Characterization of β-Primeverosidase, Being Concerned with Alcoholic Aroma Formation in Tea Leaves To Be Processed into Black Tea, and Preliminary Observations on Its Substrate Specificity

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A β-primeverosidase has been, for the first time, purified from fresh leaves of a cultivar (*Camellia sinensis* var. *assamica*) for black tea in the same way as in the case of green and oolong tea leaves previously reported. The molecular weight was shown to be 60.3 kDa by MALDI-TOFMS analysis. Its p*I*, optimum temperature, and pH are 9.5, 45 °C, and 4, respectively. The enzyme is stable below 40 °C and between pH 4 and 5. These enzymic characteristics are very similar to those of the β-primeverosidases from cvs. Yabukita and Shuixian which are exclusively processed into green and oolong tea, respectively. Each β-primeverosidase from tea leaves for green, oolong, and black teas was further purified by HPLC (ODS) and digested by trypsin to be analyzed by HPLC (ODS capillary column). Their chromatograms were not identical but very similar to each other. The molecular weight differences among these enzymes (60.5, 60.2, and 60.3 kDa from the cultivars for green, oolong, and black teas, respectively) suggest that these β-primeverosidases are enzymatically identical, but slightly different on their molecular basis. Next, several kinds of disaccharide glycosides which had been isolated as aroma precursors were reacted with endogenous β-primeverosidase and β-glucosidase fractions from fresh tea leaves (cv. Yabukita). The β-primeverosidase hydrolyzed β-primeverosides and 6-*O*-β-D-apiofuranosyl-β-D-glucopyranosides isolated as aroma precursors from tea leaves more effectively than other disaccharide glycosides to yield each disaccharide and aglycon. It also hydrolyzed both β-vicianoside and 6-*O*-α-L-arabinofuranosyl-β-D-glucopyranoside into each disaccharide and aglycon, but the amount of generated aroma was smaller than that produced by the β-glucosidase fraction.

Keywords: *β-Primeverosidase; Camellia sinensis var. assamica; aroma formation; tea aroma; glycosidase*

INTRODUCTION

Floral tea aroma is one of the most important factors in determining the quality of each made tea, especially oolong and black tea. Monoterpene alcohols (linalool, geraniol, etc.) and aromatic alcohols (benzyl alcohol, 2-phenylethanol, etc.) are known to be the major floral tea aroma constituents (Yamanishi, 1995). The alcohols having floral aroma, which are mainly produced during the processing of black and oolong tea by the action of the endogenous enzymes, are especially important.

In the course of our study on the molecular basis of the alcoholic aroma formation in oolong tea, we have isolated aroma precursors of most of the alcoholic aroma as glycosides. Most of them were β-primeverosides (6-*O*-β-D-xylopyranosyl-β-D-glucopyranosides) (Guo et al., 1993, 1994; Moon et al., 1994, 1996; Sakata et al., 1995a). But the aroma precursors of linalool oxides III and IV (*trans*- and *cis*-linalool 3,7-oxides) and (*Z*)-3-hexenol were exceptionally isolated as 6-*O*-β-D-apiofuranosyl-β-D-glucopyranosides and β-D-glucopyranoside, respectively (Moon et al., 1996).

Kobayashi et al. have, for the first time, isolated and identified (*Z*)-3-hexenyl and benzyl β-D-glucopyranosides as aroma precursors from cv. Yabukita (Kobayashi et al., 1994; Yano et al., 1991). Recently they have also found geranyl β-primeveroside as well as geranyl β-vicianoside (6-*O*-α-L-arabinopyranosyl-β-D-glucopyranoside) in cv. Yabukita (Nishikitani et al., 1996).

Quite recently we have purified β-primeverosidases from fresh tea leaves (cvs. Yabukita and Shuixian), which showed high substrate specificity toward β-primeverosides, to hydrolyze them into primeverose and the corresponding aglycons (Guo et al., 1995; Sakata et al., 1995b; Ogawa et al., 1997). We have already shown that both the amount of aroma precursors and enzymatic activity decreased with the maturity of tea leaves (Ogawa et al., 1995; Sakata et al., 1995b).

In this paper, we describe the purification of β-primeverosidase from fresh leaves of a cultivar (*Camellia sinensis* var. *assamica* (*C. sinensis* var. *assamica*)) for black tea to compare its enzymatic properties and primary structure in the molecular level with those of

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β-primeverosidases from cvs. Yabukita and Shuixian to be exclusively processed to green and oolong tea.

Next, we preliminarily observed the substrate specificity of the β-primeverosidase and/or other enzymes of tea leaves (cv. Yabukita) toward several kinds of disaccharide glycosides mainly isolated as aroma precursors from tea leaves and flowers.

