Purification of a β-Primeverosidase Concerned with Alcoholic Aroma Formation in Tea Leaves (Cv. Shuixian) To Be Processed to Oolong Tea†

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β-Primeverosidase, which is concerned with the alcoholic aroma formation in tea leaves, was purified from fresh tea leaves (*Camellia sinensis* var. *sinensis* cv. Shuixian) to be processed to oolong tea by successive precipitation with acetone and ammonium sulfate followed by column chromatography on CM-Toyopearl and Mono S-HR. The molecular mass was shown to be 61 and 60.3 kDa by SDS−PAGE and TOFMS, respectively. Its p*I*, optimum temperature, and pH are 9.5, 45 °C, and 4, respectively. The enzyme is stable below 45 °C and between pH 3 and 5. The enzyme hydrolyzes β-primeveroside into primeverose and an aglycon without further hydrolysis. These enzymic characteristics were found to be quite similar to those of the β-primeverosidase from cv. Yabukita exclusively processed to green tea. As most of the alcoholic tea aroma precursors have been isolated as β-primeverosides, the main route of the aroma formation in oolong tea has been elucidated on a molecular basis.

Keywords: *β-Primeverosidase; oolong tea; Camellia sinensis; aroma formation; glycosidase; tea aroma*

INTRODUCTION

Any fresh tea leaves (*Camellia sinensis* var. *sinensis* and *C. sinensis* var. *assamica*) can principally be processed to green, oolong, and black tea. Each made tea has its own characteristics. Particular species of tea plants have been selected to produce each type of tea. For example, the fresh tea leaves of cv. Shuixian (*C. sinensis* var. *sinensis*) and cv. Yabukita (*C. sinensis* var. *sinensis*) used in this experiment are in general exclusively processed to oolong tea in China and to Japanese type green tea in Japan, respectively.

Alcoholic floral aroma is important in oolong tea, for the quality of the tea is said to mainly depend on the aroma. Monoterpene alcohols (linalool, geraniol, etc.) and aromatic alcohols (benzyl alcohol, 2-phenylethanol, etc.) are known to contribute to the floral aroma of oolong tea (Yamanishi, 1995).

In the course of our study on the molecular basis of the alcoholic aroma formation in oolong tea, we have isolated aroma precursors in 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 (*cis*- and *trans*-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 in cv. Yabukita (Nishikitani et al., 1996).

Quite recently we have purified a β-primeverosidase from fresh tea leaves (cv. Yabukita), which showed high substrate specificity toward β-primeverosides, which are hydrolyzed into primeverose and the corresponding aglycon (Guo et al., 1995; Sakata et al., 1995b). In this study, we attempted to purify the enzyme concerned with the alcoholic aroma formation from fresh leaves of cv. Shuixian and to compare its enzymic properties with that from cv. Yabukita.

MATERIALS AND METHODS

Tea Leaves. 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 plucking, the leaves were conventionally treated to prepare acetone powder. Fresh tea leaves (cv. Maoxie; harvested in May 1991, in Fujian, People's Republic of China) for oolong tea were plucked, immediately heated by pan-firing to inactivate the enzymes, and dried. The material was transported to Japan and extracted and fractionated to give the crude aroma precursor solution (Guo et al., 1993, 1994).

Measurement of Glycosidase Activities toward Several Kinds of *p*-Nitrophenyl (*p*NP) Glycosides. During purification, the enzyme fractions were measured for the hydrolysis activities of *p*NP monosaccharide glycosides (*p*NP β-D-gluco-, β-D-xylo-, β-D-galacto-, and α-L-arabinopyranosides; purchased from Sigma Chemical Co.) and *p*NP β-primeveroside [β-*O*-Xyl-(1−6)-β-D-Glc-$OC_6H_4NO_2$-*p*); enzymatically synthe-

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sized; 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 amount of enzyme liberating 1 μmol of *p*-nitrophenol/min under the assay conditions.

Preparation of Crude Aroma Precursor Solution. The hot-water extracts of tea leaves (cv. Maoxie) were subjected to successive column chromatographies on active charcoal (water−methanol) and Amberlite XAD-2 (water−methanol) as previously reported (Guo et al., 1993). The fractions containing aroma precursors from XAD-2 column were used for the following crude enzyme assay.

