Substrate specificity of β-primeverosidase, a key enzyme in aroma formation during oolong tea and black tea manufacturing.

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Summary

In the course of our studies on molecular basis of floral tea aroma formation during the tea manufacturing, we have clarified that a β-primeverosidase is deeply concerned with the floral tea aroma formation of so-called fermented tea, oolong tea and black tea. To know in detail the substrate specificity of the purified β-primeverosidase from a tea cultivar (cv. Yabukita) as well as the apparent substrate specificity of a crude enzyme extract from the cultivar, we synthesized nine kinds of diglycosides and a monoglycoside of 2-phenylethanol. The crude enzyme extract mainly showed β-primeverosidase activity, although monoglycosidase activities are present to some extent. The purified β-primeverosidase showed very narrow substrate specificity with respect to the glycon moiety, especially for β-primeverosyl (6-*O*-β-D-xylopyranosyl-β-D-glucopyranosyl) moiety. The enzyme hydrolyzed naturally occurring diglycosides such as β-primeveroside (100), β-vicianoside (3.0), β-acuminoside (0.8), β-gentiobioside (0.25) and 6-*O*-α-L-arabinofuranosyl-β-D-glucopyranoside (0.07), all of which have 1 - 6 glycosyl linkages, but were unable to hydrolyze synthetic unnatural diglycosides. The purified enzyme was inactive toward 2-phenylethyl β-D-glucopyranoside, too. The enzyme hydrolyzed each of the diglycosides into the corresponding disaccharide and 2-phenylethanol. These results indicate that the β-primeverosidase is a major glycosidase in tea leaves and is a key enzyme involved in aroma formation during the tea manufacturing process.

Key words

substrate specificity, β-primeverosidase, diglycosidase, β-primeveroside, disaccharide glycoside, tea aroma, *Camellia sinensis*

Introduction

Aroma is one of the most important factors to determine the character and quality of each tea product. Monoterpene alcohols such as linalool and geraniol, and aromatic alcohols such as benzyl alcohol and 2-phenylethanol, which are known to contribute the floral aroma of oolong tea and black tea, have been clarified to be mainly present as diglycosides in fresh leaves of tea plants.[1-3)]

We recently purified a specific glycosidase, β-primeverosidase, which hydrolyzes those diglycosidic aroma precursors to liberate the floral tea aroma and disaccharides from fresh leaves of a tea cultivar for green tea (*C. sinensis* var. *sinensis* cv. Yabukita). This enzyme has 61 kDa of molecular weight by SDS-PAGE analysis and is also present in fresh leaves of a tea cultivar for oolong tea (*C. sinensis* var. *sinensis* cv. Shuixian) and that for black tea (*C. sinensis* var. *assamica*).[4,5)]

Preliminary studies on the β-primeverosidase have shown its ability to hydrolyze β-acuminoside and β-vicianoside as well as β-primeveroside.[5,6)] However, the enzymatic characteristics and substrate specificity of this enzyme still remains unclear, although gene cloning of the enzyme has been succeeded.[7)]

Here we describe substrate specificity of the purified β-primeverosidase from cv. Yabukita in detail using various kinds of diglycosides and monoglycosides as substrates.

Materials and Methods

Substrates. *p*-Nitrophenyl (*p*NP) β-primeveroside (**11**) was enzymatically synthesized as previously reported[13] and provided by Nihon Shokuhin Kako Co. Ltd. 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 by ourselves.[8]