MATERIALS AND METHODS

Tea Leaves. The fresh leaves (leaf shoots with up to three or four leaves) of cv. Yabukita for green tea manufacturing were plucked at the National Research Institute of Vegetables, Ornamental Plants and Tea, Kanaya, Shizuoka, Japan, in May 1995. The fresh leaves of a cultivar of *C. sinensis* var. *assamica* for black tea manufacturing were plucked at the Makurazaki Station of the National Research Institute of Vegetables, Ornamental Plants and Tea, Kagoshima, Japan, in November 1996. The fresh leaves of cv. Shuixian for oolong tea manufacturing were plucked in the Species Garden of the Department of Tea Science, Zhejiang Agricultural University, Hangzhou, Zhejiang, People's Republic of China, in May 1995. After being plucked, the leaves were conventionally treated to prepare acetone powder and stored in a freezer (−20 °C) before use.

Measurement of Glycosidase Activities toward Several Kinds of *p*-Nitrophenyl (*p*NP) Glycosides. The measurements were carried out as previously reported (Ogawa et al., 1997). The enzyme fractions were measured for the hydrolysis activities of *p*NP-monosaccharide glycosides (*p*NP β-D-gluco- and β-D-xylopyranosides; purchased from Sigma Chemical Co., St. Louis, MO) and *p*NP β-primeveroside [β-*O*-Xyl-(1−6)-β-D-Glc-$OC_6H_4NO_2$-*p*; enzymatically synthesized (Kajiura, 1994)]. The incubation mixture (1 mL) is composed of 900 μL of 20 mM citrate buffer (pH 6.0), 50 μL of an enzyme sample solution, and 50 μL of 10 mM substrate solution. Reaction was started by adding an enzyme sample at 37 °C and stopped by addition of 500 μL of 1 M Na_2CO_3. The liberated *p*-nitrophenol was determined spectrophotometrically at 405 nm. One unit was defined as the amounts of enzyme liberating 1 μmol of *p*-nitrophenol/min under the assay conditions.

Purification of β-Primeverosidase. All operations were carried out at 4 °C unless otherwise stated.

(1) From cvs. Yabukita and Shuixian. Just after being plucked, tea leaves were finely chopped, crushed in dry ice−acetone by a homogenizer, and then filtered in vacuo. The residue was washed with chilled acetone (−20 °C) until the filtrate became nearly colorless. The residue was made free from acetone in vacuo to yield acetone powder (crude enzyme). Purification of β-primeverosidases was carried out in the same manner previously reported (Guo et al., 1996; Ogawa et al., 1997). The procedures are mostly the same (DTT, EDTA, and ascorbic acid were not used in the previous experiments) as in the case from var. *assamica* described below.

(2) From var. assamica. Fresh tea leaves of *C. sinensis* var. *assamica* were subjected to the conventional preparation process for acetone powder. The acetone powder (20.0 g, equivalent to 100 g of the fresh tea leaves) was suspended in 0.1 M citrate buffer (pH 6.0, 800 mL) containing 2 mM DTT, 5 mM EDTA, Polyclar SB-100 (30 g), and ascorbic acid (1 g), stirred for 1.5 h, and centrifuged at 35000*g* for 20 min. To the combined supernatant (ca. 570 mL), chilled acetone (−4 °C) was added up to 50% with stirring and left overnight at 4 °C. The precipitate obtained by centrifugation at 35000*g* for 20 min was dissolved in 0.1 M citrate buffer (pH 6.0, 200 mL) containing 2 mM DTT and subjected to the conventional ammonium sulfate precipitation. The crude enzyme separated by 40−80% ammonium sulfate saturation was dissolved in 32 mL of 20 mM citrate buffer (pH 6.0) and then dialyzed overnight against the same buffer. The dialyzed enzyme was charged on a CM-Toyopearl 650M column (Tosoh Corp., Tokyo, Japan; 32 mm i.d. × 140 mm, ca. 110 mL) equilibrated with 20 mM citrate buffer (pH 6.0). The adsorbed protein was eluted by a linear gradient elution of NaCl from 0 to 0.5 M in 500 mL each of 20 mM citrate buffer (pH 6.0) at a flow rate of 2 mL/min. Fractions of 10 mL were collected. The primeverosidase fractions were combined and concentrated using an ultrafiltration module (Amicon PM-10, Grace Japan Co., Ltd., Tokyo, Japan). A primeverosidase fraction obtained by the CM-Toyopearl column chromatography was further purified by FPLC (Pharmacia LKB Biotechnology, Uppsala, Sweden) on a column of Mono S HR (0.5 mm i.d. × 50 mm; Pharmacia Biotech Co. Ltd., Tokyo, Japan) equilibrated with 20 mM citrate buffer (pH 6.0). The enzyme was eluted by a linear gradient elution of NaCl from 0 to 0.2 M in the same buffer at a flow rate of 1 mL/min. Fractions of 1 mL were collected.

Sodium Dodecyl Sulfate−Polyacrylamide Gel Electrophoresis (SDS−PAGE). Analysis was done in the same manner as previously reported (Ogawa et al., 1997).