Crude Enzyme Assay. The enzyme solution [1 g equivalent of fresh tea leaves (cv. Shuixian)] was reacted with the crude aroma precursor solution [0.84 mL, 20 g equivalent of fresh tea leaves (cv. Maoxie)] in 0.1 M citrate buffer (10 mL, pH 6.0) at 30 °C for 1 h. Liberated aroma was extracted with ethyl ether (5 mL × 2). The aroma extract was transferred into a sample bottle, which was placed in a desiccator and subjected to gradually reducing pressure to concentrate the extract to ca. 1 mL. Two microliters of an internal standard solution containing ethyl decanoate (10 μg) was added to the concentrate. The sample solution was passed through a column (ca. 3 mL) of anhydrous Na_2SO_4, concentrated by a stream of nitrogen, and analyzed by a Hitachi Model G-5000 gas chromatograph (Hitachi, Ltd., Tokyo, Japan) equipped with an FID and a TC-Wax capillary column (0.25 mm i.d. × 30 m; GL Science Inc., Tokyo, Japan). The GC conditions were as follows: carrier gas, N_2 (1 mL/min); split ratio, 100:1; temperature program, hold at 60 °C for 10 min and then ramp to 200 °C at 3 °C/min; injector temperature, 250 °C; detector temperature, 250 °C.

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

(1) From Cv. Shuixian. Just after plucking, 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 spread on filter paper and occasionally turned over to evaporate the acetone. The residual powder ("acetone powder") was frozen and transported to Japan.

The following chromatographic purification was carried out basically in the same manner as previously reported for the enzyme from cv. Yabukita (Guo et al., 1995, 1996). The acetone powder (36.4 g, equivalent to 200 g of the fresh tea leaves) was suspended in 0.1 M citrate buffer (pH 6.0, 800 mL), stirred for 4 h, and centrifuged at 35000*g* for 20 min. The precipitate was resuspended in 0.1 M citrate buffer (pH 6.0, 300 mL) and treated in the same way. To the combined supernatant (ca. 1000 mL) was added chilled acetone (−20 °C) 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, 400 mL) and subjected to the conventional ammonium sufate precipitation. The crude enzyme separated by 40−80% ammonium sulfate precipitation was dissolved in 40 mL of 20 mM citrate buffer (pH 6.0) and then dialyzed overnight against 20 mM citrate buffer (pH 6.0). The dialyzed enzyme was charged on a CM-Toyopearl 650M column (Tosoh Corp., Tokyo, Japan; ∅ 32 × 140 mm, ca. 110 mL) equilibrated with 20 mM citrate buffer (pH 6.0). The absorbed protein was eluted by a linear gradient of NaCl from 0 to 0.5 M in 500 mL each of 20 mM citrate buffer (pH 6.0) with 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) and Centricon 10 (Grace Japan Co.). The primeverosidase fraction obtained by the CM-Toyopearl column chromatography was placed on a column (∅ 0.5 × 5 cm) of Mono S HR (Pharmacia Biotech Co. Ltd., Tokyo, Japan) equilibrated with 20 mM citrate buffer (pH 6.0). The enzyme was eluted with a linear gradient 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.

(2) From Cv. Yabukita. Purification of the β-primeverosidase was carried out in the same manner previously reported (Guo et al., 1995, 1996).

(a) Sodium Dodecyl Sulfate−Polyacrylamide Gel Electrophoresis (SDS−PAGE). SDS−PAGE was done according to the method of Laemmli (1970) on a 7.5% acrylamide gel (PAGEL, ATTO Corp., Tokyo, Japan) in a 25 mM Tris−192 mM glycine buffer system under nonreducing conditions. Proteins on the gel were stained with 0.115% Coomassie brilliant blue R-250. Phosphorylase *b* (94 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), soybean trypsin inhibitor (20.1 kDa), and α-lactalbumin (14.4 kDa) were used as standard proteins for the mass calibration.

