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Note

Purification and Characterization of β-Glucosidase Involved in the Emission of 2-Phenylethanol from Rose Flowers

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β-Glucosidase was partially purified from *Rosa* 'Hoh-Jun' petals. The enzyme was highly specific for such β-D-glucopyranosides as 2-phenylethyl β-D-glucopyranoside. The optimal activity was observed at pH 6.0 and 35 °C. The enzymes were composed with two proteins (160 and 155 kDa) by blue native-PAGE, and were classified in a family 1 glucosidase based on LC-MS/MS analyses.

Key words: β-glucosidase; *Rosa* 'Hoh-Jun'; petal; 2-phenylethyl β-D-glucopyranoside; blue native-PAGE

Such flower scent compounds as 2-phenylethanol (2PE), geraniol and benzylalcohol have been reported to be present in the form of monoglycosides and/or diglycosides in plant tissues.[1,2] These glyco-conjugates of volatile compounds are hydrolysed by β-glucosidase or endoglycosidase, *e.g.*, β-primeverosidase, to release the volatile compounds for emission from plant tissues.[3,4] In some flowers, floral scent emission is concurrent with an increase in β-glucosidase activity.[3] 2-Phenylethyl β-D-glucopyranoside (2PE β-Glc) is one of the main glycoconjugates in such Damask roses as *Rosa damascena* Mill. and *R.* 'Hoh-Jun.'[5] In our previous study,[6] we have confirmed that 2-phenyl-*N*-glucosyl-acetamidiumbromide, a glycone-specific β-glucosidase inhibitor, inhibited the hydrolysis of 2PE β-Glc in an enzymatic reaction by using enzymes prepared from the petals of these roses. 2-Phenyl-*N*-glucosyl-acetamidiumbromide also partially inhibited the emission of 2PE from rose petals. These results indicate the involvement of β-glucosidase in the emission of 2PE from rose flowers. To elucidate the role of β-glucosidase, we purified and characterized the enzyme from *R.* 'Hoh-Jun' petals.

The β-glucosidase activity was measured in a standard assay by the method of Yamamoto *et al.*[7] with some modifications. The reaction mixture (200 µl) consisting of 5 mM 2PE β-Glc and an enzyme solution in a 50 mM citrate buffer (pH 6.0) was incubated for 15 min at 30 °C. The reaction was quenched by adding a 0.1 M trichloroacetic acid solution (100 µl). The amount of 2PE released from 2PE β-Glc was analyzed by HPLC under the following conditions: column, RP-18GP Aqua (5 µm, 4.6 × 150 mm); detection, 205 nm; column temperature, 40 °C; sample temperature, 15 °C; injection volume, 20 µl; mobile phase A, a 10 mM phosphate buffer (pH 6.0); mobile phase B, acetonitrile; gradient condition, 35–70% of B over 10 min; flow rate, 1 ml/min. 2PE was detected at 3.3 min. One unit of enzyme activity is defined as the amount releasing 1 µmole of 2PE per min. The glycosidic compounds of the volatile aglycones were chemically prepared according to the reported methods.[6] Protein concentration was measured by using a DC protein assay kit (Bio-Rad), with bovine serum albumin as a standard.

Cell free extracts were prepared from lyophilized *R.* 'Hoh-Jun' flower petals (0.5 g, stages 4 to 6) with 100 ml of buffer A (0.1 M potassium phosphate at pH 7.5, containing 0.5% 3-[(3-cholamidopropyl)-dimethylamino]-1-propanesulfonate (CHAPS), 1 mM EDTA, 2 mM DTT, 0.5 mM PMSF, and 1% glycerol) and 1 g of Polyclar 10. After centrifuging at 4000 × *g* for

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Abbreviations: 2PE, 2-phenylethanol; 2PE β-Glc, 2-phenylethyl β-D-glucopyranoside; BN-PAGE; blue native-polyacrilamide gel electrophoresis; CHAPS, 3-[(3-cholamidopropyl)-dimethylamino]-1-propanesulfonate; *p*NP, *p*-nitrophenyl

