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Purification and Partial Characterization of β-Glucosidase from Fresh Leaves of Tea Plants (*Camellia sinensis* (L.) O. Kuntze)

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Abstract β-Glucosidases are important in the formation of floral tea aroma and the development of resistance to pathogens and herbivores in tea plants. A novel β-glucosidase was purified 117-fold to homogeneity, with a yield of 1.26%, from tea leaves by chilled acetone and ammonium sulfate precipitation, ion exchange chromatography (CM-Sephadex C-50) and fast protein liquid chromatography (FPLC; Superdex 75, Resource S). The enzyme was a monomeric protein with specific activity of 2.57 U/mg. The molecular mass of the enzyme was estimated to be about 41 kDa and 34 kDa by SDS-PAGE and FPLC gel filtration on Superdex 200, respectively. The enzyme showed optimum activity at 50 °C and was stable at temperatures lower than 40 °C. It was active between pH 4.0 and pH 7.0, with an optimum activity at pH 5.5, and was fairly stable from pH 4.5 to pH 8.0. The enzyme showed maximum activity towards *p*NPG, low activity towards *p*NP-Galacto, and no activity towards *p*NP-Xylo.

Key words *Camellia sinensis*; β-glucosidase; purification; characterization

Tea aroma is one of the most important factors in determining tea quality. Among more than 500 kinds of tea aroma constituents, monoterpene alcohols and aromatic alcohols, which have a floral or fruity (fruit-like) smell, are known to contribute to the floral aroma of tea [1]. β-glucosidases (EC3.2.1.21) are widely distributed throughout the plant kingdom. They hydrolyze β-*D*-glycoside bonds to release non-reducing β-*D*-glucose residues and terminal aglycone. According to recent studies, glycosides with monoterpene alcohols and aryl alcohols as aglycone are abundant in fresh tea leaves. The alcoholic tea aroma is generated by enzymatic hydrolysis of this kind of glucoside during the manufacture of tea, which is especially important for the quality of black tea, Oolong tea and green tea [2,3]. Moreover, β-glucosidases also play a significant role in the resistance of tea plants to pathogens and herbivores by catalyzing the cleavage of defensive glucosides, such as cyanogenic glucosides [4]. Many published studies have focused on the activity assay of the β-glucosidase enzyme and its activity variations during the development of tea plants and in the process of manufacturing tea [5–7].

In this study, we describe the purification and partial enzymatic characterization of a novel β-glucosidase from tea leaves.

Materials and Methods

Materials

Fresh tea leaves (a bud and two leaves of *Camellia sinensis* (L.) O. Kuntze, cv. Fudingdabaicha) were plucked from the tea plantation in Anhui Agricultural University (Hefei, Anhui Province, China) in May 2004. *p*-Nitrophenyl-β-*D*-glucopyranoside (*p*NPG), *p*-nitrophenyl-β-*D*-xylopyranoside (*p*NP-Xylo), *p*-nitrophenyl-β-*D*-galactopyranoside (*p*NP-Galacto) and *p*-nitrophenyl-β-*D*-lactopyranoside (*p*NP-Lacto) were purchased from Sigma Chemical Company (St. Louis, MO, USA). Standard molecular mass markers (12.4–200 kDa) for fast protein liquid chromatography (FPLC) gel filtration, CM-Sephadex

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C-50 prepacked columns of Superdex 75, Superdex 200, and Resource S were all purchased form Amersham Pharmacia Biotech (Uppsala, Sweden). Reagents for gel electrophoresis were purchased from Sangon Biological Engineering Technology & Service Company Limited (Shanghai, China). All other chemicals used were of analytical grade.

Enzyme assay

According to the method described by Jiang and Li [5], one unit of enzyme activity was defined as the amount of β-glucosidase releasing 1 μmol *p*-nitrophenol per minute in the reaction mixture under the assay conditions.

Protein determination

The protein concentration was measured according to the method described by Li *et al.* [8], using bovine serum albumin as the standard. During column chromatography, the absorbance at 280 nm was measured to monitor the protein content.

Purification of β-glucosidase

All operations were performed at 4 °C unless otherwise stated.

