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Substrate Specificity of β-Primeverosidase, A Key Enzyme in Aroma Formation during Oolong Tea and Black Tea Manufacturing

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We synthesized nine kinds of diglycosides and a monoglycoside of 2-phenylethanol to investigate the substrate specificity of the purified β-primeverosidase from fresh leaves of a tea cultivar (*Camellia sinensis* var. *sinensis* cv. Yabukita) in comparison with the apparent substrate specificity of the crude enzyme extract from tea leaves. The crude enzyme extract mainly showed β-primeverosidase activity, although monoglycosidases activity was present to some extent. The purified β-primeverosidase showed very narrow substrate specificity with respect to the glycon moiety, and especially prominent specificity for the β-primeverosyl (6-*O*-β-D-xylopyranosyl-β-D-glucopyranosyl) moiety. The enzymes hydrolyzed naturally occurring diglycosides such as β-primeveroside, β-vicianoside, β-acuminoside, β-gentiobioside and 6-*O*-α-L-arabinofuranosyl-β-D-glucopyranoside, but were unable to hydrolyze synthetic unnatural diglycosides. The purified enzyme was inactive toward 2-phenylethyl β-D-glucopyranoside. The enzyme hydrolyzed each of the diglycosides into the corresponding disaccharide and 2-phenylethanol. These results indicate the β-primeverosidase, a diglycosidase, to be a key enzyme involved in aroma formation during the tea manufacturing process.

Key words: substrate specificity; β-primeverosidase; diglycosidase; β-primeveroside; *Camellia sinensis*

Aroma is one of the most important factors to determine the character and quality of a tea product. A recent review has described that more than 600 volatile compounds are concerned with tea aroma; monoterpene alcohols such as linalool and geraniol, and aromatic alcohols such as benzyl alcohol and 2-phenylethanol were reported as the major floral tea aroma constituents.[1] Many of the alcoholic aroma components have been reported to be present as glycosides in tea leaves and to be liberated from their glycosidic aroma precursors by the action of an endogenous glycosidase during the so-called fermentation process during tea manufacturing.[2–4] In addition, attractive flavor compounds such as monoterpenes, C_{13}-norisoprenoids and shikimate-derived compounds have also been reported to accumulate as glycosidic conjugates in flowers, fruits and some other parts of plants.[5–21]

We have been studying the molecular basis for the aroma formation during tea processing. We have previously isolated several kinds of glycosides [β-primeverosides (6-*O*-β-D-xylopyranosyl-β-D-glucopyranosides), β-acuminosides (6-*O*-β-D-apiofuranosyl-β-D-glucopyranosides) and β-D-glucopyranosides] as aroma precursors from fresh leaves of tea cultivars for oolong tea (*Camellia sinensis* var. *sinensis* cv. Shuixian and Maoxie) and green tea (*C. sinensis* var. *sinensis* cv. Yabukita), and found that most of the aroma precursors were present as diglycosides.[22–27] Nishikitani *et al.* have also isolated a diglycoside [β-vicianoside (6-*O*-α-L-arabinopyranosyl-β-D-glucopyranoside)] from fresh leaves of a tea cultivar of cv. Yabukita.[28] Among these diglycosides, β-primeverosides were the most commonly found glycosides. These results suggested that a specific β-glycosidase must be involved in aroma formation during tea processing. We have recently purified a specific glycosidase considered to hydrolyze the diglycosidic aroma precursors from fresh tea leaves of a tea cultivar for green tea (*C. sinensis* var. *sinensis* cv. Yabukita), and tentatively named the enzyme β-primeverosidase.[29] This enzyme had a 61 kDa molecular weight by an SDS-PAGE analysis and was also present in fresh leaves of a tea cultivar for oolong tea (*C. sinensis* var. *sinensis* cv. Shuixian) and for black tea (*C. sinensis* var. *assamica*).[29–31]

Whereas numerous monoglycosidases such as β-

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Abbreviations: ODS, octadecyl silica gel; *p*NP, *p*-nitrophenyl; HRFABMS, high resolution-fast atom bombardment mass spectrometry; BSA, bovine serum albumin

glucosidases and β-galactosidases are found in the plant kingdom and microorganisms, only a few kinds of diglycosidases have been reported in higher plants such as *Rhamnus dahurica* (rhamnodiastase),[32] *Viburnum furcatum* (furcatin hydrolase),[33] *Fagopyrum esculentum* (heteroglycosidase)[34] and *Fogopyrum tataricum* L. (rutinase),[35] and in the fungus, *Aspergillus flavus* (rutinase).[36]

β-Primeverosidase capable of hydrolyzing a β-primeveroside into primeverose and its aglycon was first found in *Primular officinalis* J.[37] This enzyme has been also reported to possibly be present in most of the higher plants containing β-primeverosides.[38] Günata *et al.* have recently detected and identified an enzyme showing β-primeverosidase- and/or rutinosidase-like activity from grape berry skins (cv. M. Alexandria).[39] Recent preliminary studies on the β-primeverosidase of tea leaves have shown its ability to hydrolyze β-acuminoside and β-vicianoside as well as β-primeveroside.[31,40] However, the enzymatic characteristics and substrate specificity of this enzyme are still unclear, because the purity of the enzyme and the number of substrates were limited.

In this paper, we describe the substrate specificity of the purified β-primeverosidase from fresh leaves of cv. Yabukita by using various diglycosides and monoglycosides as substrates. We also describe the synthesis of several kinds of diglycosides and a monoglycoside of 2-phenylethanol, a major alcoholic aroma constituent present as a glycoside in tea leaves.

Materials and Methods

General methods. Pyridine was dried over KOH pellets and stored over 4 Å molecular sieves. Flash column chromatography was performed on silica gel 60 (Merck 9385, 230–400 mesh). Reversed-phase medium-pressure column chromatography was performed with an ULTRA PACK ODS column (ODS-S-50A or 50B, 50 μm, 120 Å; Yamazen Co., Osaka, Japan), monitoring the eluate at 210 nm. ^{1}H- and ^{13}C-NMR spectra were recorded on Varian VXR-200 (200 MHz) and JEOL JNM-AL 400 (400 MHz) instruments with tetramethylsilane as an internal standard. Mass spectra (FAB, glycerol) were obtained with a JEOL JMS 700 spectrometer (JEOL, Tokyo, Japan), and elemental analyses were performed with a Yanaco MT-5 instrument (Yanaco Co., Kyoto, Japan).

Chemicals. 2-Phenylethanol, maltose, lactose, D-xylose and L-arabinose were purchased from Wako Pure Chemical Industries (Osaka, Japan). 2-Phenylethanol was distilled and stored over 4 Å molecular sieves in ampoules. D-Glucose, cellobiose, gentiobiose, melibiose and *p*NP β-D-glucopyranoside were purchased from Sigma-Aldrich Co. Primeverose obtained by transglycosylation between xylobiose and D-glucose with a β-xylosidase from *Aspergillus pulverulentus*[41] was kindly provided by Dr. Okada of Pola Chemical Industries, Japan.

Substrates. *p*NP β-Primeveroside (**11**) was kindly provided by Amano Enzyme Co., Japan. It was also obtained by transglycosylation between xylobiose and *p*NP β-D-glucopyranoside with a β-xylosidase from *A. pulverulentus*. Four other *p*NP monogycosides [*p*NP β-D-glucopyranoside (**12**), β-D-galactopyranoside (**13**), β-D-xylopyranoside (**14**) and β-L-arabinopyranoside (**15**)] were purchased from Sigma-Aldrich Co. The 2-phenylethyl glycosides (**1–10**) were synthesized as described next.