20 min, the supernatant was brought to 20–75% saturation with solid ammonium sulfate at 4 °C. The pellet was collected after centrifuging at 4000 × *g* for 20 min, then dissolved in buffer B (10 mM citrate at pH 5.0, containing 0.1% CHAPS and 1% glycerol), and desalted on a PD-10 column that had been equilibrated with buffer B. The desalted solution was applied to an SP Sepharose FF column (3 ml) that had been equilibrated with buffer B. The column was washed with 9 ml of the same buffer, and then eluted with 9 ml of buffer B containing 0.5 M NaCl. The eluted fraction was desalted on a PD-10 column with buffer B. The desalted fraction was applied to a Mono S 4.6/100 PE column that had been equilibrated with buffer B. Proteins with β-glucosidase activity were eluted by a linear gradient of NaCl from 0 M to 0.5 M in buffer B at a flow rate of 1 ml/min. The β-glucosidase activity was detected in a single peak at 0.4 M NaCl. The β-glucosidase-active fractions were combined and concentrated, and the concentrate was dissolved in buffer C (10 mM potassium phosphate at pH 7.0, containing 0.1% CHAPS and 1% glycerol) by ultrafiltration (Nanocep 30K Omega, Pall Corporation). Finally, β-glucosidase was purified 36-fold from the cell free extract from rose petals with a specific activity of 1.1 U/mg and 13% yield. The β-glucosidase sample achieved the maximal activity at pH 6.0 when 2PE β-Glc was used as a substrate. The enzyme was most stable at pH 7.5, but slowly inactivated at below pH 5.0. The optimum temperature for the enzyme reaction was 35 °C, and inactivation occurred at temperatures above 45 °C.

Table 1 shows the substrate specificity of the partially purified enzyme toward various substrates. The enzyme showed high activity toward 2PE β-Glc, (*S*)-citronellyl β-D-glucopyranoside, and *Z*-3-hexenyl β-D-glucopyranoside, moderate activity toward *p*NP β-D-glucopyranoside, and weak activity toward *p*NP β-D-galactopyranoside and 2PE β-D-galactopyranoside. The enzyme showed no activity toward the other substrates. These results suggest that the partially purified enzyme was highly specific to β-glucopyranosides. In addion, the enzyme activity was inihibited by 2-phenyl-*N*-glucosylacetamidiumbromide, a glycone-specific β-glucosidase inhibitor.

Table 1. Substrate Specificity of the Ppartially Purified Enzymes toward the Glycoconjugates of *p*-Nitorophenol and Volatile Compounds

Each substrate was used at a 5 mM concentration. The activity of the 2PE β-Glc sample (1.1 μmol/mg of protein/h) is regarded as 100%. The activities toward the *Z*-3-hexenyl and (*S*)-citronellyl glycosides were also assessed by HPLC. Each value is the mean of duplicate experiments. The enzyme activity was determined spectrophotometrically (405 nm) for *p*-nitrophenol when *p*-nitrophenyl (*p*NP) glycosides were used as substrates.

Substrate	Relative activity (%)
p-nitrophenyl β-D-glucopyranoside	58
p-nitrophenyl β-D-galactopyranoside	18
p-nitrophenyl α-D-glucopyranoside	0
p-nitrophenyl-α-D-galactopyranoside	0
2-phenylethyl β-D-glucopyranoside	100
2-phenylethyl β-D-galactopyranoside	2
2-phenylethyl β-D-xylopyranoside	0
(*S*)-citronellyl β-D-glucopyranoside	88
(*S*)-citronellyl β-D-galactopyranoside	0
(*S*)-citronellyl β-D-xylopyranoside	0
Z-3-hexenyl β-D-glucopyranoside	105
Z-3-hexenyl β-D-galactopyranoside	0
Z-3-hexenyl β-D-xylopyranoside	0