Tea leaf acetone powder (150 g), which was prepared by traditional method and stored at –20 °C, was suspended in cold 0.1 M citrate buffer (pH 6.0), stirred slowly for 4 h and centrifuged at 16,000 *g* for 35 min. Chilled acetone (–20 °C) was added to the supernatant (the crude extract) to 50% (*V*/*V*), and the mixture was stirred and then incubated overnight at 4 °C. The precipitate obtained from the crude extract by centrifugation at 16,000 *g* for 35 min was dissolved in the citrate buffer and separated by ammonium sulfate gradient precipitation. The enzyme precipitate was obtained by 45%–75% ammonium sulfate gradient precipitation, redissolved in 20 mM citrate buffer (pH 6.0), and then dialyzed using the same buffer. The desalted enzyme solution was loaded on CM-Sephadex C-50 (1.6 cm×60 cm) previously equilibrated with 20 mM citrate buffer (pH 6.0). The column was eluted with a linear gradient of 0–0.6 M NaCl in 20 mM citrate buffer (pH 6.0), with a flow rate of 18 ml/h. The β-*D*-glucosidase containing enzyme fractions were dialyzed and concentrated by freeze drying.

The concentrated enzyme fraction was loaded on a fast protein liquid chromatography (FPLC) Superdex 75 column (HR10/30, flow rate of 1.2 ml/min) previously equilibrated with 50 mM citrate buffer (pH 6.0) and was eluted with the same buffer. The eluted fractions with β-*D*-glucosidase activity were pooled, concentrated and loaded on a Resource S FPLC column (1 ml) that had been equilibrated with 20 mM citrate buffer (pH 6.0), and then eluted with a linear gradient of NaCl from 0 to 0.5 M in the same buffer at a flow rate of 3 ml/min. The fractions with β-*D*-glucosidase activity were combined to give a total volume of 1 ml.

Purity assay

SDS-polyacrylamide gel electrophoresis (PAGE) was carried out as described by Li *et al*. [8] with 12.5% separating gel and 4.5% stacking gel to determine the purity of the enzyme. The gel was stained with Coomassie brilliant blue R-250 and destained in a solution of 7.5% acetic acid and 5% methanol.

The purified enzyme was also loaded on the Superdex 75 FPLC column to analyze its purity.

Molecular mass determination

The molecular mass of the native enzyme was estimated by FPLC gel filtration on a Superdex 200 HR 10/30 column. The markers were β-amylase (200 kDa), alcohol dehydrogenase (150 kDa), bovine serum albumin (66 kDa), carbonic anhydrase (29 kDa) and cytochrome C (12.4 kDa). The purified enzyme and the markers were loaded on the column, and eluted with 0.50 mM phosphate buffer (pH 7.0) containing 0.15 M NaCl. The linear equation of the standard curve was y=–1.6095x+7.6152 (R^2=0.9902), with log(MW) representing the y-axis and V_e/V_o representing the x-axis.

The subunit molecular mass was determined by SDS-PAGE.

Effect of temperature

The optimum temperature and the thermal stability of the enzyme were analyzed by measuring the enzyme activity at various temperatures (30–60 °C) in the 0.1 M citrate buffer. The thermal stability of the enzyme was determined by incubating it for 30 min and then cooling it to 4 °C.

Effect of pH

The optimum pH level of the enzyme was analyzed by measuring the enzyme activity at various pH levels (pH 3.0–8.0). The pH stability of the enzyme was determined from the residual activity after incubating the enzyme in buffers of pH 3.0–8.0 at 20 °C for 12 h.