2-Phenylethyl β-primeveroside (6-O-β-D-xylopyranosyl-β-D-glucopyranoside; 1). 1,2,3,4,2′,3′,4′-hepta-*O*-benzoyl-primeverose (**1b**; 1.57 g, 1.51 mmol) prepared from primeverose (**1a**) by the known method[42] was dissolved in DMF (15 ml), and hydrazine acetate (140 mg, 1.51 mmol) was added. After stirring at room temperature for 24 h, the mixture was diluted with $CHCl_3$ and successively washed with sat. aq. NaCl and H_2O. The organic layer was applied to flash chromatography ($CHCl_3$-MeOH, 96:4) to afford 2,3,4,2′,3′,4′-hexa-*O*-benzoyl-primeverose (**1c**; 789 mg, 56%). To a solution of **1c** (789 mg, 0.84 mmol) and CCl_3CN (0.26 ml, 1.26 mmol) in dry CH_2Cl_2 (20 ml) was added dried K_2CO_3 (120 mg, 0.84 mmol). After stirring at room temperature for 12 h, the mixture was filtered and evaporated *in vacuo*. The residue was purified by flash chromatography ($CHCl_3$-MeOH, 97:3) to give 2,3,4,2′,3′,4′-hexa-*O*-benzoyl-primeverosyl trichloroacetimidate (**1d**; 493 mg, 55%). A solution of **1d** (493 mg, 0.46 mmol) and 2-phenylethanol (110 mg, 0.9 mmol) in dry MeCN (15 ml) was treated with trimethylsilyl trifluoromethanesulfonate (TMSOTf; 0.12 ml, 0.46 mmol) at −20°C in an argon atmosphere. After 10 min, the mixture was diluted with CH_2Cl_2 (40 ml), and successively washed with 1 M HCl and sat. aq. $NaHCO_3$. The organic layer was applied to flash chromatography ($CHCl_3$-MeOH, 99:1) to afford 2-phenylethyl 2,3,4,2′,3′,4′-hexa-*O*-benzoyl-β-primeveroside (**1e**; 273 mg, 56%). Compound **1e** (273 mg, 0.26 mmol) was dissolved in MeOH (5 ml), and a 1 M sodium methoxide soln. (2 ml) was added. The mixture was stirred at room temperature for 12 h. After neutralizing with Dowex-50 (H^+-form), the mixture was filtered and evaporated *in vacuo*. Freeze-drying followed by flash chromatography ($CHCl_3$-MeOH, 4:1) gave analytically pure 2-phenylethyl β-primeveroside (**1**) as white powder (82 mg, 77%; total yield from **1a**, 12.5%). Compound **1**: ^{1}H-NMR δ_H (CD_3OD): 2.93 (2H, t, J=7.8 Hz, CH_2CH_2Ph), 3.15–3.21 (3H, H-2, 2′ and 5′a), 3.27–3.34 (3H, H-3, 4 and 3′), 3.43 (1H, m, H-5), 3.48 (1H, m, H-4′), 3.73 (1H, dd, J=11.7 and

5.9, H-6a), 3.77 (1H, dt, J=9.7 and 7.8, OCHH_aCH$_2$Ph), 3.85 (1H, dd, J=11.0 and 5.2, H-5'b), 4.05 (1H, dt, J=9.7 and 7.8, OCH_bHCH$_2$Ph), 4.08 (1H, dd, J=11.7 and 2.2, H-6b), 4.29 (1H, d, J=7.8 Hz, H-1), 4.30 (1H, d, J=7.5 Hz, H-1'), 7.14–7.26 (5H, m, phenyl); ^{13}C-NMR δ_C (CD$_3$OD): 37.2 (CH$_2$CH$_2$Ph), 66.8 (C-5'), 69.6 (C-6), 71.0 (C-4), 71.3 (C-4'), 71.7 (OCH$_2$CH$_2$Ph), 74.7 (C-2), 74.9 (C-2'), 76.8 (C-5), 77.5 (C-3'), 77.8 (C-3), 104.2 (C-1), 105.3 (C-1'), 126.9, 129.1, 129.7, 139.8 (phenyl). Elemental analysis. Found: C, 52.73; H, 6.86%. Calcd. for $C_{19}H_{28}O_{10}\cdot H_2O$: C, 52.53; H, 6.96%. HRFABMS m/z [M+H]$^+$: calcd. for $C_{19}H_{29}O_{10}$, 417.1760; found, 417.1741.

2-phenylethyl β-vicianoside (6-O-α-L-arabinopyranosyl-β-D-glucopyranoside; 2) and 2-phenylethyl-6-O-α-L-arabinofuranosyl-β-D-glucopyranoside; 5). A mixture of 2,3,4-tri-*O*-benzoyl-L-arabinopyranosyl bromide (**2b**) and 2,3,5-tri-*O*-benzoyl-L-arabinofuranosyl bromide (**5b**) was prepared from L-arabinose (**2a**) by the known method.[43] To a solution of the mixture of **2b** and **5b** (1.4 g, 2.1 mmol) and Hg(CN)$_2$ (0.6 g, 2.4 mmol) in dry MeCN (10 ml) was added 2-phenylethyl 2',3',4'-tri-*O*-benzoyl-β-D-glucopyranoside (0.83 g, 1.4 mmol) which had been prepared from 2-phenylethyl β-D-glucopyranoside (**10**) by the reported procedure.[42] The reaction mixture was stirred at room temperature in an argon atmosphere for 36 h, diluted with CH$_2$Cl$_2$ and washed with a 1 M NaHCO$_3$ soln. The organic layer was evaporated and subjected to deacylation by the same procedure as that described for the synthesis of **1** to afford a mixture (850 mg, 39%) of 2-phenylethyl β-vicianoside (**2**) and 6-*O*-α-L-arabinofuranosyl-β-D-glucopyranoside (**5**). This mixture (630 mg, 0.60 mmol) was separated by medium-pressure chromatography (ODS-S-50B column, 26×300 mm; gradient elution by 10–35% MeCN in H$_2$O) and then freeze-dried to give analytically pure **2** (110 mg, 0.27 mmol, 44%; total yield from **2a**, 12.6%) and **5** (78 mg, 0.19 mmol, 31%; total yield from **2a**, 8.8%) as white powder. Compound **2**: ^{1}H-NMR δ_H (CD$_3$OD): 2.93 (2H, t, J=7.6 Hz, CH$_2$CH_2Ph), 3.19 (1H, dd, J=9.2 and 7.6 Hz, H-2), 3.34 (1H, t, J=9.2, H-4), 3.35 (1H, t, J=9.2, H-3), 3.44 (1H, m, H-5), 3.49 (1H, dd, J=8.9 and 3.4, H-3'), 3.49 (1H, dd, J=12.4 and 1.9, H-5'a), 3.58 (1H, dd, J=8.9 and 7.6, H-2'), 3.73 (1H, dd, J=11.4 and 5.8, H-6a), 3.77–3.79 (2H, OCHH_aCH$_2$Ph and H-4'), 3.85 (1H, dd, J=12.4 and 3.2, H-5'b), 4.06 (1H, dt, J=10.1 and 7.6, OCH_bHCH$_2$Ph), 4.08 (1H, dd, J=11.4 and 2.5, H-6b), 4.30 (1H, d, J=7.6, H-1), 4.36 (1H, d, J=7.6, H-1'), 7.16–7.26 (5H, m, phenyl); ^{13}C-NMR δ_C (CD$_3$OD): 37.2 (CH$_2$CH$_2$Ph), 66.7 (C-5'), 69.5 (C-4'), 69.6 (C-6), 71.6 (C-4), 71.9 (OCH$_2$CH$_2$Ph), 72.4 (C-2'), 74.2 (C-3'),75.1 (C-2), 77.0 (C-5), 78.0 (C-3), 104.4 (C-1), 105.2 (C-1'), 127.2, 129.4, 130.1, 140.2 (phenyl). Elemental analysis. Found: C, 52.83; H, 6.81%. Calcd. for $C_{19}H_{28}O_{10}\cdot H_2O$: C, 52.53; H, 6.96%. HRFABMS m/z [M+H]$^+$: calcd. for $C_{19}H_{29}O_{10}$, 417.1760; found, 417.1748.

Compound **5**: ^{1}H-NMR δ_H (CD$_3$OD): 2.98 (2H, t, J=7.3 Hz, CH$_2$CH_2Ph), 3.18 (1H, dd, J=9.2 and 7.9, H-2), 3.29 (1H, t, J=9.2, H-4), 3.35 (1H, t, J=9.2, H-3), 3.44 (1H, m, H-5), 3.61 (1H, dd, J=11.0 and 6.1, H-6a), 3.63 (1H, dd, J=11.9 and 5.5, H-5'a), 3.73 (1H, dd, J=11.9 and 3.4, H-5'b), 3.76 (1H, dt, J=10.1 and 7.3, OCHH_aCH$_2$Ph), 3.82 (1H, dd, J=6.0 and 3.2, H-3'), 3.97 (1H, m, H-4'), 3.99 (1H, dd, J=3.2 and 1.2, H-2'), 4.02 (1H, dd, J=11.0 and 2.5, H-6b), 4.05 (1H, dt, J=10.1 and 7.3, OCH_bHCH$_2$Ph), 4.31 (1H, d, J=7.9, H-1), 4.96 (1H, d, J=1.2, H-1'), 7.14–7.26 (5H, m, phenyl); ^{13}C-NMR δ_C (CD$_3$OD): 37.3 (CH$_2$CH$_2$Ph), 63.1 (C-5'), 68.1 (C-6), 71.9 (OCH$_2$CH$_2$Ph), 72.0 (C-4), 75.1 (C-2), 76.7 (C-5), 78.0 (C-3), 78.9 (C-3'), 83.1 (C-2'), 85.9 (C-4'), 104.5 (C-1), 110.0 (C-1'), 127.2, 129.4, 130.0, 140.0 (phenyl). Elemental analysis. Found: C, 52.70; H, 6.88%. Calcd. for $C_{19}H_{28}O_{10}\cdot H_2O$: C, 52.53; H, 6.96%. HRFABMS m/z [M+Na]$^+$: calcd. for $C_{19}H_{28}O_{10}$Na, 439.1581; found, 439.1580.

