

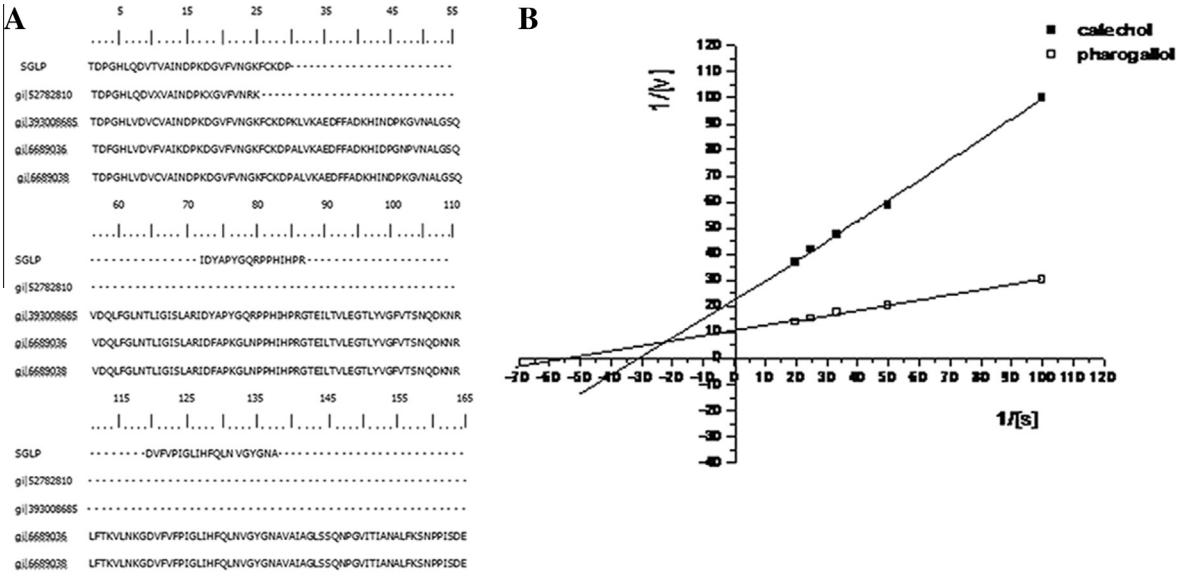

Fig. 2. (A) Alignment of sequenced peptides of SGLP gained from *Satasuma mandarine*. (B) K_m of (-■-) catechol and (-□-) pyrogallol substrates for GLP. The vertical intercept was $1/V_{max}$ while the horizontal intercept was $1/K_m$.

proteases [12,16]. The characteristic feature of this group can vary from one GLP to another based on the source and the protein sequence. Although the functions of GLPs are various, a GLP with PPO activity has not been detected so far.

As the optimal reaction conditions are essential to understand the basis of a functional protein, relevant enzymatic characteristics like acidic/alkaline stability, thermal stability, and structure characteristics were measured in this study.

3.4. Enzymatic analysis of GLP

According to Sener and Ünal [25], K_m value represents the affinity of enzymes to substrates with lower values reflect greater affinity. V_{max} is rate constant for the formation of product from the enzyme substrate (ES) complex. The K_m values of GLP with catechol and pyrogallol substrates were 0.03657 M and 0.01961 M, respectively (Table 1). Pyrogallol showed a higher affinity to the GLP protein. The K_m values were also measured for the substrates resorcin, gallic acid, and chlorogenic acid; the binding of GLP to these substrates was in the millimolar range (data not shown), suggesting weaker substrate binding. The V_{max} values suggested that the reaction rates for GLP were higher using the pyrogallol substrate, indicating that GLP with PPO activity was more active toward pyrogallol. Besides, phenolic-hydroxyl in ortho-position shows higher activity than that of para- or meta-positions [26]. Therefore, pyrogallol is the most appropriate substrate to GLP. However, pyrogallol was prone to spontaneous oxidation in presence of air making it challenging to determine accurate enzyme activity. Therefore, catechol was selected as the main substrate in this study to further characterize GLP.

The optimum reaction pH of GLP with PPO activity was found to be 6.5 (Fig. 3(A)). This result is similar to that of the previous studies [27,28] that used catechol as the substrate for apple and potato PPOs and reported the optimal pH of 6.5 and 6.6, respectively. Results of residual activity (Fig. 3(B)) indicated that GLP incubated at pH 6.0 and 6.5 retained more than 80% of its original activity and dropped to less than 50% of the original activity when incubated at pH below 5.0 or above 7.5. Considering GLP's pI (4.48), it can be inferred that a large proportion of GLP precipitated at acidic condition resulting in low PPO activity. The affinity contact between GLP and its substrate may be affected by the amount of proton in the environment; the weak acidic environment resulted in better substrate binding and higher activity suggesting that certain proteins at a weak acidic pH can be more open or unfolded, exposing the hydrophobic groups resulting in greater binding.