Results

The β-*D*-glucosidase (45%–75% ammonium sulfate

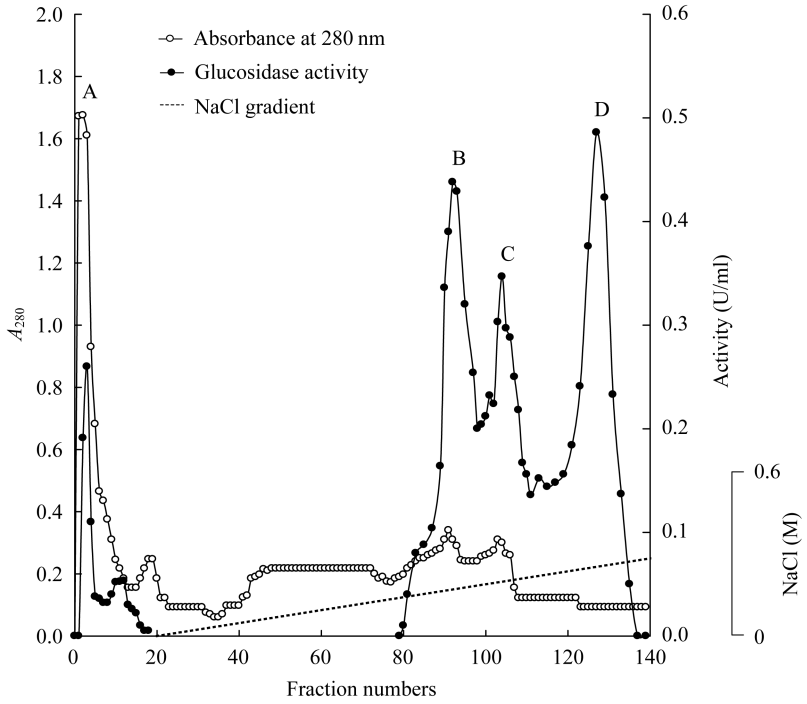

Fig. 1 CM-Sephadex C-50 column chromatogram of β-glucosidase preparation from 45%–75% ammonium sulfate precipitation

precipitation) was chromatographed on a CM-Sephadex C-50 column. The activity was measured using *p*NPG and four active peaks (A–D) were found (**Fig. 1**). Fraction A contained non-absorbed protein (flow-through) whereas the fractions B, C and D were absorbed by CM-Sephadex and eluted using a 0.2–0.4 M NaCl gradient. The specific activity of peak D was the highest among the four fractions and, of the three substrates (*p*NP-Xylo, *p*NP-Galacto and *p*NP-Lacto), fraction D showed only slight activity towards *p*NP-Galacto (**Table 1**). Therefore, fraction D was chosen for further purification. It was loaded on the Superdex 75 FPLC column, and the results showed several protein peaks. Only the first main protein peak had β-glucosidase activity (**Fig. 2**). The fractions were then combined and loaded on a Resource S FPLC column. After that, the enzyme was finally purified (**Fig. 3**). The non-absorbed fractions had no activity, and only the main protein peak obtained during the elution with the NaCl gradient had β-glucosidase activity.

The results of each purification step are summarized in **Table 2**. The β-glucosidase at the final step was purified by about 117-fold to homogeneity, with a yield of 1.26% from the crude enzyme. The purified enzyme showed a single protein band on the SDS-PAGE (**Fig. 4**) and only

Table 1 β-glucosidase activities of fractions from CM-Sephadex C-50 column chromatography

Fractions	Relative activity (%)				Specific activity (U/mg)	Purification (fold)
	*p*NPG	*p*NP-Xylo	*p*NP-Galacto	*p*NP-Lacto		
A	100	27	110	5.25	0.05	2.27
B	100	243	702	38	0.18	5.45
C	100	0	42.3	0	0.80	36.30
D	100	0	21.5	0	1.05	47.70

Relative activity was measured at 20 mM substrate concentrations. Enzyme activity was assayed as described in "Materials and Methods". Activities are expressed as a percentage of the activity calculated with *p*NPG as the substrate.