2-phenylethyl β-acuminoside (6-O-β-D-apiofuranosyl-β-D-glucopyranoside; 3). Peracetylated 2-phenylethyl β-acuminoside (4 mg, 6 μmol), which had been kindly provided by Dr. Z. Günata of Institut des Products de la Vigne (France), was treated with 5 ml of sat. methanolic ammonia at room temperature for 24 h and purified by medium-pressure chromatography (ODS-S-50A column, 11×300 mm; gradient elution by 10–35% MeCN in H$_2$O) and freeze-dried to give analytically pure 2-phenylethyl β-acuminoside (**3**) as white powder (2 mg, 80%). Compound **3**: ^{1}H-NMR δ_H (CD$_3$OD): 2.93 (2H, t, J=7.8 Hz, CH$_2$CH_2Ph), 3.23 (1H, dd, J=9.2 and 7.9 Hz, H-2), 3.27 (1H, dd, J=9.2 and 7.9 Hz, H-4), 3.31 (1H, dd, J=9.2 and 7.9 Hz, H-3), 3.37 (1H, m, H-5), 3.59 (2H, s, H-5'), 3.62 (1H, dd, J=11.3 and 6.2, H-6a), 3.75 (1H, dt, J=9.7 and 7.8, OCHH_aCH$_2$Ph), 3.78 (1H, d, J=9.7, H-4'a), 3.93 (1H, d, J=2.7, H-2'), 3.99 (1H, d, J=9.7, H-4'b), 4.00 (1H, dd, J=11.3 and 1.9, H-6b), 4.05 (1H, dt, J=9.7 and 7.8, OCH_bHCH$_2$Ph), 4.32 (1H, d, J=7.9 Hz, H-1), 5.04 (1H, d, J=2.7 Hz, H-1'), 7.14–7.26 (5H, m, phenyl); ^{13}C-NMR δ_C (CD$_3$OD): 37.2 (CH$_2$CH$_2$Ph), 65.5 (C-5'), 68.6 (C-6), 71.7 (C-4), 71.7 (OCH$_2$CH$_2$Ph), 74.9 (C-2), 75.0 (C-4'), 76.9 (C-5), 77.9 (C-2'), 77.9 (C-3), 80.4 (C-3'), 104.2 (C-1), 110.8 (C-1'), 126.9, 129.0, 129.7, 139.7 (phenyl). HRFABMS m/z [M+Na]$^+$: calcd. for $C_{19}H_{28}O_{10}$Na, 439.1581; found, 439.1570.

2-Phenylethyl β-gentiobioside (4). To a solution of 2,3,4,2',3',4',6'-hepta-*O*-benzoyl-α-getiobiosyl

bromide (**4b**; 4.4 g, 3.9 mmol), which had been prepared[43] from commercially available gentiobiose (**4a**) in dry CH_2Cl_2 (10 ml), were added 2-phenylethanol (710 mg, 5.8 mmol), dry silver trifluoromethanesufonate (AgOTf, 1.0 g, 3.9 mmol) and tetramethylurea (TMU, 0.64 g, 3.9 mmol), and the mixture was stirred under in argon atmosphere at −20°C for 12 h. The reaction was stopped by adding $NaHCO_3$, and the mixture evaporated. The residue was extracted with EtOAc and washed with sat. aq. $NaHCO_3$. The organic layer was applied to flash chromatography (eluted with CH_2Cl_2) to afford 2-phenylethyl 2,3,4,2′,3′,4′,6′-hepta-*O*-benzoyl-β-gentiobioside (**4c**; 1.5 g, 53%). The benzoylated 2-phenylethyl gentiobioside (**4c**; 1.4 g, 1.2 mmol) was deacylated by the same procedure as that described for the synthesis of **3**. The product was purified by flash chromatography (stepwise elution with $CHCl_3$-MeOH; 10, 30 and 50% MeOH) and by medium-pressure chromatography (ODS-S-50B column, 26× 300 mm; gradient elution with 10–35% MeCN in H_2O). Freeze-drying gave analytically pure 2-phenylethyl β-gentiobioside (**4**) as white powder (470 mg, 95%; total yield from **4a**, 38%). Compound **4**: ^{1}H-NMR δ_H (CD_3OD): 2.93 (2H, t, J=7.4, CH_2CH_2Ph), 3.20 (1H, dd, J=8.9 and 7.8 Hz, H-2), 3.31 (1H, dd, J=9.2 and 8.9 Hz, H-4′), 3.35 (1H, dd, J=9.2 and 8.9 Hz, H-2′), 3.37 (1H, t, J=8.9 Hz, H-3), 3.42 (1H, dd, J=8.9 and 7.8 Hz, H-4), 3.45 (1H, m, H-5), 3.65 (1H, t, J=9.2 Hz, H-3′), 3.67 (1H, m, H-5′), 3.68–3.74 (3H, OCH_aCH_2Ph, H-6a and 6′a), 3.78 (1H, dd, J=11.7 and 2.1 Hz, H-6′b), 3.97 (1H, dd, J=10.7 and 4.1 Hz, H-6b), 4.08 (1H, dt, J=9.7 and 7.4 Hz, OCH_bHCH_2Ph), 4.33 (1H, d, J=7.8 Hz, H-1), 4.84 (1H, d, J=8.9 Hz, H-1′), 7.14–7.26 (5H, m, phenyl); ^{13}C-NMR δ_C (CD_3OD): 37.1 (CH_2CH_2Ph), 62.4 (C-6′), 67.0 (C-6), 71.2 (C-4), 71.5 (C-4′), 71.9 (OCH_2CH_2Ph), 73.4 (C-5′). 73.7 (C-2′), 74.9 (C-2), 75.1 (C-3′), 76.1 (C-5), 77.9 (C-3), 99.7 (C-1′), 104.4 (C-1), 126.9, 129.1, 129.7, 139.6 (phenyl). Elemental analysis. Found: C, 51.82; H, 6.85%. Calcd. for $C_{20}H_{30}O_{11}\cdot H_2O$: C, 51.73; H, 6.95%. HRFABMS m/z $[M+Na]^+$: calcd. for $C_{20}H_{30}O_{11}Na$, 469.1686; found, 469.1673.

2-Phenylethyl β-lactoside (6), β-cellobioside (7), β-maltoside (8), β-melibioside (9) and β-D-glucopyranoside (10). The diglycosides (**6**, **7**, **8** and **9**) and β-D-glucopyranoside (**10**) were prepared from commercially available lactose, cellobiose, maltose, melibiose and D-glucose, respectively, by the same procedure as that used for **4**.

Compound **6** (white powder, 580 mg; total yield from lactose, 41%): ^{1}H-NMR δ_H (CD_3OD): 2.93 (2H, t, J=7.4 Hz, CH_2CH_2Ph), 3.26 (1H, t, J= 7.9 Hz, H-2), 3.40 (1H, m, H-5), 3.47 (1H, dd, J= 9.2 and 3.2 Hz, H-3′), 3.49 (1H, dd, J=9.2 and 8.7 Hz, H-2′), 3.53 (1H, t, J=7.9 Hz, H-3), 3.56 (1H, dd, J=9.4 and 7.9 Hz, H-4), 3.59 (1H, m, H-5′), 3.69 (1H, dd, J=11.4 and 4.6 Hz, H-6′a), 3.75–3.82 (3H, OCH_aCH_2Ph, H-4′ and 6′b), 3.83 (1H, dd, J=12.1 and 4.1 Hz, H-6a), 3.89 (1H, dd, J=12.1 and 2.4 Hz, H-6b), 4.08 (1H, dt, J=9.7 and 7.4 Hz, OCH_bHCH_2Ph), 4.33 (1H, d, J=7.9 Hz, H-1), 4.34 (1H, d, J=8.7 Hz, H-1′), 7.15–7.27 (5H, m, phenyl); ^{13}C-NMR δ_C (CD_3OD): 37.2 (CH_2CH_2Ph), 61.8 (C-6), 62.4 (C-6′), 70.1 (C-4′), 71.6 (OCH_2CH_2Ph), 72.4 (C-2′), 74.6 (C-3′), 74.7 (C-2), 76.3 (C-5), 76.3 (C-3), 76.9 (C-5′), 80.5 (C-4), 104.0 (C-1), 104.8 (C-1′), 126.9, 129.0, 129.7, 139.7 (phenyl). Elemental analysis. Found: C, 53.01; H, 6.75%. Calcd. for $C_{20}H_{30}O_{11}\cdot 0.5H_2O$: C, 52.95; H, 6.86%. HRFABMS m/z $[M+Na]^+$: calcd. for $C_{20}H_{30}O_{11}Na$, 469.1686; Found, 469.1697.