Molecular Weight Measurement. The molecular weight of each β-primeverosidase was measured by MALDI-TOFMS (Hillenkamp, 1991). The most purified enzyme solution (20−30 pmol/μL in 0.1% TFA) was mixed with the same volume of a saturated solution of recrystallized sinapic acid in 0.1% TFA−Me_3CN (2:1). A 1 mL aliquot of the mixture was analyzed on a REFREX II (Bruker-Franzen Analytik, Bremen, Germany) mass spectrometer equipped with an N_2 laser (337 nm). The mass spectrometer was operated in the laser mode with an accelerating voltage of 28.5 kV. Mass calibration was accomplished using the singly and doubly charged molecular ions of bovine albumin (Sigma) as an external standard. The spectra were recorded in the positive-ion mode.

Isoelectric Point Measurement. The measurement was performed as previously reported (Ogawa et al., 1997).

Effects of Temperature and pH on the Enzymic Activity and Stability. The optimum temperature for the enzyme activity was measured with *p*NP β-primeveroside in 20 mM citrate buffer (pH 6.0) at various temperatures (30−70 °C). The thermal stability of the enzyme was examined from the residual activities after incubation in the same conditions for 1 h. As for the optimum pH, it was determined in buffers with various pHs (pH 2.0−9.0) at 37 for 1 h. The pH stability was examined from the residual activities after incubation in the buffers at various pHs [pH 2.0−9.0; 20 mM Clark and Lubs buffer (pH 2.0), 20 mM citrate buffer (pH 3.0−6.0), and 20 mM Tris-HCl buffer (pH 7.0−9.0)] at 37 °C for 1 h.

Digestion of β-Primeverosidases by Trypsin. Each β-primeverosidase purified by FPLC was further purified by HPLC (Column, YMC-Pack C8; 4.6 mm i.d. × 150 mm; equilibrated with H_2O containing 0.1% TFA). The adsorbed protein was eluted by a gradient elution of MeCN from 0 to 80% at a flow rate of 0.8 mL/min at 40 °C. Each primeverosidase was eluted at 64 min, while a β-glucosidase from almond was at 38 min in this conditions. Each primeverosidase fraction was evaporated to dryness by a centrifugal concentrator. The β-primeverosidase dissolved in a mixture of H_2O (120 μL) and PBS (40 μL) was denatured for 10 min at 100 °C and reacted with 2−5 μg of trypsin (Wako, Trypsin Cryst; 5400 USP unit/mg) for 24 h at 37 °C.

HPLC Analysis of the Trypsin Hydrolysate of β-Primeverosidases. The hydrolysate of each β-primeverosidase obtained by the reaction with trypsin was concentrated with a centrifugal concentrator and analyzed by HPLC (BMLC-10, Shimadzu Co. Ltd., Kyoto, Japan) equipped with a capillary column (FUS-15-03-C18; 0.3 mm i.d. × 150 mm, equilibrated with 5% MeCN containing 0.1% TFA). Eluates were obtained by a gradient elution of MeCN from 5 to 90% at a flow rate of 2.5 μL/min at 40 °C monitoring at 220 nm.

Preliminary Observations on the Substrate Specificity of the Primeverosidase Fraction and Other Enzymic Samples. *(1) GC Analysis of the Liberated Alcoholic Aroma.* Each of the glycosides [(*S*)-linalyl and benzyl β-primeverosides (500 μg each) isolated from *C. sinensis* var. *sinensis* cv. Yabukita (Ijima, 1994), (*R*)-linalyl β-vicianoside (6-*O*-α-L-arabinopyranosyl-β-D-glucopyranoside; 200 μg) isolated from Gardenia jasminoides (Watanabe et al., 1994), geranyl 6-*O*-α-L-arabinofuranosyl-β-D-glucopyranoside (50 μg) isolated from *Rosa damascena* var. *bulgaria* (Oka, 1997) and benzyl

6-*O*-β-D-apiofuranosyl-β-D-glucopyranoside (1000 μg) from *Epimedium grandiflorum* MORR var. *thunbergianum* (Miyase et al., 1988)] was hydrolyzed with each enzyme sample (equivalent to 5 g of fresh tea leaves) in 0.1 M citrate buffer (1 mL, pH 6.0) at 37 °C for 24 h. The reaction mixture without any enzyme sample and without any substrate was also subjected to the same operation to confirm the stability of the substrate and contamination of any substrate in the enzymic sample preparations, respectively. Liberated aroma was analyzed by GC in the same way as previously reported (Ogawa et al., 1997) as follows:

The aroma extract was analyzed by a Hitachi Model G-5000 gas chromatograph (Hitachi, Ltd., Tokyo) equipped with FID and a TC-WAX capillary column (0.25 mm i.d. × 30 m; GL Science Inc., Tokyo). The GC conditions were as follows: carrier gas, N_2 (1 mL/min); split ratio, 100:1; temperature program, holding at 60 °C for 10 min and then ramping to 200 °C at 3 °C/min; injector temperature, 250 °C; detector temperature, 250 °C. All the glycosides were stable enough under the reaction conditions not to liberate significant amounts of any aroma volatiles. No significant amount of aroma volatiles was also detected from the reaction mixture without any substrate.