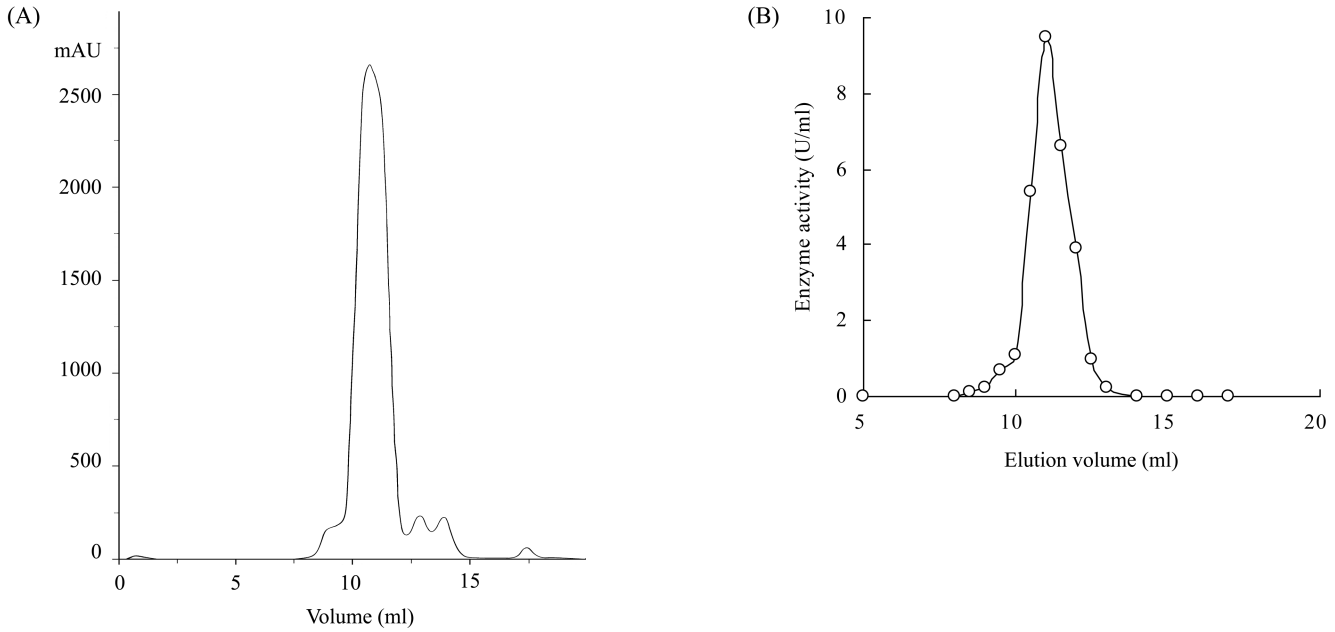

Fig. 2 FPLC results for β-glucosidase using the Superdex 75 column

(A) Elution curve of β-glucosidase protein. (B) Enzyme activity curve of β-glucosidase. The eluting buffer was 50 mM citrate buffer (pH 6.0) and the flow rate was 1.2 ml/min.

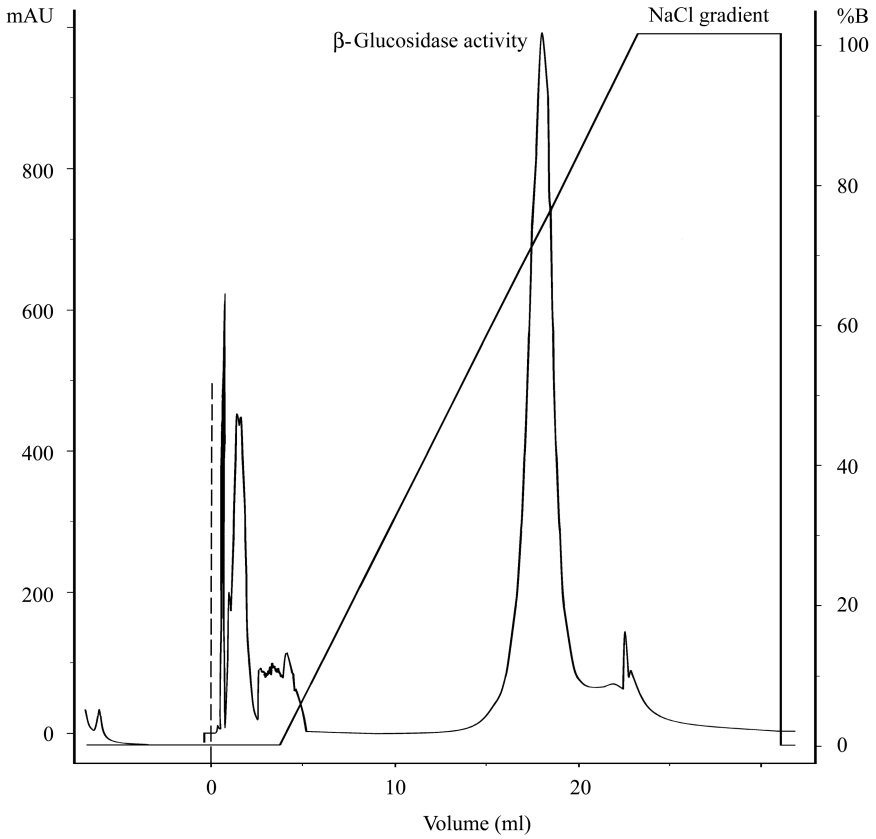

Fig. 3 FPLC results for β-glucosidase using the Resource S column

0–0.5 M NaCl gradient elution, with 20 mM citrate buffer (pH 6.0) as the eluting buffer at flow rate of 3.0 ml/min.