Molecular Mass Measurement. The molecular mass of the enzyme was measured by matrix assisted laser desorption ionization time of flight mass spectrometry (MALDI-TOFMS). The most purified enzyme solution [0.1% trifluoroacetic acid (TFA)] was mixed with the same volume of matrix: a saturated sinapic acid (0.1% TFA/CH_3CN = 2:1). One microliter of the mixture was analyzed on a REFREX II (Bruker Co. Ltd., Karlsruhe, Germany) mass spectrometer. The mass spectrometer was operated in the positive mode, and ions were accelerated to an energy of 30 keV before entering the MALDI-TOF mass spectrometer. The matrix and analyte were ionized with N_2 laser beam.

Isoelectric Point Measurement. The isoelectric point of the enzyme was measured with a Pharmacia Phast System using Phast Gel Dry IEF (Pharmacia Biotech Co. Ltd.) reswelled by a reswelling solution containing water (2 mL), glycerol (300 μL), arginine (3 mg), and Ampholine 7−9 (100 μL) and 9−11 (80 μL). Proteins were stained with Coomassie brilliant blue R-350. Cytochrome *c* (p*I* 10.25), trypsinogen (p*I* 9.30), lentil lectin basic band (p*I* 8.65), lentil lectin middle band (p*I* 8.45), lentil lectin acidic band (p*I* 8.15), horse myoglobin basic band (p*I* 7.35), horse myoglobin acidic band (p*I* 6.85), human carbonic anhydrase B (p*I* 6.55), bovine carbonic anhydrase B (p*I* 5.85), and β-lactoglobulin A (p*I* 5.20) were used as standard proteins.

Sugar Analysis. Eugenyl β-primeveroside isolated from *Camellia sasanqua* Thumb. (Yamada et al., 1967) was hydrolyzed by the enzyme sample in 20 mM citrate buffer (pH 6.0) at 37 °C for 24 h, and then proteins in the reaction mixture were removed by Centricon 10 (Grace Japan Co. Ltd.). The supernatant (10 μL) was injected into a CarboPac PAI (∅ 4 × 250 mm) (Dionex Corp., Sunnyvale, CA) and eluted with a linear gradient from 55 to 100 mM NaOH at flow rate of 1 mL/min at room temperature. Into a solution postcolumn 300 mM sodium hydroxide was added at room temperature. Sugars were detected by using a pulsed amperometric detector (Dionex Corp.).

The molecular mass of the liberated sugar was measured with a LC-MS: a JMS-SX 102A mass spectrometer (JEOL Co. Ltd., Tokyo, Japan) with JMA-DA 7000 (JEOL Co. Ltd.) mass data system linked with a Hitachi-L6000 series HPLC (Hitachi, Ltd.). LC-frit FAB-MS conditions were as follows: column, Asahipak NH2-50P 4E (∅ 4.6 × 250 mm; Asahikasei Kogyo Co. Ltd., Tokyo, Japan); solvent, 80% MeCN; flow rate, 1 mL/min; injection volume, 20 μL; accelerated volt, −10 keV; ion multivolt, 2.2 keV; matrix, diethanolamine.

Effect of Temperature and pH on Enzymic Activity and Stability. The optimum temperature of the enzyme was measured with *p*-nitrophenyl 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 20 mM citrate buffer (pH 6.0) at various temperatures (30−60 °C) for 1 h. The optimum pH of the enzyme was examined in buffers with various pH values (2.0−9.0) at 37 °C for 1 h. The pH stability of the enzyme was examined from the residual activities after incubation in the buffers with various pH values (2.0−9.0) at 37 °C for 1 h. The buffers used were 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).

Table 1. Aroma Amounts Liberated from a Crude Aroma Precursor Solution by Treatment with Each Enzyme Preparation from Cv. Shuixian

purification step	alcoholic tea aroma compounds				
	LO[a] I	IO II	linalool	geraniol	2-phenylethanol
buffer extracts	20[b]	16	170	120	43
50% acetone ppt	8.5[c]	7.2[c]	93[c]	61[c]	21[c]
40% $(NH_4)_2SO_4$ ppt	1.0	0.80	26	26	3.1
40−80% $(NH_4)_2SO_4$ ppt	9.3	6.5	89	66	20
80−100% $(NH_4)_2SO_4$ ppt	1.0	0.70	34	23	3.0

[a] LO, linalool oxide. [b] Relative amounts were calculated from the peak area by comparison with that of an internal standard (ethyl decanoate). [c] Lower activities than the sum of that of each $(NH_4)_2SO_4$ ppt sample are likely to be caused by the remaining acetone.