(2) Determination of the Liberated Sugars. The resulting aqueous phase after the extraction of the liberated aroma compounds was subjected to centrifugation at 35 000*g* for 20 min with Centricon-10 (Grace Japan, Co. Ltd., Tokyo) to remove proteins. The supernatant was analyzed by HPLC equipped with a Shodex SUGAR KS-801 (8 mm i.d. × 300 mm) column (H_2O at flow rate of 0.5 mL/min; 70 °C). Detection of sugars was performed at 415 nm by the postlabeling method using 0.1 M H_3BO_3, 50 mM guanidine hydrochloride, 0.5 mM $NaIO_4$, and 20% MeCN (pH 12) at a flow rate of 0.5 mL/min at 170 °C.

RESULTS AND DISCUSSION

Purification of β-Primeverosidase from *C. sinensis* var. *assamica* and Comparison with Those from cvs. Yabukita and Shuixian. At this stage we obtained *p*-nitrophenyl (*p*NP) β-primeveroside by the application of enzymatic transglycosylation from xylobiose to *p*NP β-D-glucopyranoside (Kajiura, 1994). As oxidative enzyme activity is known to be more potent in black tea leaves (var. *assamica*) than in oolong and green tea leaves, crude enzymes from black tea leaves were extracted from the acetone powder with 0.1 M citrate buffer (pH 6.0), containing 2 mM DTT, 5 mM EDTA, Polyclar SB100, and ascorbic acid, and subjected to acetone precipitation followed by 40−80% ammonium sulfate precipitation. The crude enzymes prepared from var. *assamica* and both cvs. Shuixian and Yabukita were applied on the same column of CM-Toyopearl in the same way, and glycosidase activities of each fraction were measured with *p*NP β-D-glucoside and *p*NP β-primeveroside. The β-primeverosidase from var. *assamica* was eluted just before the main glucosidase, and the obtained elution pattern (Figure 1) was similar to those of cvs. Yabukita and Shuixian (Guo et al., 1996; Ogawa et al., 1997). The β-primeverosidase fractions which were almost devoid of other glycosidases were purified by FPLC on a column of Mono S-HR (Figure 2). The peak showing high primeverosidase activity and very low apparent β-glucosidase activity overlapped with the peak of the absorption at 280 nm. The β-primeverosidase from var. *assamica* was shown to be a single protein of 61 kDa by SDS−PAGE analysis (Figure 3) and identical to those of cvs. Yabukita (Guo et al., 1995, 1996; Sakata et al., 1995b) and Shuixian (Ogawa et al., 1997). The β-primeverosidase showed maximal activity at 45 °C and pH 4.0. It was stable at temperatures up to 40 °C and between pH 4 and 5. The specific activity

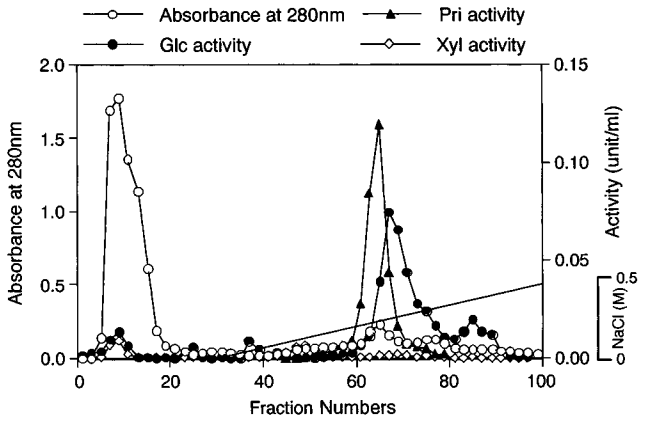

Figure 1. CM-Toyopearl 650M column chromatography of enzymes from black tea leaves (*Camellia sinensis* var. *assamica*). Elution: 20 mM citrate buffer (pH 6.0); NaCl gradient elution; flow rate, 2 mL/min.

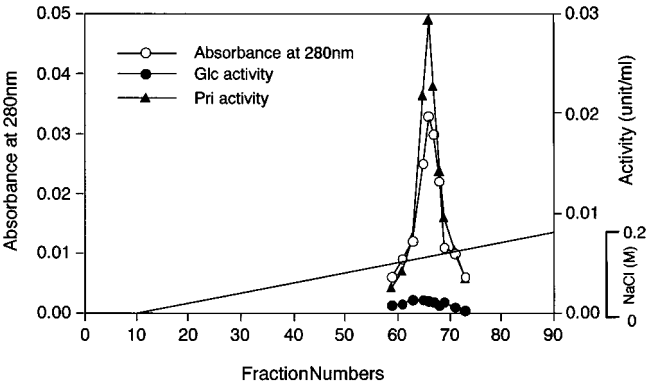

Figure 2. FPLC of a primeverosidase fraction on a Mono S column from black tea leaves (*Camellia sinensis* var. *assamica*); column, Mono S HR 5/5; elution, 20 mM citrate buffer (pH 6.0); NaCl gradient elution; flow rate, 1 mL/min.