Table 2 Summary of the purification of the β-glucosidase from tea leaves

Purification step	Total protein (mg)	Total activity (U)	Recovery (%)	Specific activity (U/mg)	Purification (fold)
Crude extract	13860.0	304.49	100.00	0.022	1.0
Acetone fraction 50%	3311.0	182.10	59.80	0.055	2.9
45%–75% ammonium sulfate fraction	922.6	154.07	50.60	0.170	7.6
CM-Sephadex C-50 fraction D	15.0	15.67	5.15	1.050	47.5
Superdex 75 FPLC	8.5	12.24	4.00	1.430	65.0
Resource S FPLC	1.5	3.86	1.26	2.570	117.0

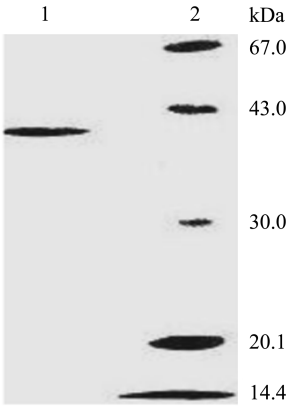

Fig. 4 SDS-PAGE pattern of purified β-glucosidase
1, purified β-glucosidase; 2, molecular mass marker.

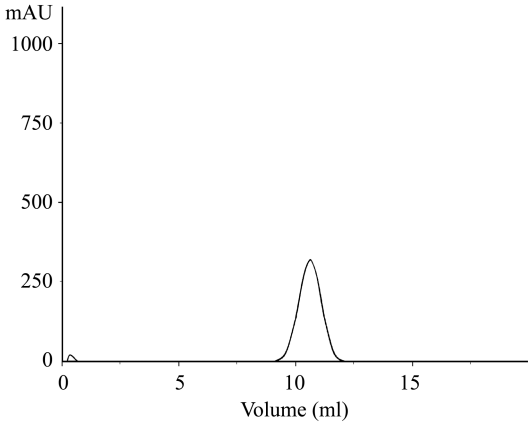

Fig. 5 FPLC of purified β-glucosidase on Superdex 75
The elution conditions are the same as that in **Fig. 2**.

one symmetric eluted protein peak after the final purification step of Superdex 75 FPLC (**Fig. 5**). These findings confirmed the homogeneity of the purified β-glucosidase.

The molecular mass of the native enzyme was estimated to be about 34 kDa, according to the V_e/V_o of the purified enzyme and the linear equation. Based on the mobility of the enzyme protein in SDS-PAGE, the molecular mass of the enzyme was about 41 kDa. These similar results indicate that the β-glucosidase in tea leaves may be a monomeric protein.

The effect of temperature on the activity and stability of the purified β-glucosidase is shown in **Fig. 6**. The enzyme showed maximal activity at 50 °C. The enzyme in 20 mM citrate buffer was fairly stable at temperatures below 40 °C for 30 min. It became inactive when incubated at 60 °C for 30 min. Our further investigation on the enzyme's thermal stability indicated that the enzyme

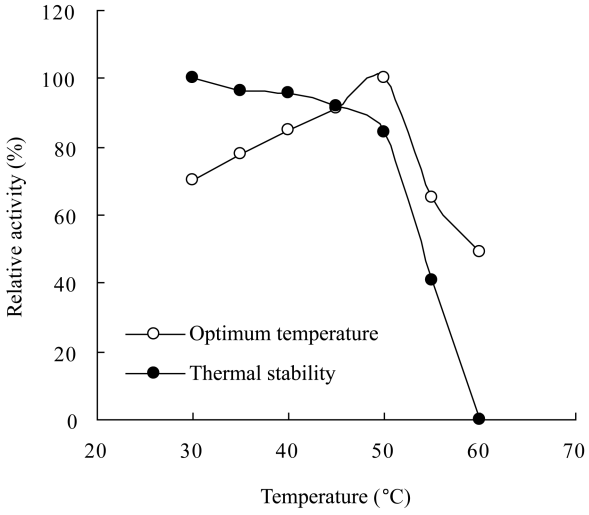

Fig. 6 Effect of temperature on the activity and stability of purified β-glucosidase