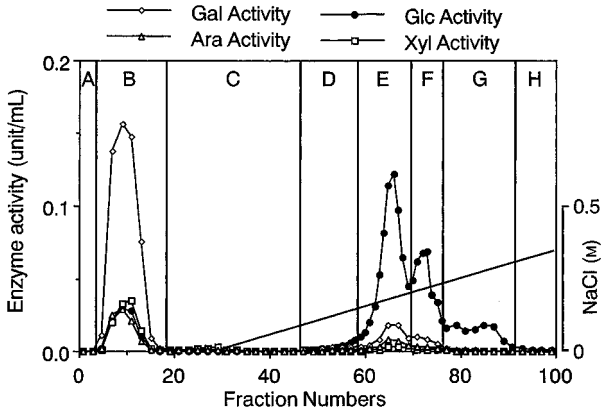

Figure 1. CM-Toyopearl 650M column chromatography of enzymes from tea leaves (cv. Shuixian), *p*NP β-Glycosides hydrolytic activities of each fraction. Elution: 20 mM citrate buffer (pH 6.0); NaCl gradient elution; flow rate, 2 mL/min. Glucosidase (Glc), xylosidase (Xyl), galactosidase (Gal), and arabinosidase (Ala) activities were measured with *p*NP β-D-gluco-, β-D-xylo-, β-D-galacto-, and α-L-arabinopyranosides, respectively.

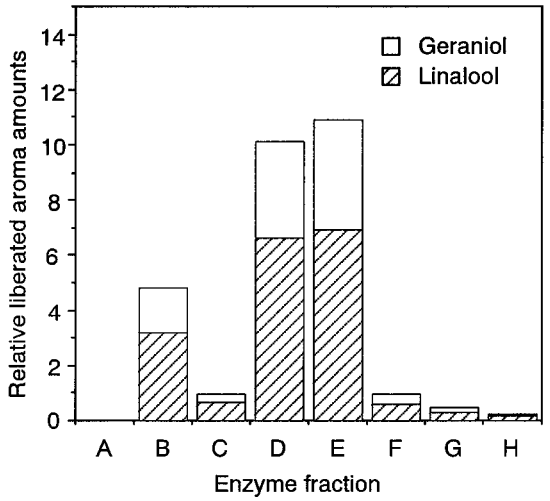

Figure 2. Linalool and geraniol liberated from a crude tea aroma precursor solution by the action of the enzyme fractions A−H (Figure 1). Relative amounts of geraniol and linalool were calculated from the peak area by comparison with that of an internal standard (ethyl decanoate).

RESULTS AND DISCUSSION

An acetone powder (a crude enzyme) was prepared from the fresh tea leaves (cv. Shuixian) by the conventional method. Glycosidases were extracted from the acetone powder with 0.1 M citrate buffer (pH 6.0) and subjected to acetone precipitation followed by ammonium sulfate precipitation. Enzyme samples of each purification step were reacted with a crude aroma precursor solution prepared from tea leaves (cv. Maoxie) as previously reported (Guo et al., 1993). The generated aromas were analyzed by a capillary GC (Table 1). Most of the enzyme activities concerned with the alcoholic aroma formation were contained in 40−80% ammonium sulfate precipitate, although considerable amounts of geraniol and linalool were liberated by the action of the other enzyme preparations (40 and 80−100% ammonium sulfate ppt).

The main crude enzyme (40−80% ammonium sulfate precipitate) was chromatographed on a CM-Toyopearl column (20 mM citrate buffer, pH 6.0, 0−0.5 M NaCl gradient). Each fraction was measured for glycosidase activities with several kinds of monosaccharide glycosides and combined into fractions A−H according to the β-glucosidase activity that was the most prominent (Figure 1). This elution pattern is similar to that in the case of cv. Yabukita (Figure 4) (Guo et al., 1996).