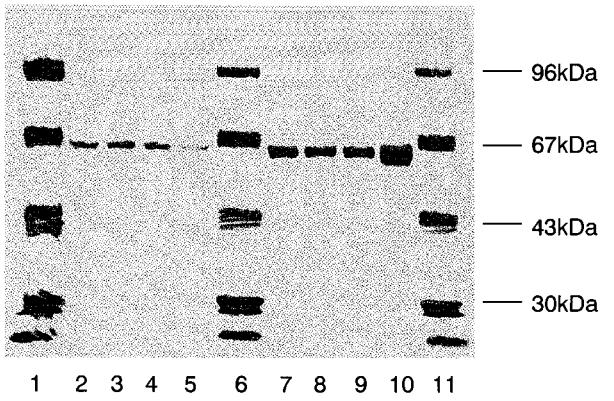

Figure 3. SDS−PAGE analytical results of β-primeverosidases from black, oolong, and green tea leaves. Lanes 1, 6, and 11, molecular mass marker; lanes 2−5, β-primeverosidase from black tea leaves (var. *assamica*); lanes 7−9, β-primeverosidase from oolong tea leaves (cv. Shuixian); lane 10, β-primeverosidase from green tea leaves (cv. Yabukita).

of the β-primeverosidase is 0.99 unit/mg which was very close to those of β-primeverosidases from cvs. Yabukita and Shuixian (Table 1). The isoelectric point of the β-primeverosidase from var. *assamica* was found to be 9.5, which is a value very similar to those of the primeverosidases from cvs. Yabukita (9.4) and Shuixian (9.5) (Guo et al., 1996; Ogawa et al., 1997). These analytical results suggest that β-primeverosidase from var. *assamica* is identical or quite similar to the enzymes from cvs. Yabukita and Shuixian.

Table 1. Specific Activity of Each β-Primeverosidase

origin of β-primeverosidase	specific activity[a] (unit/mg)	
	β-primeverosidase	β-glucosidase
black tea (var. *assamica*)	0.99	0.03
oolong tea (cv. Shuixian	0.98	0.10
green tea (cv. Yabukita)	0.90	0.03

[a] Apparent activity: one unit was defined as the amounts of enzyme liberating 1 μmol of *p*-nitrophenol from *p*NP β-primeveroside or β-D-glucopyranoside per min under the assay conditions.

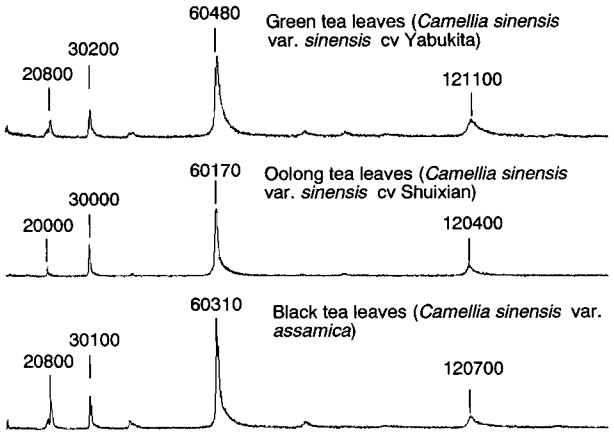

Figure 4. TOFMS spectra of β-primeverosidases from fresh tea leaves.

The slight molecular weight differences among these three kinds of β-primeverosidases were determined by TOFMS analysis (60.3, 60.5, and 60.2 kDa, respectively; Figure 4). This suggests that these molecular weight differences are ascribable to one or two amino acid residues based on the error range of 15−80 mass units in this mass region around 0−70 000.

Each β-primeverosidase purification by FPLC was finally performed by HPLC with a YMC-Pack C8 column (0−80% MeCN in 0.1% TFA). Each β-primeverosidase, which showed the same retention time, was digested by trypsin at 37 °C for 24 h and analyzed by HPLC equipped with a capillary column of FUS-15-03-C18 (5−90% MeCN in 0.1% TFA). Each chromatogram showed a very similar elution profile (Figure 5); however, that of black tea (var. *assamica*) was a little different from those of green and oolong tea (cvs. Yabukita and Shuixian, respectively). This indicates that each enzyme from the green, oolong, or black tea cultivar has very similar molecular composition.

On the basis of the foregoing, each β-primeverosidase was confirmed to be enzymatically identical, but different on its molecular level (differences of one or two amino acids) from species to species in tea plants.