activity was 100% of the original level at 4 °C, 94% at 15 °C and 76% at 25 °C for a week (data not shown). This shows that the enzyme is temperature-sensitive and has better stability at low temperature.

The effects of pH on the activity and stability of purified β-glucosidase are shown in **Fig. 7**. From the results, the optimal activity of β-glucosidase was at around pH 5.5. Relatively high activity was observed in the range of pH 4.5 to pH 6.5, with no activity at pH 3.0 and pH 8.0. The enzyme was fairly stable from pH 4.5 to pH 8.0, with 100% activity at pH 6.0, but became unstable at pH levels lower than 4.0, with complete loss of enzyme activity at pH 3.0 for 12 h.

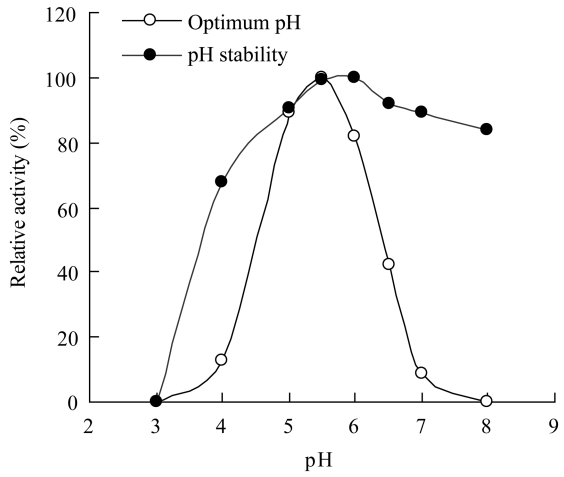

Fig. 7 Effect of pH on the activity and stability of purified β-glucosidase

Substrate specificity of the tea leaf β-glucosidase was determined using different *p*NP glycosides. As shown in **Table 3**, the hydrolytic activities towards other substrates are expressed as a percentage of the activity calculated with *p*NPG (100%). Among all the *p*NP glycosides used here, the purified enzyme specifically hydrolyzed *p*NPG with high efficiency, but displayed low activity towards *p*NP-Galacto and no measurable activity towards *p*NP-α-*D*-glucoside, *p*NP-Xylo and *p*NP-Lacto. However, the crude extract showed different activities towards different glycosides and, among different glycosides, the highest activity was shown towards *p*NP-Galacto. This indicates that β-galactosidase may be abundant in tea leaves.

Table 3 Substrate specificity of β-glucosidase for *p*NP glycosides

Substrate	Relative activity (%)	
	Purified β-glucosidase	Crude extract
*p*NPG	100	100
*p*NP-α-*D*-glucoside	0	10
*p*NP-Xylo	0	26
*p*NP-Galacto	10	244
*p*NP-Lacto	0	12

Measurement of relative activity is as described in **Table 1**.

Discussion

This is our first report on the purification and characterization of a novel β-glucosidase from fresh tea leaves. A number of reports on the β-glucosidases from plants, bacteria and fungi suggest that they play important roles in many biotransformation processes. However, few attempts have been made to investigate the physicochemical characteristics of the β-glucosidases in tea plants.

The specific activity of the purified enzyme is 2.57 U/mg, and the yield is only 1.26%. The low recovery of the enzyme is mainly a result of the presence of various β-glycosidases in the crude extract. Their broad substrate specificity and similar properties make it difficult to choose the correct fraction of β-glucosidase during the isolation and purification process. In addition, there might be other β-glucosidases (isozyme) in tea leaves, which require more purification steps. These factors present significant obstacles to the recovery of the purified enzyme.