Each fraction A−H was reacted with a crude aroma precursor solution, and the generated aroma constituents were analyzed by GC (Figure 2). Amounts of the alcoholic aroma generated increased in the order of fraction E, D, and B. Since there was little glycosidase activity toward monosaccharide glycosides in fraction D, we expected the presence of primeverosidase in

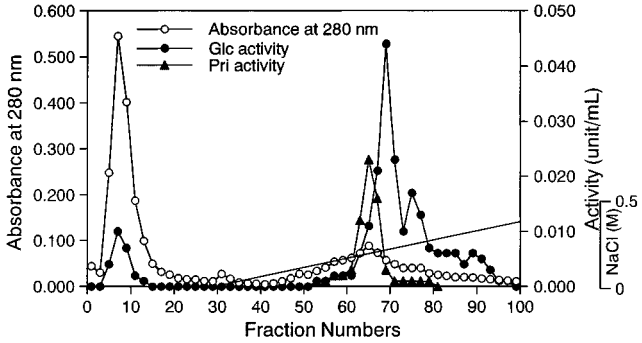

Figure 3. CM-Toyopearl 650M column chromatography of crude enzymes from tea leaves (cv. Shuixian). The elution conditions were the same as in Figure 1. Glucosidase (Glc) and primeverosidase (Pri) activities were measured with *p*NP β-D-glucopyranoside and β-primeveroside, respectively.

fractions D and E, because we had isolated several kinds of β-primeverosides as aroma precursors (Sakata et al., 1995a).

At this stage we obtained *p*NP β-primeveroside by enzymatic transglycosylation from xylobiose to *p*NP β-D-glucopyranoside (Kajiura, 1994). The crude enzymes prepared from both cvs. Shuixian and Yabukita were applied on the same column of CM-Toyopearl in the same way, and the glycosidase activity of each fraction was measured with *p*NP β-D-glucoside and *p*NP β-primeveroside. In both cases each β-primeverosidase was eluted just before the main glucosidase (Figures 3 and 4), suggesting that the β-primeverosidases were identical or quite similar to each other. From this fact the β-primeverosidase from cv. Shuixian was supposed to be eluted mainly in fraction E and only partly in fraction

Table 2. Purification of β-Primeverosidase from Fresh Tea Leaves (Cv. Shuixian)

purification step	total act. (units)	sp act. (units/mg)	purification fold	recovery (%)
buffer extract	7.8[a]			100
40−80% $(NH_4)_2SO_4$ ppt	2.6			33
CM-Toyopearl (fractions 61−69)	1.1	0.16	1	14
Mono S (fractions 61−73)	0.10	0.98	6.1	1.3

[a] The enzyme activities were measured with *p*NP primeveroside.

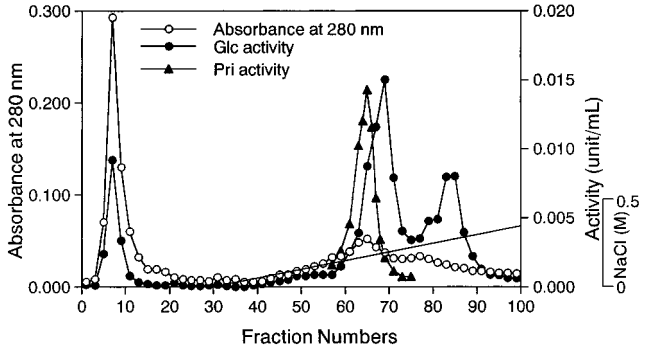

Figure 4. CM-Toyopearl 650M column chromatography of crude enzymes from tea leaves (cv. Yabukita). The elution conditions and measurements of enzymic activities were the same as in Figure 3.

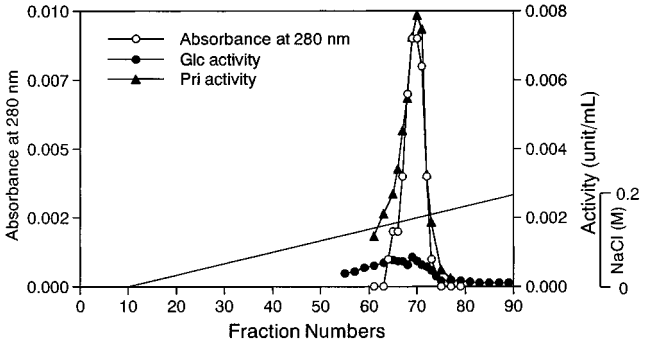

Figure 5. FPLC of primeverosidase fraction on a Mono S column. Column, Mono S HR 5/5; elution, 20 mM citrate buffer (pH 6.0); NaCl gradient (0−0.2 M); flow rate, 1 mL/min.