Compound **7** (white powder, 450 mg; total yield from cellobiose, 42%): ^{1}H-NMR δ_H (CD_3OD): 2.93 (2H, t, J=7.8 Hz, CH_2CH_2Ph), 3.22 (1H, dd, J=8.0 and 7.8 Hz, H-2′), 3.25 (1H, dd, J=9.0 and 7.8 Hz, H-2), 3.33–3.38 (3H, H-3′, 4′ and 5′), 3.40 (1H, m, H-5), 3.50 (1H, t, J=9.0, H-3), 3.56 (1H, t, J=9.0, H-4), 3.65 (1H, dd, J=11.9 and 6.3, H-6a), 3.75 (1H, dt, J=9.7 and 7.8, OCH_aCH_2Ph), 3.84–3.89 (3H, H-6b and 6′), 4.07 (1H, dt, J=9.7 and 7.8 Hz, OCH_bHCH_2Ph), 4.33 (1H, d, J=7.8 Hz, H-1), 4.40 (1H, d, J=7.8 Hz, H-1′), 7.14–7.26 (5H, m, phenyl); ^{13}C-NMR δ_C (CD_3OD): 37.2 (CH_2CH_2Ph), 61.7 (C-6), 62.3 (C-6′), 71.2 (C-4′), 71.6 (OCH_2CH_2Ph), 74.7 (C-2), 74.8 (C-2′), 76.3 (C-3), 76.3 (C-5), 77.7 (C-3′), 77.9 (C-5′), 80.5 (C-4), 104.0 (C-1), 104.4 (C-1′), 126.9, 129.0, 129.7, 139.7 (phenyl). Elemental analysis. Found: C, 51.65; H, 6.96%. Calcd. for $C_{20}H_{30}O_{11}\cdot H_2O$: C, 51.73; H, 6.95%. HRFABMS m/z $[M+Na]^+$: calcd. for $C_{20}H_{30}O_{11}Na$, 469.1686; found: 469.1688.

Compound **8** (white powder, 200 mg; total yield from maltose, 36%): ^{1}H-NMR δ_H (CD_3OD): 2.93 (2H, t, J=7.4 Hz, CH_2CH_2Ph), 3.23 (1H, dd, J=9.2 and 7.8 Hz, H-2), 3.26 (1H, dd, J=10.2 and 9.2 Hz, H-4′), 3.36 (1H, m, H-5), 3.44 (1H, dd, J=7.8 and 3.9 Hz, H-2′), 3.53 (1H, t, J=9.2 Hz, H-4), 3.60 (1H, t, J=9.2 Hz, H-3), 3.63 (1H, dd, J=10.2 and 3.9 Hz, H-3′), 3.65–3.69 (2H, H-5′and 6′a), 3.74 (1H, dt, J=10.2 and 7.4 Hz, OCH_aCH_2Ph), 3.77–3.82 (2H, H-6a and 6′b), 3.88 (1H, dd, J=12.1 and 2.1 Hz, H-6b), 4.07 (1H, dt, J=10.2 and 7.4 Hz, OCH_bHCH_2Ph), 4.32 (1H, d, J=7.8 Hz, H-1), 5.15 (1H, d, J=3.9 Hz, H-1′), 7.15–7.27 (5H, m, phenyl); ^{13}C-NMR δ_C (CD_3OD): 37.2 (CH_2CH_2Ph), 62.1 (C-6), 62.6 (C-6′), 71.4 (C-4′), 71.6 (OCH_2CH_2Ph), 74.0 (C-2′), 74.5 (C-2), 74.6 (C-5′), 74.9 (C-3), 76.5 (C-5), 77.7 (C-3′), 81.1 (C-4), 102.7 (C-1′), 104.4 (C-1), 126.9, 129.0, 129.7, 139.6 (phenyl). Elemental analysis. Found: C, 51.77; H, 6.68%. Calcd. for $C_{20}H_{30}O_{11}\cdot H_2O$: C, 51.73; H, 6.95%. HRFABMS m/z $[M+Na]^+$: calcd. for $C_{20}H_{30}O_{11}Na$, 469.1686; found, 469.1676.

Compound **9** (white powder, 35 mg; total yield from melibiose, 31%): ^{1}H-NMR δ_H (CD_3OD): 2.94 (2H, t, J=7.8 Hz, CH_2CH_2Ph), 3.19 (1H, dd, J=9.2 and 7.8 Hz, H-2), 3.35 (1H, dd, J=9.2 and 7.8 Hz, H-3), 3.38 (1H, dd, J=9.2 and 7.8 Hz, H-4), 3.47 (1H, m, H-5), 3.68–3.74 (5H, H-6a, 2′, 3′, 4′ and 6′a), 3.78 (1H, dt, J=9.2 and 7.8 Hz, OCH_aCH_2Ph), 3.85–3.90 (2H, H-5′ and 6′b), 3.96 (1H, dd, J=7.8 and 4.7 Hz, H-6b), 4.04 (1H, dt, J=9.2 and 7.8 Hz, OCH_bHCH_2Ph), 4.33 (1H, d, J=7.8 Hz, H-1), 4.86 (1H, d, J=3.9 Hz, H-1′), 7.15–7.26 (5H, m, phenyl); ^{13}C-NMR δ_C (CD_3OD): 37.2 (CH_2CH_2Ph), 62.6 (C-6′), 67.1 (C-6), 70.4 (C-4′), 70.9 (C-5′), 71.3 (C-4), 71.5 (C-3′), 72.0 (OCH_2CH_2Ph), 72.1 (C-2′). 75.0 (C-2), 76.2 (C-5), 77.9 (C-3), 99.9 (C-1′), 104.4 (C-1), 127.0, 129.1, 129.7, 139.6 (phenyl). Elemental analysis. Found: C, 52.00; H, 6.81%. Calcd. for $C_{20}H_{30}O_{11}\cdot H_2O$: C, 51.73; H, 6.95%. HRFABMS m/z $[M+Na]^+$: calcd. for $C_{20}H_{30}O_{11}Na$, 469.1686; found, 469.1692.

Compound **10** (hygroscopic white powder, 50 mg; total yield from D-glucose, 36%): ^{1}H-NMR δ_H (CD_3OD): 2.93 (2H, t, J=7.8 Hz, CH_2CH_2Ph), 3.14 (1H, dd, J=8.6 and 7.8 Hz, H-2), 3.24 (1H, m, H-5), 3.30 (1H, dd, J=8.6 and 7.8 Hz, H-4), 3.34 (1H, dd, J=8.9 and 7.8 Hz, H-3), 3.67 (1H, dd, J=11.9 and 5.5 Hz, H-6a), 3.75 (1H, dt, J=9.7 and 7.8 Hz, OCH_aCH_2Ph), 3.86 (1H, dd, J=11.9 and 5.5 Hz, H-6b), 4.05 (1H, dt, J=9.7 and 7.8 Hz, OCH_bHCH_2Ph), 4.33 (1H, d, J=7.8 Hz, H-1), 7.14–7.26 (5H, m, phenyl); ^{13}C-NMR δ_C (CD_3OD): 37.2 (CH_2CH_2Ph), 62.9 (C-6), 71.7 (OCH_2CH_2Ph), 71.8 (C-4), 75.3 (C-2), 77.8 (C-5), 78.2 (C-3), 103.7 (C-1), 126.9, 129.0, 129.7, 139.7 (phenyl). Elemental analysis. Found: C, 57.83; H, 7.08%. Calcd. for $C_{14}H_{20}O_6\cdot 0.6H_2O$: C, 57.74; H, 7.19%. HRFABMS m/z $[M+H]^+$: calcd. for $C_{14}H_{21}O_6$, 285.1338; found, 285.1329.