Using the β-glucosidase cDNA of tea plant, Li has successfully expressed the mature β-glucosidase protein with the molecular mass of 37 kDa, which is very similar to our purified enzyme, in a pET-32 system [9]. Compared to β-glucosidases from other plants with molecular masses ranging from 56 to 300 kDa [10,11], the tea plant glucosidase is smaller. The molecular mass of the purified enzyme is similar to that of *Candia peltata* (43 kDa) [12] and *Pseudomonas* (33 kDa) [13]. Like many other plants, the β-glucosidase of the tea plant is a monomer. However, there are also other β-glucosidases which exist as oligomers, such as dimers for the grape [14], maize [15] and soybean [16], and tetramers for wheat [11] and vanilla bean [17].

The β-glucosidase reported for other plants shows maximal hydrolytic activity towards *p*NPG, with the

optimal pH level and temperature ranging from pH 4.5 to pH 6.5 and 30 °C to 60 °C, respectively. The optimal activity of our purified enzyme is observed at pH 5.5. The optimum pH level of the purified enzyme is the same as that for β-glucosidases from wheat, rye and citrus [11, 18,19], higher than that for β-glucosidases from rice (pH 4.5), soybean (pH 4.5), barley (pH 5.0) [10,16,20], and lower than that for β-glucosidases from maize (pH 5.8) and vanilla bean (pH 6.5) [15,17]. The optimal temperature (50 °C) of our enzyme is lower than that for β-glucosidases from barley (60 °C) [20], higher than that for β-glucosidases from soybean (45 °C), vanilla bean (40 °C), rye (25–30 °C) and *Citrus sinensis* var. Valencia fruit (40–45 °C) [16–19], and similar to that for β-glucosidases from rice and maize [10,15].

The broad specificity of β-glucosidases for *p*NP glycosides has been observed in many plants. No activity was found for *p*NP-α-*D*-glucoside, *p*NP-Xylo and *p*NP-Galacto in rice β-glucosidases [10]. *p*NP-Galacto and *p*NP-Xylo are hardly hydrolyzed, and *p*NP-α-glucopyranoside is poorly catalyzed by the β-glucosidase of *Citrus sinensis* var. Valencia fruit [19]. Furthermore, the β-glucosidases of rye [18] and sweet cherry [21] respectively hydrolyze *p*-nitrophenyl-β-*D*-fucopyranoside and *o*-nitrophenyl-β-*D*-glucopyranoside more efficiently than *p*NPG. The purified enzyme only hydrolyzes β-linked, synthetic monoglycosides, and shows maximum activity towards *p*NPG, low activity towards *p*NP-Galacto and no activity towards *p*NP-Xylo. The substrate specificities of the tea enzyme for *p*NP glycosides are similar to those of the vanilla bean β-glucosidase [17]. The purification and characterization of the natural substrates (tea aroma precursors) of tea leaf β-glucosidases will help to better clarify the function of the purified β-glucosidase *in vivo* in future.

During the traditional manufacturing processes for tea (such as withering for green tea; withering, rolling and fermentation for black tea; and withering and bruising for Oolong tea), the β-glucosidase is stable below 40 °C, which contributes to the formation and development of the floral tea aroma. However, it has been shown that heat completely inactivates the enzyme activity at 60 °C for 30 min, so the β-glucosidase shows little heat resistance. After the green tea, black tea or Oolong tea leaves are heated by pan-firing or steaming, the β-glucosidase has no activity, so it may have nothing to do with the formation of the tea aroma at the later stages of processing for these types of tea.

The quantitative analysis of glycosidic aroma precursors has revealed that disaccharide glycosides, especially β-primeverosides, are about three times more abundant in tea leaves than β-glucosides in each tea cultivar. β-primeverosidase, a key disaccharide glycosidase, is thought to be the main glycosidase for the formation of tea aroma [22,23]. The sequential mechanism of the hydrolysis of diglycosides suggests that diglycosides are firstly cleaved to yield rhamnose or arabinose and a glucoside that is then hydrolyzed by β-glucosidase [24]. In the case of the formation of the tea aroma, glycosidase has been reported to hydrolyze diglycosides through a sequential mechanism [25], although the hydrolysis of diglycosides mainly occurs in one step with the release of the aglycone moiety and disaccharide moiety catalyzed by a specific β-primeverosidase. This implies that β-glucosidase plays a role in the formation of the tea aroma.

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