E (Figure 1). This explains why a lot of tea aromas were liberated (Figure 2) when the enzyme sample of fraction D was reacted with a crude tea aroma precursor solution, although fraction D did not show any glycosidase activities toward any *p*NP monosaccharide glycosides (Figure 1). In other words, the potent β-glucosidase activity in fraction E is likely to have almost nothing to do with the tea aroma formation.

Considerable amounts of aroma were generated from fraction B (Figure 2), in which many kinds of monosaccharide glycosidases were eluted (Figure 1). This can be rationalized as follows. Some of them possibly hydrolyze the xylosyl moiety of geranyl and linalyl β-primeverosides to some extent, and the liberated β-glucosides can be hydrolyzed by β-glucosidase in the fraction to liberate the aroma.

The β-primeverosidase fractions almost devoid of other glycosidic enzymes (fractions 62−64) were finally purified by Fast Protein liquid chromatography (FPLC; Pharmacia LKB Biotechnology, Uppsala, Sweden) with a column of Mono S-HR (20 mM citrate buffer, pH 6.0, 0−0.2 M NaCl gradient) (Figure 5). Primeverosidase activity with a very low level of the apparent β-glucosidase activity overlapped with the absorption at 280 nm. The purification procedures are summarized in Table 2. The molecular mass was found to be 61 kDa by the SDS−PAGE analysis and identical to that of cv. Yabukita (Guo et al., 1995, 1996; Sakata et al., 1995b). It was confirmed to be 60.3 kDa by TOFMS (Figure 6). The repurified β-primeverosidase from cv. Yabukita gave a molecular mass identical with that of the β-primeverosidase from cv. Shuixian in the TOFMS analysis (Figure 6), although the sample was not very pure. The isoelectric point of the β-primeverosidase from cv. Shuixian was found to be 9.5. This value is also quite similar to that (p*I* 9.4) of the β-primeverosidase from cv. Yabukita (Guo et al., 1996). These analytical results suggest that both enzymes are identical or quite similar to each other.

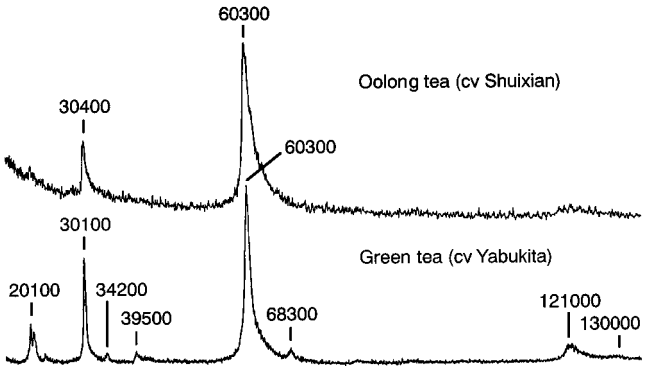

Figure 6. TOFMS spectra of β-primeverosidases from fresh tea leaves (cvs. Shuixian and Yabukita).

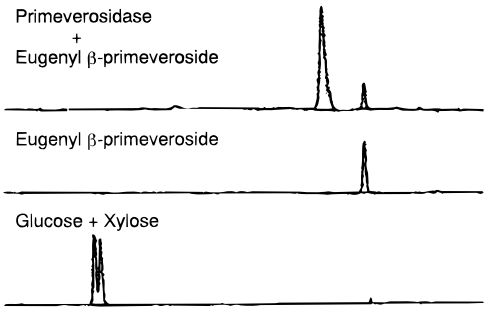

Figure 7. HPLC analysis of the hydrolysate of eugenyl β-primeveroside by the β-primeverosidase from tea leaves (cv. Shuixian).