Measurement of the glycosidase activity. Acetone powder (8.8 unit/g) was prepared from fresh leaves of the tea cultivar of cv. Yabukita by the conventional method.[30] The crude enzyme extract (0.44 unit/ml) was prepared by extracting the acetone powder (100 g) with 2 liter of a 20 mM citrate buffer (pH 6.0). β-Primeverosidase was purified from the crude enzyme extract as described previously.[29] The purified enzyme (36 unit/mg) was dissolved in a 20 mM citrate buffer (pH 6.0) and was used as a purified β-primeverosidase stock solution (0.54 unit/ml). The glycosidase activity was determined by measuring the liberation of *p*-nitrophenol or 2-phenylethanol from each glycoside. Each reaction mixture (150 μl) contained a 10 mM substrate, 20 mM citrate buffer (pH 6.0), 0.1% BSA and 60 μl of the enzyme solution. A mixture without the enzyme was preincubated at 37°C, and the reaction was started by adding the enzyme. An aliquot (20 μl) of each enzyme reaction mixture was taken after various time intervals during the incubation. This aliquot of a reaction mixture with *p*NP glycoside was added to 60 μl of a 0.2 M Na_2CO_3 solution, and liberated *p*-nitrophenol was determined at 405 nm. In the reaction with 2-phenylethyl glycosides, 20 μl of the reaction mixture was added to 2 μl of 1 N NaOH, and then 8 μl of an MeCN solution containing 3 μg of benzyl alcohol was added as an internal standard. A sample (20 μl) from each mixture (30 μl) was injected for HPLC (*vide infra*), and 2-phenylethanol (t_R 8.7 min) liberated from each glycoside was detected at 210 nm. The hydrolysis rate was determined from the linear portion (within 10% hydrolysis of the substrate) of each assay curve.

The protein content was measured by the Bradford method[44] with the Coomassie® protein assay reagent (PIERCE, Rockford, Illinois, USA), using BSA as a standard. One unit of activity is defined as the amount of enzyme liberating 1 μmol of *p*-nitrophenol from *p*NP β-primeveroside per min in a 20 mM citrate buffer (pH 6.0) at 37°C.[29]

The kinetic parameters of the purified β-primeverosidase for 2-phenylethyl β-primeveroside (**1**) were determined by the reaction of 0.5–4 mM of **1** and 5 μl of the enzyme solution (0.54 unit/ml) in 150 μl of a 20 mM citrate buffer (pH 6.0) containing 0.1% BSA for 25 min. For β-vicianoside (**2**), 15 μl of the enzyme solution and 5–25 mM of **2** were reacted for 50 min under the same conditions as those for **1**. Aliquots (20 μl) were taken at regular intervals and applied to HPLC as just described. The kinetic parameters and standard deviation were analyzed by the Kaleida graph and estimated by fitting the initial velocity data to the Michaelis-Menten equation.

HPLC analysis. Analysis of liberated 2-phenylethanol was performed under the following conditions: column, YMC-pack ODS-AQ (250×4.6 mm) (YMC Co., Kyoto, Japan); detection, 210 nm with a Waters 996 photodiode array detector; column temperature, 40°C; mobile phase, 33% MeCN; flow rate, 1.0 ml/min.

TLC analysis. TLC was carried out on silica gel 60 F_{254} plates (Merck 5715, 0.25 mm), using a solvent system of butanol-pyridine-water-acetic acid (6:4:3:1, v/v). Glycosides and sugars were detected by heating at 120°C after spraying with 0.2% naphthoresorcinol in H_2SO_4-EtOH (1:19, v/v).[45]

Results and Discussion

Synthesis of the substrates

In order to clarify the substrate specificity of β-primeverosidase for the glycon moiety, nine kinds of diglycosides and a glucoside of 2-phenylethanol were synthesized (Fig. 1). These glycosides were selected

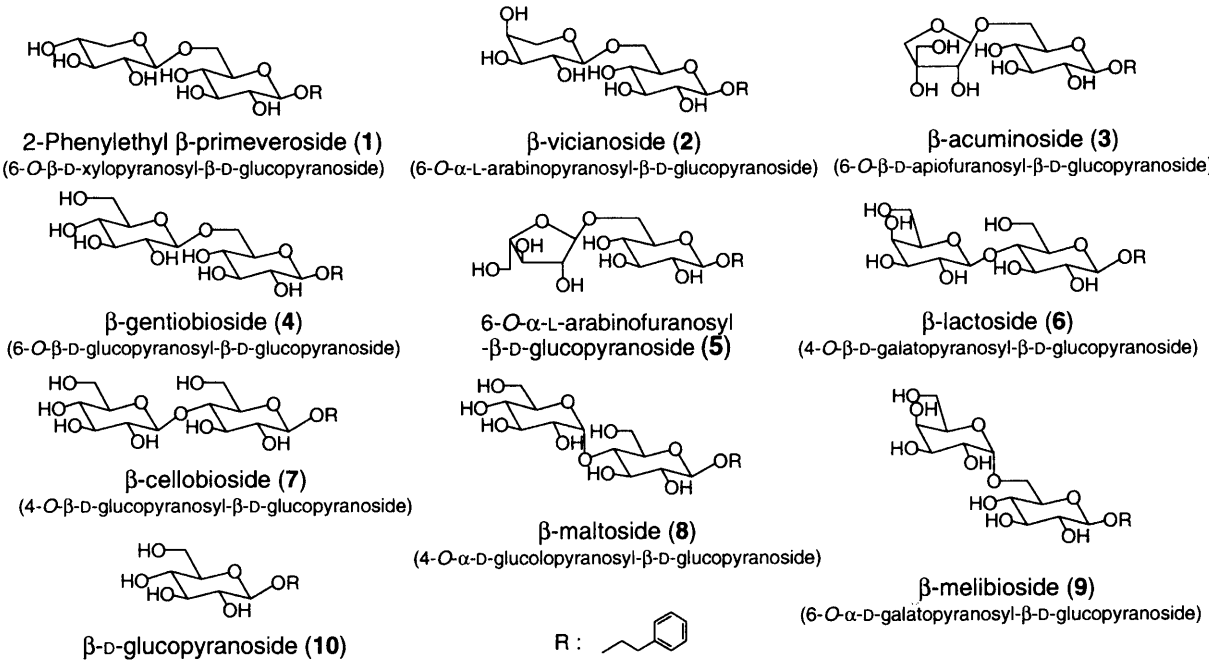
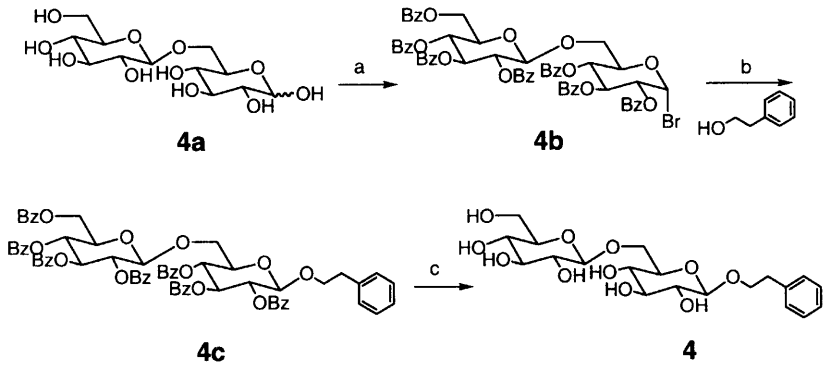
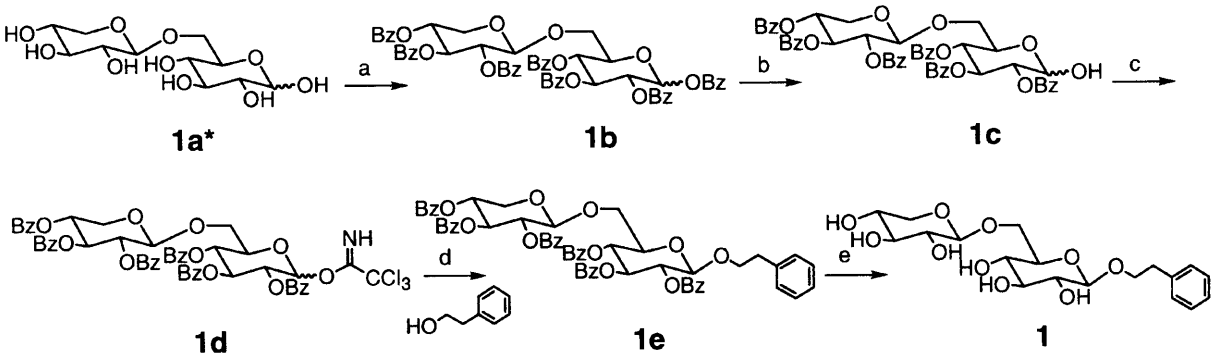


Fig. 1. 2-Phenylethyl Glycosides Designed to Study the Substrate Specificity of β-Primeverosidase.