The partially purified enzyme was further separated by blue native-polyacrilamide gel electrophoresis (BN-PAGE), beeing performed by the method of Schägger and Von Jagow[8] with some modifications. After electorophoresis, the gel was stained with 5 mM 4-methylumbelliferyl β-D-glucopyranoside (pH 5.0) for 10 min at 30 °C. Two activity bands were detected at 160 and 155 kDa (Fig. 1A and B, bands 1 and 2, respectively). A gel piece containg two activity bands was cut out, and the proteins were extracted with buffer A to show activities toward 2PE β-Glc and *p*NP β-D-glucopyranoside.

To identify the protein complexes and resolve their composition, these bands were further subjected by two-dimensional BN/SDS–PAGE.[9] The strip from first-dimension BN-PAGE were cut out and then applied to second-dimension SDS–PAGE. Spots 1 and 2 derived from bands 1 and 2, respectively, were detected on the SDS gel (Fig. 1C). The molecular masses of spots 1 and 2 were both approximately 54 kDa. These native proteins were thought to be trimers. Spots 1 and 2 were excised and digested with trypsin, and the resulting peptides were analyzed by LC/Linear Ion Trap-Time of Flight MS. A MASCOT database search of the MS/MS data for the several tryptic peptides derived from spots 1 and 2 indicated that some of the sequences of the peptides were identical to the theoretical tryptic peptides of the prunasin hydrolase isoform PHA precursor in *Prunus serotina* (AAF34650), and β-glucosidase in *Hordeum vulgare* (AAA87339) (Table 2). Three peptides, FGINYVDYDNGLKR, YKEDVGIMK and NMGFDAYR, were identified from both spots 1 and 2, and FSISWSR was identified from spot 1. Although details of the differences in the proteins of spots 1 and 2 were not clear, the sequence motifs detected in the amino acid sequences were those of plant members of the glycoside hydrolase family 1, including *P. avium* β-glucosidase (AAA91166), *P. serotina* amygdalin hydrolase isoform AH I precursor (AAA93234), and *P. serotina* prunasin hydrolase isoform PH B precursor (AAL39079). Many of the β-glucosidases hydrolizing the glycosides of secondary metabolites in planta belong to family 1. The β-glucosidase is therefore suggested to be classified in the family 1 glucosidase.

To clarify the mode of hydrolysis of the β-glucosidases and the relationship between the β-glucosidase and 2PE emission from rose flowers, cloning and characterization of these enzymes are now in progress.

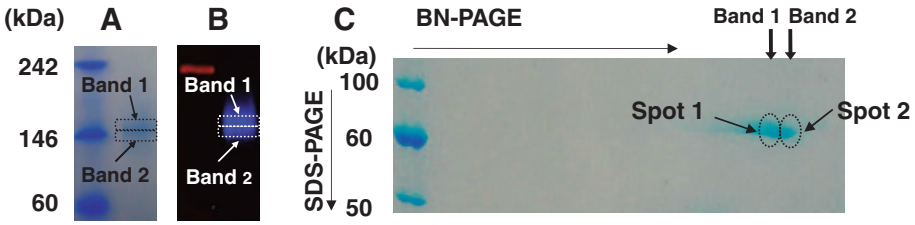

Fig. 1. Identification of the β-Glucosidases in Rose Petals by BN-PAGE and BN/SDS–PAGE.

A, BN-PAGE, Coomassie staining. B, BN-PAGE, activity staining with 4MUG. C, BN/SDS–PAGE, Coomassie staining. BN-PAGE: The sample was prepared by mixing 6.5 µl of the enzyme solution, 2.5 µl of native PAGE sample buffer (Invitrogen), and 1 µl of 5% Coomassie G-250. The sample was electrophoresed on 4–16% polyacrylamide gel with 0.002% Coomassie G250 in the cathode buffer. Native gel protein standards were obtained from Invitrogen.