After authentic eugenyl β-primeveroside (Yamada et al., 1967) was hydrolyzed with the β-primeverosidase, generated sugars were analyzed by HPLC. Only primeverose and unreacted eugenyl β-primeveroside were detected and neither glucose nor xylose (Figure 7), indicating that the enzyme hydrolyzes β-primeveroside to release aglycon and primeverose (6-*O*-β-D-xylopyranosyl β-D-glucopyranose). The molecular mass of the peak expected to be primeverose was confirmed to be *m/z* 311 [$(M - H)^-$] by LC/MS analysis. The β-primeverosidase showed maximal activity at 45 °C and pH 4.0. It was stable at temperatures up to 45 °C and between pH 3 and 5 (Figure 8).

On the basis of the foregoing, most of the alcoholic tea aroma in oolong tea leaves has been confirmed to be generated from β-primeverosides as aroma precursors by the action of the endogenous β-primeverosidase. The β-primeverosidase from tea leaves for oolong tea

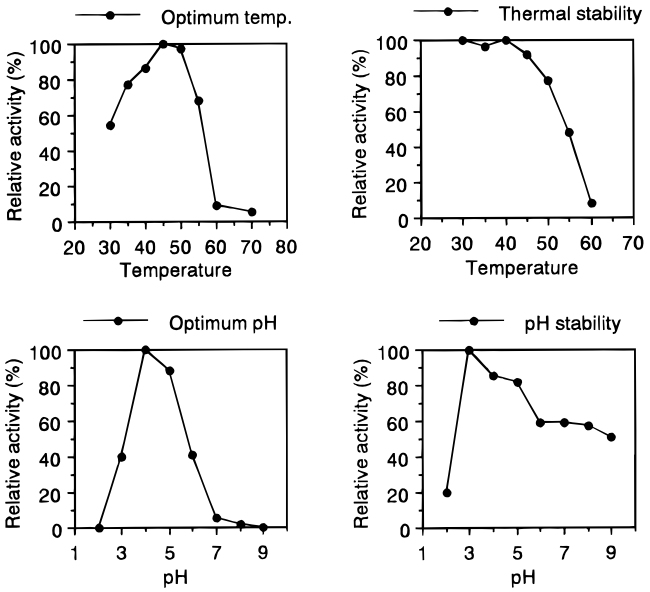

Figure 8. Effects of temperature and pH on the activity and stability of β-primeverosidase from oolong tea leaves (cv. Shuixian).

(cv. Shuixian) was shown above to have the same molecular mass as and quite similar characteristics to β-primeverosidase from tea leaves for green tea (cv. Yabukita). Elution patterns of glycosidases from the CM column were slightly different from each other between cvs. Shuixian and Yabukita (Figures 3 and 4), suggesting that glycosidase activities are different in detail from species to species in tea plants, although both cultivars contain β-primeverosidase as a main glycosidase.

We have isolated most of alcoholic aroma precursors as β-primeverosides [geranyl, (*S*)-linalyl, 2-phenylethyl, benzyl, methyl salicylate, linalool oxides I and II β-primeverosides] (Guo et al., 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*)-hexenyl β-D-glucopyranoside (Moon et al., 1996). On the other hand, geranyl β-primeveroside and β-vicianoside have been isolated as aroma precursors of geraniol from cv. Yabukita by Kobayashi's group (Nishikitani et al., 1996), while only the primeveroside has been isolated as its sole aroma precursor from cv. Shuixian by us (Guo et al., 1993). Kobayashi et al. reported the isolation of benzyl β-D-glucopyranoside as an aroma precursor from cv. Yabukita (Yano et al., 1991), but we isolated only benzyl β-primeveroside as a sole aroma precursor of benzyl alcohol. Then we tried to confirm the aroma precursor(s) of cv. Yabukita by applying the same isolation procedure in the case of cv. Maoxie and found that benzyl alcohol is stored as an aroma precursor of a ca. 1:1 mixture of β-primeveroside and β-D-glucopyranoside in cv. Yabukita (Ijima, 1994).

Thus, different aroma precursors of the same aroma components are likely to be present in tea leaves of different cultivars. It will be interesting and important for breeding of new tea plants to obtain made tea with higher quality to know varieties and amounts of alcoholic aroma precursors in each tea cultivar as well as those of glycosidases concerned with the alcoholic aroma formation.

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