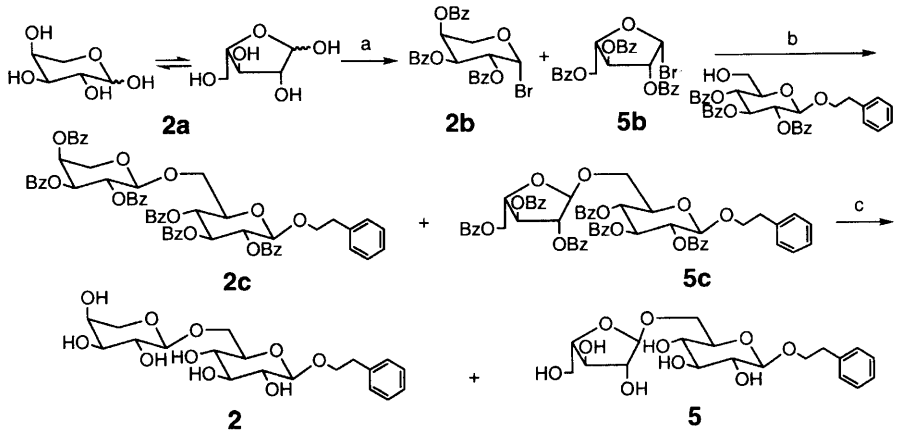


Scheme 1. Synthesis of 2-Phenylethyl β-Gentiobioside (**4**).
a) i. BzCl, pyridine/$CHCl_3$, 24 h. ii. HBr in AcOH, dry CH_2Cl_2, 3 h (76%, 2 steps). b) AgOTf, TMU, dry CH_2Cl_2, −20°C, 12 h (53 %). c) NH_3/MeOH, 24 h (95%).

according to the natural aroma precursors isolated from plants and on the ease of synthesis. β-Primeverosides,[5,6,22-26] β-vicianosides[6,12,28] and β-acuminosides[7,13,22,27] have been found in tea leaves, flowers and fruits, while β-gentiobiosides have been found in fruits,[8,15] and 6-*O*-α-L-arabinofuranosyl-β-D-glucopyranoside found in flowers and fruits.[5,7,9-11] The aglycon moiety was fixed as the 2-phenylethyl group, since 2-phenylethanol is one of the common aroma compounds, and its diglycosides (β-primeveroside and 6-*O*-α-L-arabinofuranosyl-β-D-glucopyranoside) have been isolated from tea leaves, flowers and fruits as aroma precursors.[5,11,25] Five kinds of diglycosides [2-phenylethyl β-gentiobioside (**4**), β-lactoside (**6**), β-cellobioside (**7**), β-maltoside (**8**) and β-melibioside (**9**)] and a monoglycoside [2-phenylethyl β-D-glucopyranoside (**10**)] were synthesized from commercially available disaccharides and D-glucose by the typical synthetic procedure as shown in Scheme 1. The Koenig-Knorr reaction was used in the glycosidation between brominated benzoyl saccharides and the alcohol, except for the synthesis of 2-phenylethyl β-primeveroside (**1**). For the Koenig-Knorr reaction, 1-bromo derivatives of benzoyl saccharides have to be prepared as common intermediates. However, the standard method for the bromination of perbenzoyl primeverose with HBr was accompanied by a side reaction of cleaving the internal glycosidic linkage between xylose and glucose. Although titanium tetrabromide has been used to avoid this side reaction, the yield is relatively low (33%).[46] Therefore, the imidate method with 2,3,4,2′,3′,4′ - hexa - *O* - benzoyl - primeverosyl trichloroacetimidate was used for the synthesis of **1**



Scheme 2. Synthesis of 2-Phenylethyl β-Primeveroside (**1**).
a) BzCl, pyridine/$CHCl_3$, 24 h (94%). b) NH_2-NH_2 in AcOH, dry DMF, 24 h (56%). c) CCl_3CN, K_2CO_3, CH_2Cl_2, 12 h (55%). d) TMSOTf, dry MeCN/CH_2Cl_2, −20°C, 10 min (56%). e) MeONa/MeOH, 12 h (77%).
* Obtained by the transglycosylation reaction with a β-xylosidase from *Aspergillus pulverulentus*.[41]



Scheme 3. Synthesis of 2-Phenylethyl β-Vicianoside (**2**) and 6-*O*-β-L-Arabinofuranosyl-β-D-glucopyranoside (**5**).
a) i. BzCl, pyridine/$CHCl_3$, 36 h. ii. HBr in AcOH, dry CH_2Cl_2, 3 h (73%, 2 steps). b) $Hg(CN)_2$, dry MeCN, 36 h (39%). c) MeONa/MeOH, 12 h (75%).

(Scheme 2). Compounds **2** and **5** were synthesized as a mixture from L-arabinose, which was present as a mixture of the pyranose and furanose forms, and were separated in the last step (Scheme 3). All of the synthesized glycosides (**1**–**10**) were stable for at least 24 h under our assay conditions (data not shown).

Substrate specificity of β-primeverosidase

The hydrolysis pattern of a crude enzyme extract from tea leaves on two synthetic 2-phenylethyl glycosides (**1** and **10**) and on five kinds of commonly used *p*NP glycosides (**11**–**15**) was examined for a preliminary characterization of the enzyme extract (Table 1). The crude enzyme (0.18 unit/ml) was incubated with each glycoside (10 mM) in a 20 mM citrate buffer (pH 6) at 37°C. Liberated 2-phenylethanol was analyzed by HPLC (ODS; 33% acetonitrile), and *p*-nitrophenol was determined at 405 nm. The activity is based on the amount of 2-phenylethanol or *p*-nitrophenol liberated during the assay. The crude enzyme hydrolyzed most of the glycosides: the relative activity on **10**, **11**, **12**, **13**, **14** and **15** was 2.6, 52, 11, 12, 1.2 and 0.4%, respectively, relative to the activity on **1** (100%), although the activity on **14** and **15** was very low. The crude enzyme showed 5–250-fold

Table 1. Hydrolytic Activities of the Crude Enzyme Extract and Purified β-Primeverosidase from Tea Leaves

Substrate	Relative activity (%)*	
	Crude enzyme extract	Purifed β-primeverosidase
2-phenylethyl β-primeveroside (**1**)	100	100
2-phenylethyl β-D-glucopyranoside (**10**)	2.6	ND
*p*NP β-primeveroside (**11**)	52	54
*p*NP β-D-glucopyranoside (**12**)	11	0.2
*p*NP β-D-galactopyranoside (**13**)	12	ND
*p*NP β-D-xylopyranoside (**14**)	1.2	ND
*p*NP β-L-arabinopyranoside (**15**)	0.4	ND

ND., not detected.
* Substrates: 2-phenylethyl and *p*NP glycosides (10 mM); enzymes: the crude enzyme extract (0.18 unit/ml) and purified β-primeverosidase (0.22 unit/ml) from tea leaves; relative activity was calculated after incubating in a 20 mM citrate buffer (pH 6) at 37°C. The liberated 2-phenyethanol was analyzed by HPLC, and *p*-nitrophenol was determined at 405 nm.

Table 2. Substrate Specificity of the Crude Enzyme Extract and the Purified β-Primeverosidase from Tea Leaves toward 2-Phenylethyl Diglycosides

Substrate	Crude enzyme extract		Purified β-primeverosidase	
	Hydrolysis activity* (μmol/min/mg of protein)	Relative activity (%)	Hydrolysis activity* (μmol/min/mg of protein)	Relative activity (%)
2-Phenylethyl β-primeveroside (**1**)	2.33	100	39.1	100
2-Phenylethyl β-vicianoside (**2**)	0.056	2.42	1.16	2.97
2-Phenylethyl β-acuminoside (**3**)	0.014	0.60	0.301	0.77
2-Phenylethyl β-gentiobioside (**4**)	0.008	0.34	0.098	0.25
2-Phenylethyl 6-*O*-α-L-arabinofuranosyl-β-D-glucopyranoside (**5**)	0.001	0.05	0.027	0.07
2-Phenylethyl β-lactoside (**6**)	ND	ND	ND	ND
2-Phenylethyl β-cellobioside (**7**)	ND	ND	ND	ND
2-Phenylethyl β-maltoside (**8**)	ND	ND	ND	ND
2-Phenylethyl β-melibioside (**9**)	ND	ND	ND	ND

ND., not detected.

* Hydrolysis activity is expressed by the initial velocity (μmol/ml/min) per protein concentration (mg/ml). The activity was determined by the liberation of 2-phenyethanol from each glycoside. The liberated 2-phenyethanol was analyzed by HPLC. Each reaction mixture contained 10 mM of a substrate, an enzyme (the crude enzyme extract, 0.18 unit/ml or purified β-primeverosidase, 0.22 unit/ml) and a 20 mM citrate buffer (pH 6), and the mixture was incubated at 37°C.