Preliminary Observations on the Substrate Specificity of β-Primeverosidase. Enzymes were extracted from the acetone powder prepared from the fresh leaves (cv. Yabukita) were extracted with 0.1 M citrate buffer (pH 6.0) and subjected to acetone precipitation followed by ammonium sulfate precipitation. The crude enzymes (40−80% ammonium sulfate precipitate) were chromatographed on a CM-Toyopearl column (20 mM citrate buffer, pH 6.0, 0−0.5 M NaCl gradient). Each fraction was measured for β-glucosidase and β-primeverosidase activities and then combined into fractions A−H according to the glycosidase activities (Figure 6).

Several kinds of disaccharide glycosides (Figure 7), which were mostly isolated as aroma precursors from

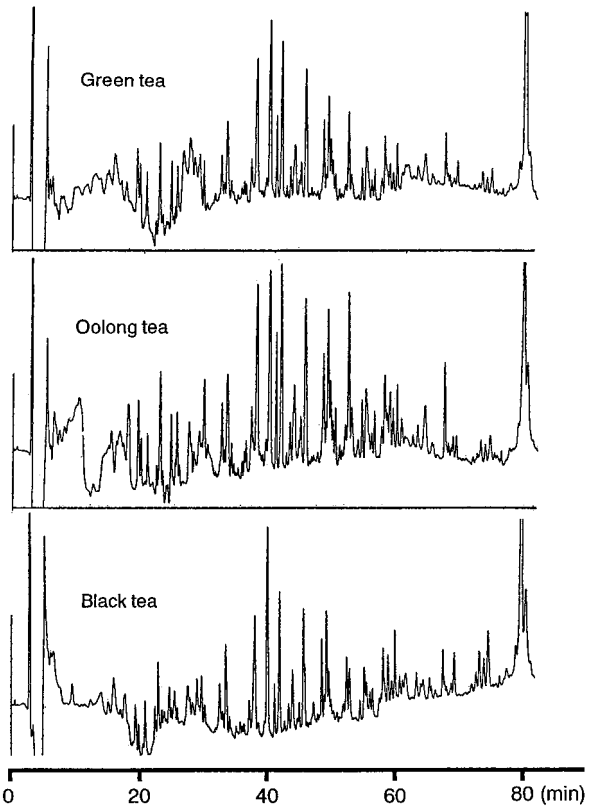

Figure 5. Peptide mapping of each primeverosidase from cultivars for green, oolong, and black tea.

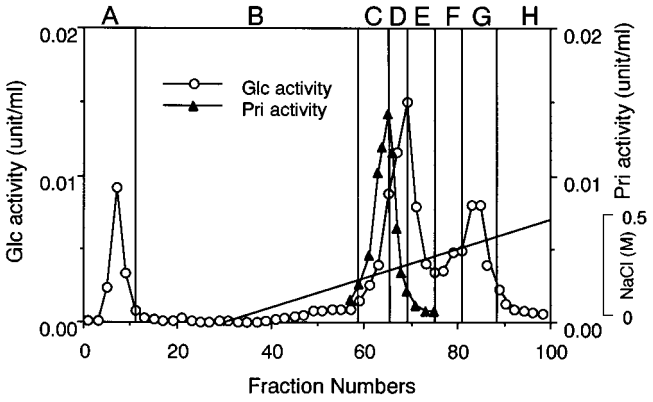

Figure 6. CM-Toyopearl 650M column chromatography of enzymes from tea leaves (cv. Shuixian): elution, 20 mM citrate buffer (pH 6.0); NaCl gradient elution; flow rate, 2 mL/min. Glucosidase (Glc) and primeverosidase (Pri) activities were measured using *p*NP β-D-glucopyranoside and *p*NP β-primeveroside, respectively.

tea leaves and some flower buds, were reacted with fraction C [primeverosidase (Pri)], fraction E [glucosidase (Glc) II)], fractions A + G (Glcs I and III), and fractions A + E + G (Glcs I−III), respectively (Figure 6), and the generated aroma constituents were analyzed by GC (Table 2). Because the sample amount was not enough to measure the initial velocity of each enzymic sample, the experiment was designed to compare the amount of aroma volatiles generated from each disaccharide glycoside sample after the enzymic reaction for 24 h (Table 2). And the resulting sugar(s) and unreacted substrate in each reaction mixture was analyzed by HPLC (Figures 8 and 9). Although the experiment was very preliminary, the following interesting characteristics of the β-primeverosidase were suggested.