Fig. 3(C) shows the effect of various temperatures on the PPO activity of GLP. Results showed that the relative activity of GLP increased with the increase of temperature up to 65 °C and decreased with a further increase in temperature. The activity was reduced by 62.60% at 85 °C as compared with the highest relative activity. The PPO residual activity (Fig. 3(D)) after incubating the enzyme at different temperatures increased with time at 25 °C and 35 °C. The activity at 45 °C and 55 °C reached to the maxima within 30 min and 15 min, respectively, indicating that higher temperature gives more energy to the transference of PPO active center from the inside to the surface of GLP particle and induces the partial active center of GLP to expose to form a favorable conformation for exhibiting higher PPO activity. The activity decreased with increasing time at 65 °C and 75 °C. The above results indicated that GLP with PPO activity was stable in a temperature range of 20–50 °C and partly denatured at temperatures above 50 °C. This may be due to the unfolding of the tertiary structure of GLP and exposing the active center of the protein at higher temperatures, which resulted in the reduction of the relative activity.

Table 1
Determination of K_m and V_{max} for GLP.

Protein	Substrate	K_m (mol/l)	V_{max} (U/min)	Slope	R^2
GLP	Catechol	0.03657	0.0468	0.7814	0.9986
	Pyrogallol	0.01961	0.09755	0.201	0.9969

3.5. Structure analysis of GLP

As shown in Fig. 4(A), the CD spectra of GLP exhibited a positive peak at 192 nm and two negative minima at 208 nm and 220 nm, a characteristic of the α-helix structure. The conformation contents based on the CONTIN algorithms are shown in Table 2. The contributions of α-helix, β-sheet, and β-turn in GLP were 64.4%, 17%, and 0%, respectively. In proteins, Trp has the highest the molar extinction coefficient and could function as the receptor for energy

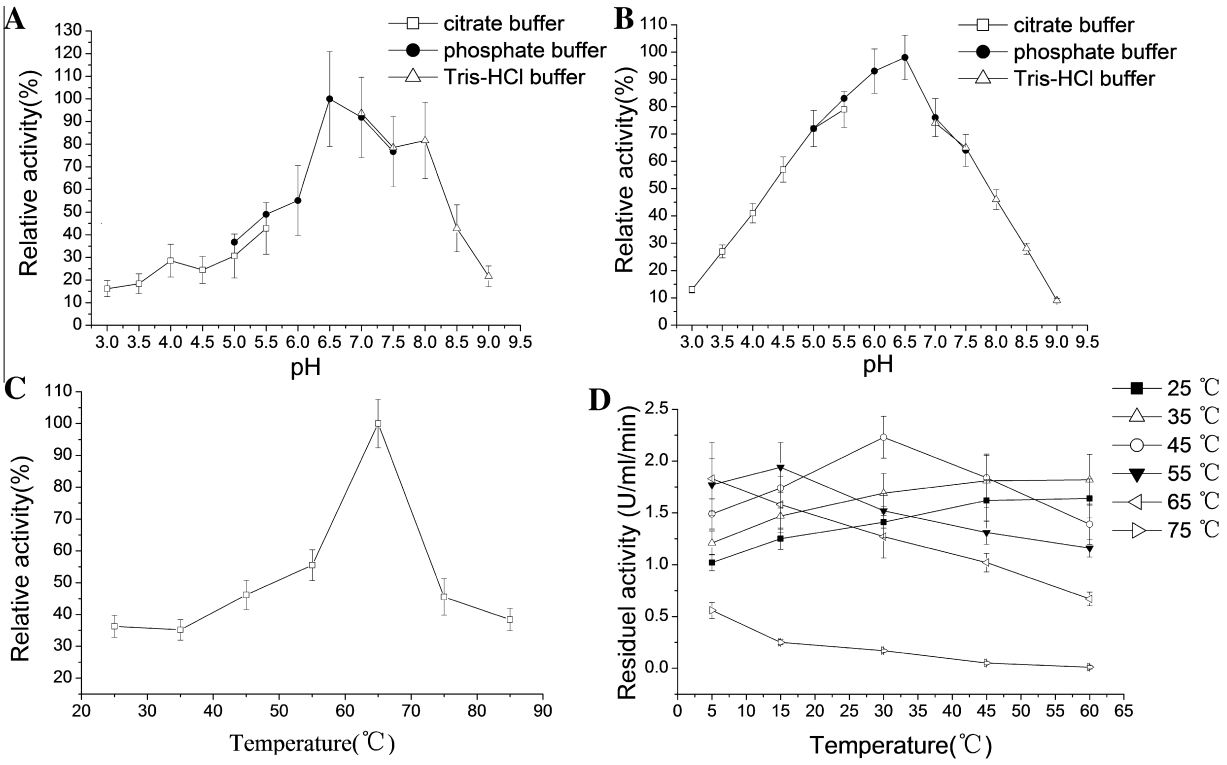

Fig. 3. (A) The PPO activity of GLP as a function of pH value. (-□-) the acetate buffer, pH 3.5–5.5; (-○-) the phosphate buffer, pH 5.0–7.5; (-△-) the Tris–HCl buffer, pH 7.0–9.0. (B) The PPO activity of GLP incubated at different pH value. (-□-) the acetate buffer, pH 3.5–5.5; (-○-) the phosphate buffer, pH 5.0–7.5; (-△-) the Tris–HCl buffer, pH 7.0–9.0. In order to avoid the interference of different buffer systems on protein activities, three pH buffer systems were chosen to made the pH value overlapping by 2 units each other. (C) The PPO activity of GLP at different temperature; (D) The PPO activity of GLP incubated at different temperature. In order to investigate the temperature stability, the proteins were incubated for 5, 15, 30, 45 and 60 min at 25 °C, 35 °C, 45 °C, 55 °C, 65 °C and 75 °C, respectively. The PPO activity was detected after cooling to room temperature.