Table 3. Kinetic Parameters for the Hydrolysis of 2-Phenylethyl β-Primeveroside (**1**) and 2-Phenylethyl β-Vicianoside (**2**) by Purified β-Primeverosidase

Substrate	K_m	k_{cat}^*	k_{cat}/K_m
	mM	s^{-1}	$s^{-1}\,mM^{-1}$
2-Phenylethyl β-primeveroside (**1**)	2.00±0.08	43.7±0.8	21.9
2-Phenylethyl β-vicianoside (**2**)	14.9±1.2	5.85±0.19	0.391

* Protein content was calculated by using BSA as a standard.

higher activity on the β-primeverosides (**1** and **11**) than on the monoglycosides (**10** and **12–15**). The activity on **1** was about 2-fold higher than that on **11**. The β-glucosidase activity (11%) was almost at the same level as the β-galactosidase activity (12%). The activity of these monoglycosidases has been reported in tea leaves, although the enzymatic characterization was limited.[29,30,47]

To ascertain whether the activity on the monoglycosides was solely from monoglycosidases or included in the activity of β-primeverosidase, the relative activity of β-primeverosidase (0.22 unit/ml) purified from tea leaves was determined on the same substrates under the same conditions as that of the crude enzyme extract (Table 1). The purified enzyme showed highly selective activity toward the diglycosidic substrates, β-primeverosides (**1** and **11**). The hydrolysis rate of **1** was about 2-fold higher than that of **11**, which is almost identical to the results with the crude enzyme extract. However, in contrast to the activity of the crude enzyme, most of the monoglycosides (**13**, **14** and **15**) were not hydrolyzed at all, while **12** was hydrolyzed only to a limited extent. These results indicate β-primeverosidase to be the major glycosidase in tea leaves, although other monoglycosidases such as β-glucosidase and β-galactosidase are present to some extent. The 2-phenylethyl group seems to be the more preferred aglycon moiety for β-primeverosidase than the *p*NP group. This is due in part to the fact that *p*NP glycoside is not a natural substrate of the glycosidases in tea leaves.

To investigate the substrate specificity of the tea leaf glycosidases with respect to the disaccharide moiety in more detail, nine synthetic diglycosides (**1–9**) of 2-phenylethanol were tested against the crude enzyme extract as well as the purified β-primeverosidase. Their relative activity toward each 2-phenylethyl diglycoside is summarized in Table 2. The crude enzyme showed 2.42, 0.60, 0.34 and 0.05% of the activity toward **2**, **3**, **4** and **5** relative to the activity of **1**, respectively, but **6–9** were not hydrolyzed at all. The activity of the crude enzyme toward the 2-phenylethyl diglycosides was most likely to have come from β-primeverosidase, because the purified β-primeverosidase showed almost the same hydrolysis rate toward the same substrates. Interestingly, all of the hydrolyzed diglycosides (**1–5**) are naturally occurring and have a β(1→6) or an α(1→6) glycosidic linkage in the glycon moiety. None of the unnatural diglycosides (**6–9**) with a β(1→4) or an α(1→4) glycosidic linkage were hydrolyzed.

To define the substrate specificity with respect to the glycon moiety more quantitatively, the kinetic parameters were determined for two kinds of 2-phenylethyl diglycosides (**1** and **2**), on which the enzyme showed relatively high activity (Table 3). The affinity of **1** (K_m = 2.00 mM) was 7.5-fold higher than that of **2** (K_m = 14.9 mM), and the specificity constant for **1** (k_{cat}/K_m = 21.9 s^{-1} mM) was 56-fold higher than that for **2** (k_{cat}/K_m = 0.391 s^{-1} mM). These results indicate that the substrate specificity of the tea leaf β-primeverosidase is highly specific for β-primeveroside, although β-primeverosidase can hydrolyze not

only β-primeveroside but also β-vicianoside and β-acuminoside. The hydrolysis of β-primeveroside as well as of β-vicianoside and β-acuminoside is likely to occur during the fermentation process while manufacturing oolong tea and black tea. During this fermentation process, β-primeveroside can be expected to be mainly hydrolyzed, because the relative activity toward β-primeveroside is much higher than that toward other glycosides, and the β-primeveroside content is higher than others in tea leaves.[48] This is supported by the report that the amount of β-primeverosides decreasing greatly during the manufacturing process of black tea.[49] These results indicate that β-primeverosidase is deeply concerned in the floral aroma formation during the tea manufacturing process. The substrate specificity of β-primeverosidase may also account for the amount and composition of the floral aroma in oolong tea and black tea. An investigation of the aglycon specificity of this enzyme will give further understanding of this aspect.

Hydrolysis mode of the diglycosides by β-primeverosidase

The hydrolysates of diglycosides **1**, **2** and **11** were analyzed by TLC (silica gel, BuOH-pyridine-H_2O-AcOH = 6:4:3:1). Spots corresponding to disaccharides (primeverose, R_f 0.39; vicianose, R_f 0.28) were clearly observed, but no spots for glucose (R_f 0.48), xylose (R_f 0.60) or arabinose (R_f 0.50) were apparent when the reaction had been completed. This confirms that the β-primeverosidase is a very specific enzyme for disaccharide glycosides to hydrolyze them into aglycons and disaccharides without cleaving the interglycosidic bond between the sugars.

In conclusion, β-primeverosidase was found to be a key enzyme responsible for the floral aroma formation during the manufacturing process of oolong tea and black tea.

Many kinds of disaccharide glycosides, including β-primeverosides with various kinds of aglycons, have been isolated from various species of plants.[5-21] Since β-primeverosidase from tea leaves was clarified to be a highly specific diglycosidase for β-primeverosides, a number of diglycosidases specific to each kind of diglycoside might be present in nature. We have succeeded in gene cloning of the β-primeverosidase,[50] and further plant molecular biological and biochemical studies on it are now in progress to elucidate the real role of the enzyme in tea plants as well as its distribution throughout the plant kingdom.

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References

1) Yamanishi, T., Special issue on tea: Flavor of tea. *Food Review Int.*, **11**, 477–525 (1995).
2) Takeo, T., Production of linalool and geraniol by hydrolytic breakdown of bound forms in disrupted tea shoots. *Phytochemistry*, **10**, 2145–2147 (1981).
3) Fischer, N., Nitz, S., and Drawert, F., Bound flavour compounds in plants: Free and bound flavour compounds in green and black tea. *Z. Lebensm. Unters. Forsch.*, **185**, 195–201 (1987).
4) Morita, K., Wakabayashi, M., Kubota, K., Kobayashi, A., and Herath, N. L., Aglycone constituents in fresh tea leaves cultivated for green and black tea. *Biosci. Biotechnol. Biochem.*, **58**, 687–690 (1994).
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) Watanabe, N., Nakajima, R., Watanabe, S., Moon, J.-H., Inagaki, J., and Sakata, K., Linalyl and bornyl disaccharide glycosides from *Gardenia jasminoides* flowers. *Phytochemistry*, **37**, 457–459 (1994).
7) Oka, N., Ohki, M., Ikegami, A., Sakata, K., and Watanabe, N., First isolation of geranyl disaccharide glycosides as aroma precursors from rose flowers. *Natural Product Letters*, **10**, 187–192 (1997).
8) Winterhalter, P., Harmsen, S., and Trani, F., A C_{13}-norisoprenoid gentiobioside from quince fruit. *Phytochemistry*, **30**, 3021–3025 (1991).
9) Skouroumounis, G. K., and Winterhalter, P., Glycosidically bound norisoprenoids from *Vitis vinifera* cv. *riesling* leaves. *J. Agric. Food Chem.*, **42**, 1068–1072 (1994).
10) Pabst, A., Barron, D., Semon, E., and Schreier, P., An α-ionol disaccharide glycoside from raspberry fruit. *Phytochemistry*, **31**, 2043–2046 (1992).
11) Williams, P. J., Strauss, C. R., Wilson, B., and Massy-Westropp, R. A., Glycosides of 2-phenylethanol and benzyl alcohol in *Vitis vinifera* grapes. *Phytochemistry*, **22**, 2039–2041 (1982).
12) Vorin, S. G., Baumes, R. L., Bitteur, S. M., Günata, Z. Y., and Bayonove, C. L., Novel monoterpene disaccharide glycosides of *Vitis vinifera* grapes. *J. Agric. Food Chem.*, **38**, 1373–1378 (1990).
13) Williams, P. J., Strauss, C. R., Wilson, B., and Massy-Westropp, R. A., Novel monoterpene disaccharides of *Vitis vinifera* grapes and wines. *Phytochemistry*, **21**, 2013–2020 (1982).
14) Pabst, A., Barron, D., Semon, E., and Schreier, P., Isolation of a novel disaccharide glycoside from raspberry fruit. *Tetrahedron Letters*, **32**, 4885–4888 (1991).
15) Güldner, A., and Winterhalter, P., Structures of two new ionone glycosides from quince fruit (*Cydonia ob-*

longa Mill.). *J. Agric. Food Chem.*, **39**, 2142–2146 (1991).