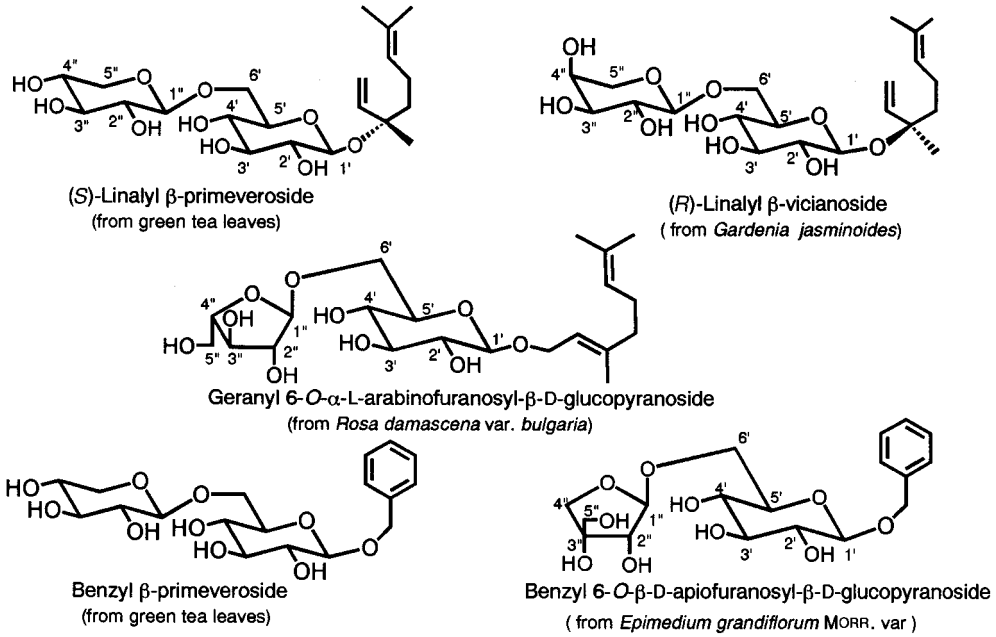

Figure 7. Structures of disaccharide glycosides tested for the enzymatic hydrolysis.

Table 2. Hydrolysis Activities of the Glycosidases from Tea Leaves (cv. Yabukita) toward Several Kinds of Disaccharide Glycosides

			enzymes[a]			
aroma precursor (MW)	substrate concn (mM)	alcoholic aroma	Pri (fraction C)	Glc II (fraction E)	Glc I + III (fractions A + G)	Glc I + II + III (fractions A + E + G)
benzyl β-primeveroside (402)	1.2	benzyl alcohol	100[b]			45
linalyl β-primeveroside (448)	1.1	linalool	100	69	18	87
benzyl 6-*O*-β-D-apiofuranosyl-β-D-glucopyranoside (402)	2.5	benzyl alcohol	100	4	5	9
geranyl 6-*O*-α-L-arabinofuranosyl-β-D-glucopyranoside (448)	0.11	geraniol	50			100
linalyl β-vicianoside (6-*O*-α-L-arabinopyranosyl-β-D-glucopyranoside) (448)	0.45	linalool	39			100

[a] Pri, β-primeverosidase fraction; Glc, β-glucosidase fraction. [b] Activities are expressed as relative activities. Each glycoside was reacted with each enzymic sample and the liberated aroma was measured by GC. The highest amount of the liberated aroma was expressed as 100.

The Pri fraction was suggested to show rather high substrate specificity toward linalyl and benzyl β-primeverosides as well as benzyl 6-*O*-β-D-apiofuranosyl-β-D-glucopyranoside (Table 2). The Glc-fractions (fractions A, E, and G) hydrolyzed them much less effectively than the Pri fraction. The Pri fraction hydrolyzed linalyl β-primeveroside as well as benzyl 6-*O*-β-D-apiofuranosyl-β-D-glucopyranoside more effectively than other fractions, suggesting that the main glycosidase concerned with the alcoholic aroma formation in tea leaves is β-primeverosidase. It is worthwhile to note that linalyl vicianoside and geranyl 6-*O*-α-L-arabinopyranosyl-β-D-glucopyranoside were hydrolyzed much less effectively by Pri fraction than by Glc fractions (fractions A + E + G) containing arabinosidase (Guo et al., 1995; Table 2).

Even from this very preliminary experiment, we can guess that the β-primeverosidase recognizes a disaccharide unit, in which the second monosaccharide is attached to the C-6′ hydroxyl group of the glucose moiety, to show a rather broad substrate specificity to these disaccharide glycosides.

Next, the remaining saccharide constituents in the residual aqueous layer in the enzymatic hydrolysates were analyzed by HPLC (Figures 8 and 9). The peak observed at t_R = 22 min in all of the chromatograms was found to be a contaminated glycerol from a centrifugation equipment. The β-primeverosidase was clearly shown to recognize disaccharide units of benzyl β-primeveroside and benzyl 6-*O*-β-D-apiofuranosyl-β-D-glucopyranoside to hydrolyze these disaccharide glycosides into each aglycon and disaccharide unit (Figure 8). Both disaccharides were hardly further hydrolyzed into monosaccharides by the β-primeverosidase. When benzyl β-primeveroside was reacted with a mixture of Glcs I−III, considerable amounts of monosaccharide (glucose and xylose) were liberated. But a lot of primeverose still remained, suggesting that the xylose moiety in either β-primeveroside or primeverose is difficult to be cleaved by other glycosidases which were found to be mainly present in the Glc I fraction (Figure 6; Guo et al., 1996; Ogawa et al., 1997). The same sort of things were observed in the case of linalyl β-vicianoside (Figure 9). In this case a disaccharide (vicianose) was liberated and not susceptible to further hydrolysis (Figure 9). On the basis of these experimental results, the β-primeverosidase can be concluded to be a glycosidase which shows fairly broad substrate specificity toward disaccharide glycosides which contain a 1−6 glycosyl linkage.