Table 2. Identified Peptide Fragments in Spots 1 and 2

The peptide sequences were identified by using the Mascot search engine.

Observed m/z	Charge	Sequence	Matched protein	Ion score
Spot 1				
558.6	3+	FGINYVDYDNGLKR	Prunasin hydrolase isoform PHA precursor [*Prunus serotina*]	90
541.7	2+	YKEDVGIMK	Prunasin hydrolase isoform PHA precursor [*Prunus serotina*]	66
495.2	2+	NMGFDAYR+Oxidation	β-Glucosidase [*Hordeum vulgare*]	41
441.7	2+	FSISWSR	β-Glucosidase [*Hordeum vulgare*]	44
Spot 2				
558.6	3+	FGINYVDYDNGLKR	Prunasin hydrolase isoform PHA precursor [*Prunus serotina*]	80
541.7	2+	YKEFVGIMK	Prunasin hydrolase isoform PHA precursor [*Prunus serotina*]	66
487.2	2+	NMGFDAYR	β-Glucosidase [*Hordeum vulgare*]	45

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References

1) Loughrin, J. H., Hamilton-Kemp, T. R., Burton, H. R., Andersen, R. A., and Hildebrand, D. F., Glycosidically bound volatile components of *Nicotiana sylvestris* and *N. suaveolens* flowers. *Phytochemistry*, **31**, 1537–1540 (1992).
2) Schulz, G., and Stahl-Biskup, E., Essential oils and glycosidic bound volatiles from leaves, stems, flowers and roots of *Hyssopus officinalis* L. (Lamiaceae). *Flavour Fragr. J.*, **6**, 69–74 (1991).
3) Reuveni, M., Sagi, Z., Evnor, D., and Hetzroni, A., β-Glucosidase activity is involved in scent production in Narcissus flowers. *Plant Sci.*, **147**, 19–24 (1999).
4) Guo, W., Ogawa, K., Yamauchi, K., Watanabe, N., Usui, T., Luo, S., and Sakata, K., Isolation and characterization of a β-primeverosidase concerned with alcoholic aroma formation in tea leaves. *Biosci. Biotechnol. Biochem.*, **60**, 1810–1814 (1996).
5) Watanabe, S., Hashimoto, I., Hayashi, K., Yagi, K., Asai, T., Knapp, H., Straubinger, M., Winterhalter, P., and Watanabe, N., Isolation and identification of 2-phenylethyl disaccharide glycosides and mono glycosides from rose flowers, and their potential role in scent formation. *Biosci. Biotechnol. Biochem.*, **65**, 442–445 (2001).
6) Hayashi, S., Yagi, K., Ishikawa, T., Kawasaki, M., Asai, T., Picone, J., Turnbull, C., Hiratake, J., Sakata, K., Takada, M., Ogawa, K., and Watanabe, N., Emission of 2-phenylethanol from its β-D-glucopyranoside and the biogenesis of these compounds from [$^2H_8$] L-phenylalanine in rose flowers. *Tetrahedron*, **60**, 7005–7013 (2004).
7) Yamamoto, S., Okada, M., Usui, T., and Sakata, K., Isolation and characterization of a β-primeverosidase-like endo-manner β-glycosidase from *Aspergillus fumigatus* AP-20. *Biosci. Biotechnol. Biochem.*, **66**, 801–807 (2002).
8) Schägger, H., and von Jagow, G., Blue native electrophoresis for isolation of membrane protein complexes in enzymatically active form. *Anal. Biochem.*, **199**, 223–231 (1991).
9) Zerbetto, E., Vergani, L., and Dabbeni-Sala, F., Quantification of muscle mitochondrial oxidative phosphorylation enzymes *via* histochemical staining of blue native polyacrylamide gels. *Electrophoresis*, **18**, 2059–2064 (1997).