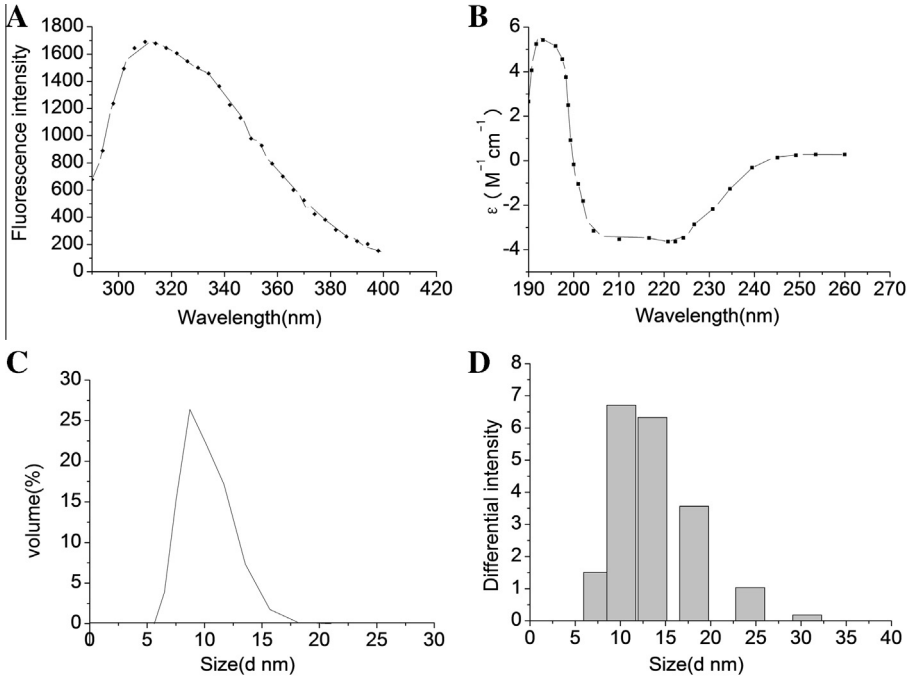

Fig. 4. (A) CD spectra of GLP; (B) Fluorescence spectra of GLP. Samples were dissolved in 50 mmol/l Tris–HCl buffer (pH 6.8), using protein-free Tris–HCl buffer as blank. (C) Particle size distributions of GLP (volume). (D) Particle size distributions of GLP (intensity). Protein solution of 0.3 mg/ml was prepared at 25 °C in 50 mM Tris–HCl buffers (pH 6.8).

transfer among the aromatic residues that contribute to the intrinsic fluorescence [29,30]. Fig. 4(B) shows λ_{max} of GLP was 314 nm with the high intensity of 1695, suggesting that Trp residues in GLP are residing in non-polar hydrophobic environment. As shown in Fig. 4(C), the particle size distribution (PSD) pattern of GLP was characterized with a relatively narrow span of 12 nm in particle

Table 2
Secondary structure contents of GLP.

Protein	α-Helix	β-Sheet	β-Turn	Random coil
GLP	0.646	0.17	0	0.184

diameter and a steep peak value of 26.37% in volume fraction with the corresponding particle diameter of 8.721 nm suggesting GLP in the aqueous system to be monodisperse. Combining the truth of the secondary structure of PPOs is primarily α-helical with the core of the enzyme formed by helix-bundle composed of α-helices [31]. It can be inferred that the high α-helix structure content and several short β-sheets of GLP constitute the helical bundle that can accommodate the catalytic center responsible for PPO activity in monodisperse state.

3.6. The possible defense mechanism involving GLPs

Over the years, studies support the involvement of GLPs in plant defense systems. For citrus GLPs the following defense mechanism is likely to exist: The phenolic compounds accumulate in response to infection, as reported in infected grapevine leaves [32]. Phenolic compounds were supposed to play a role in cell wall reinforcement [33], which may be used as the substrate for citrus GLPs for the generation of high level of quinones and ultimate browning. These quinones and brown pigments are unsavory mass with oxidation products. Furthermore, as the citrus GLPs have five subunits, the phenolic compounds accumulate but could not reach the active center of the protein due to the compact gathering state upon infection. The catalytic process proved to be very slow but permanent, giving the plant a darker outlook and lesser nutrients in response to infections.

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