We have already isolated and identified most of the alcoholic aroma precursors as β-primeverosides [geranyl, (*S*)-linalyl, 2-phenylethyl, benzyl, methyl salicylate, linalool oxides I and II β-primeverosides] (Guo et al.,

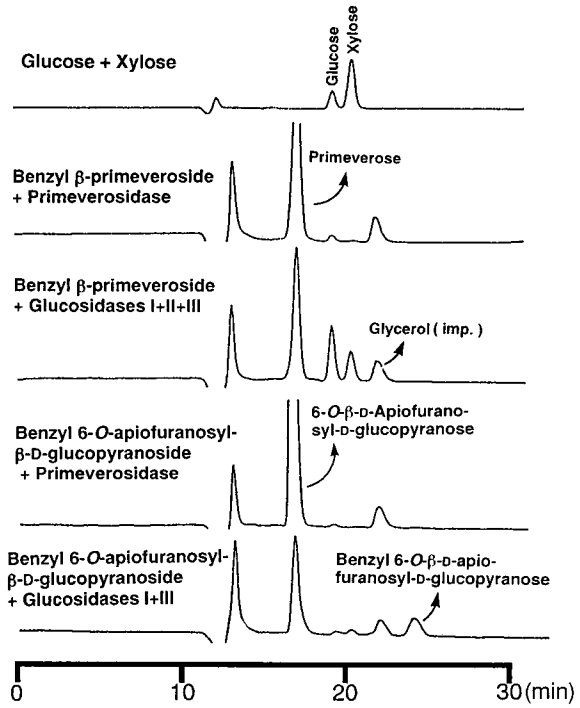

Figure 8. HPLC sugar analysis of the enzymatic hydrolysates of each disaccharide glycoside (1).

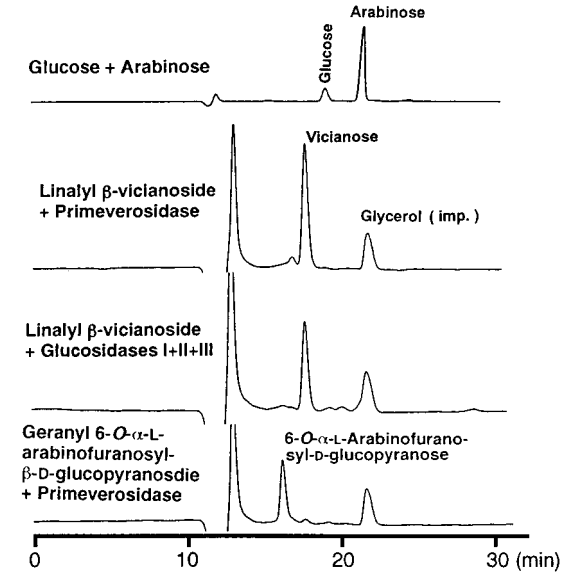

Figure 9. HPLC sugar analysis of the enzymatic hydrolysates of each disaccharide glycoside (2).

1993, 1994; Moon et al., 1994, 1996; Sakata et al., 1995a) together with a few exceptions of linalool oxides III and IV 6-*O*-*β*-D-apiofuranosyl-*β*-D-glucopyranosides and (*Z*)-3-hexenyl *β*-D-glucopyranoside (Moon et al., 1996). In this study, *β*-primeverosidase was clearly shown to be the most important glycosidase in the floral alcoholic aroma formation from the main alcoholic aroma precursors (*β*-primeveroside and 6-*O*-*β*-D-apiofuranosyl-*β*-D-glucopyranoside) (Figure 10).

ABBREVIATIONS USED

*p*NP, *p*-nitrophenyl; DTT, dithiothreitol; PBS, phosphate buffered saline; FPLC, fast protein liquid chromatography; MALDI-TOFMS, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry; TFA, trifluoroacetic acid.

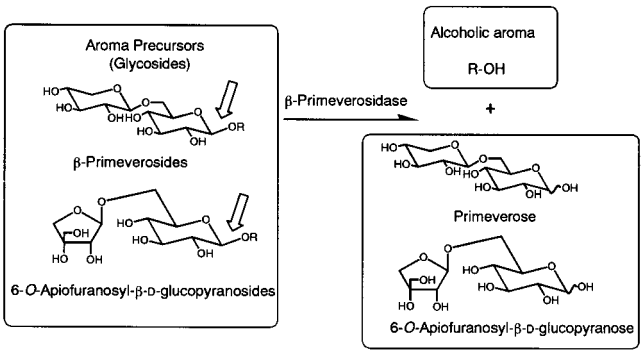

Figure 10. Proposed mechanism for the main alcoholic aroma formation in tea leaves.

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