16) Baumes, R. L., Aubert, C. C., and Bayonove, C. L., Absolute configuration of free and bound 3-hydroxy-β-damascone and of 3-oxo-α-ionol β-D-glucopyranoside in tobacco. *J. Essent. Oil Res.*, **6**, 601–606 (1994).
17) Andersson, R., and Lundgren, L. N., Monoaryl and cyclohexenone glycosides from needles of *Pinus sylvestris*. *Phytochemistry*, **27**, 559–562 (1988).
18) Otsuka, H., Takeda, A., and Yamaski, K., Xyloglucosides of benzyl and phenyl alcohols and (*Z*)-hex-3-en-1-ol from leaves of *Alagium platanifolium* var. *trilobum*. *Phytochemistry*, **29**, 3681–3683 (1990).
19) Shiraga, Y., Okano, K., Akira, T., Fukaya, C., Yokoyama, K., Tanaka, S., Fukui, H., and Tabata, M., Structures of potent antiulcerogenic compounds from *Cinnamomun cassia*. *Tetrahedron*, **44**, 4703–4711 (1988).
20) Shimizu, S., Miyase, T., Ueno, A., and Usmanghani, K., Sesquiterpene lactone glycosides and ionone derivative glycosides from *Sonchus asper*. *Phytochemistry*, **28**, 3399–3402 (1989).
21) Adinolfi, M., Parrilli, M., and Zhu, Y., Terpenoids from *Ophiopogon japonicus* root. *Phytochemistry*, **29**, 1696–1699 (1990).
22) Moon, J.-H., Watanabe, N., Ijima, Y., Yagi, A., Ina, K., and Sakata, K., *cis*- and *trans*-Linalool 3,7-oxides and methyl salicylate glycosides and (*Z*)-3-hexenyl β-D-glucopyranoside as aroma precursors from tea leaves for oolong tea. *Biosci. Biotechnol. Biochem.*, **60**, 1815–1819 (1996).
23) Guo, W., Sakata, K., Watanabe N., Nakajima, R., Yagi, A., Ina, K., and Luo, S., Geranyl 6-*O*-β-D-xylopyranosyl-β-D-glucopyranoside isolated as an aroma precursor from tea leaves for oolong tea. *Phytochemistry*, **33**, 1373–1375 (1993).
24) Moon, J.-H., Watanabe, N., Sakata, K., Yagi, A., Ina, K., and Luo, S., *trans*- and *cis*-Linalool 3,6-oxides 6-*O*-β-D-xylopyranosyl-β-D-glucopyranosides isolated as aroma precursors from leaves for oolong tea. *Biosci. Biotechnol. Biochem.*, **58**, 1742–1744 (1994).
25) Guo, W., Hosoi, R., Sakata, K., Watanabe, N., Yagi, A., Ina, K., and Luo, S., (*S*)-Linalyl, 2-phenylethyl, and benzyl disaccharide glycosides isolated as aroma precursors from oolong tea leaves. *Biosci. Biotechnol. Biochem.*, **58**, 1532–1534 (1994).
26) Ogawa, K., Moon, J.-H., Guo, W., Yagi, A., Watanabe, N., and Sakata, K., A study on tea aroma formation mechanism: Alcoholic aroma precursor amounts and glycosidase activity in parts of the tea plant. *Z. Naturforsch.*, **50c**, 493–498 (1995).
27) Ma, S.-J., Watanabe, N., Yagi, A., and Sakata, K., The (3*R*,9*R*)-3-hydroxy-β-ionol disaccharide glycoside is an aroma precursor in tea leaves. *Phytochemistry*, **56**, 819–825 (2001).
28) Nishikitani, M., Kubota, K., Kobayashi, A., and Sugawara, F., Geranyl 6-*O*-α-L-arabinopyranosyl-β-D-glucopyranoside isolated as an aroma precursor from leaves of a green tea cultivar. *Biosci. Biotechnol. Biochem.*, **60**, 929–931 (1996).
29) 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).
30) Ogawa, K., Ijima, Y., Guo, W., Watanabe, N., Usui, T., Dong, S., Tong, Q., and Sakata, K., Purification of a β-primeverosidase concerned with alcoholic aroma formation in tea leaves (cv. Shuixian) to be processed to oolong tea. *J. Agric. Food Chem.*, **45**, 877–882 (1997).
31) Ijima, Y., Ogawa, K., Watanabe, N., Usui, T., Ohnishi-Kameyama, M., Nagata, T., and Sakata, K., Characterization of β-primevrosidase, being concerned with alcoholic aroma formation in tea leaves to be processed into black tea, and preliminary observation on its substrate specificity. *J. Agric. Food Chem.*, **46**, 1712–1718 (1998).
32) Suzuki, H., Hydrolysis of flavonoid glycosides by enzymes (Rhamnodiastase) from *Rhamnus* and other sources. *Arch. Biochem. Biophys.*, **99**, 476–483 (1962).
33) Imaseki, H., and Yamamoto, T., A furcatin hydrolyzing glycosidase of *Viburnum furcatum* blume. *Arch. Biochem. Biophys.*, **92**, 467–474 (1961).
34) Bourbouze, R., Pratviel-Sosa, F., and Percheron, F., Rhamnodiastase and α-L-rhamnosidase de *Fagopyrum esculentum*. *Phytochemistry*, **14**, 1279–1282 (1975).
35) Yasuda, T., and Nakagawa, H., Purification and characterization of the rutin-degrading enzymes in tartary buckwheat seeds. *Phytochemistry*, **37**, 133–136 (1994).
36) Hay, G. W., Westlake, D. W. S., and Simpson, F. J., Degradation of rutin by *Aspergillus flavus*. Purification and characterization of rutinase. *Can. J. Microbiol.*, **7**, 921–93227 (1962).
37) Bridel, M., Primeverose, primeverosides and primeverosidase. *Comptes Rendus* (in French), **180**, 1421–1425 (1925).
38) Plouvier, V., Study and distribution of primeverosidases and gentiobiosidase. *Comptes Rendus Ser. D.* (in French), **290**, 1071–1074 (1980).
39) Günata, Z., Blondeel, C., Vallier, M. J., Lepoutre, J. C., Sapis, J. C., and Watanabe, N., An endoglycosidase from grape berry skin of cv. M. Alexandria hydrolyzing potentially aromatic disaccharide glycosidase. *J. Agric. Food Chem.*, **46**, 2748–2753 (1998).
40) Matsumura, S., Takahashi, S., Nishikitani, M., Kubota, K., and Kobayashi, A., The role of diglycosides as tea aroma precursors: Synthesis of tea diglycosidase and specificity of glycosidase in tea leaves. *J. Agric. Food Chem.*, **45**, 2674–2678 (1997).
41) Murata, T., Shimada, M., Watanabe, N., Sakata, K., and Usui, T., Practical enzymatic synthesis of primeverose and its glycoside. *J. Appl. Glycosci.*, **46**, 431–437 (1999).
42) Fletcher, H. G., and Hudson, L. S., 1,5-Anhydroxylitol. *J. Am. Chem. Soc.*, **69**, 921–925 (1947).
43) Sone, Y., and Misaki, A., Syntheses of *p*-nitrophenyl 6-*O*-α- and 6-*O*-β-xylopyranosyl-β-D-glucopyranoside. *J. Carbohydrate Chemistry*, **5**, 671–682 (1986).

44) Bradford, M., A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.*, **72**, 248–254 (1976).
45) Touchstone, J. C., in "Practice of Thin Layer Chromatography", John Wiley & Sons, New York, pp. 184 (1978).
46) Matsumura, S., Takahashi, S., Nishikitani, M., Kubota, K., and Kobayashi, A., The role of diglycosides as tea aroma precursors: Synthesis of tea diglycosidase and specificity of glycosidases in tea leaves. *J. Agric. Food Chem.*, **45**, 2674–2678 (1997).
47) Halder, J., and Bhaduri, A., Glycosidases from tea-leaf (*Camellia sinensis*) and characterization of β-galactosidase. *Nutritional Biochemistry*, **8**, 378–384 (1997).
48) Wang, D., Yoshimura, T., Kubota, K., and Kobayashi, A., Analysis of glycosidically bound aroma precursors in tea leaves. 1. Qualitative and quantitative analyses of glycosides with aglycons as aroma compounds. *J. Agric. Food Chem.*, **48**, 5411–5418 (2000).
49) Wang, D., Kurasawa, E., Yamaguchi, Y., Kubota, K., and Kobayashi, A., Analysis of glycosidically bound aroma precursors in tea leaves. 2. Changes in glycoside contents and glycosidase activities in tea leaves during the black tea manufacturing process. *J. Agric. Food Chem.*, **49**, 1900–1903 (2001).
50) Mizutani, M., Nakanishi, H., Ema, J., Ma, S-J., and Sakata, K., β-Primeverosidase: A unique diglycosidase concerned with the floral aroma formation during tea manufacturing. In "Proceedings of 2001 International Conference on O-CHA (Tea) Culture and Science," Shizuoka, October 5-8, 2001, in press.