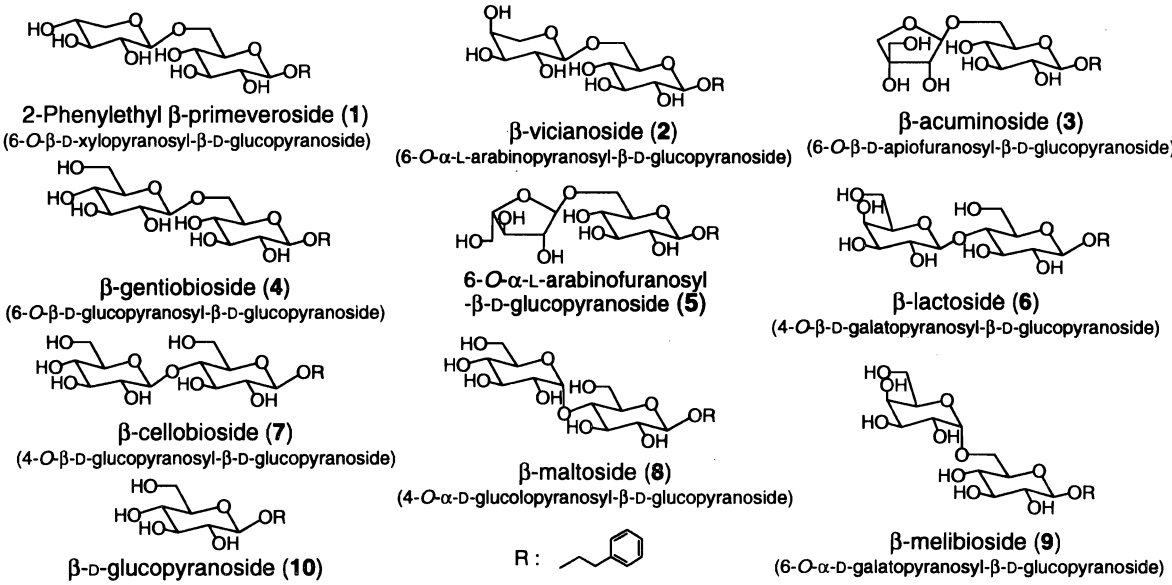

Fig. 1. 2-Phenylethyl glycosides designed to study the substrate specificity of β-primeverosidase

Measurement of glycosidase activities. A crude enzyme extract (0.44 unit/ml) and a purified β-primeverosidase (36 unit/mg) were prepared from an acetone powder (8.8 unit/g) obtained from cv. Yabukita as previously reported.[9] The purified enzyme was dissolved in 20 mM citrate buffer (pH 6.0) and was used as a purified β-primeverosidase stock solution (0.54 unit/ml).

Glycosidase activities were determined by measuring the liberation of *p*-nitrophenol or 2-phenylethanol from each glycoside. Each reaction mixture (150 μl) contained 10 mM substrate, 20 mM citrate buffer (pH 6.0), 0.1% BSA (bovine serum albumin) and 60 μl of the enzyme solution. A mixture without enzyme was preincubated at 37 °C and adding the enzyme started the reaction. An aliquot (20 μl) of each enzyme reaction mixture was taken with various time intervals during the incubation. The aliquot of a reaction mixture with *p*NP glycoside was added to 60 μl of 0.2 M Na_2CO_3 solution, and the 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 MeCN solution containing 3.0 μg of benzyl alcohol was added as an internal standard. A sample (20 μl) from each mixture (30 μl) was injected to HPLC [YMC-pack ODS-AQ (250 × 4.6 mm) (YMC Co., Ltd., Kyoto, Japan); column temperature, 40 °C; mobile phase, 33% MeCN; flow rate, 1.0 ml/min]. Liberated 2-phenylethanol (t_R 8.7 min) was detected at 210 nm. The hydrolysis rate was determined from the linear portion (within 10 % hydrolysis of substrates) of each assay curve.

Protein content was measured with the Coomassie® protein Assay Reagent (PIERCE, Rockford, USA), using BSA as a standard. One unit of activity was defined as the amount of enzyme liberating 1 μmol of *p*-nitrophenol from *p*NP β-primeveroside per min in 20 mM citrate buffer (pH 6.0) at 37 °C.

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 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 in the same conditions as in the case of **1**. Aliquots (20 μl) were taken at regular intervals and applied to HPLC as described above. The kinetic parameters and standard deviations were analyzed by the Kaleida Graph and were estimated by fitting the initial velocity data to the Michaelis-Menten equation.

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).

Results and Discussion

Substrate specificity of β-primeverosidase from fresh tea leaves (cv. Yabukita)

Initially, the hydrolysis pattern of a crude enzyme extract from tea leaves on two synthetic 2-phenylethyl glycosides (**1** and **10**) and on *p*NP β-primeveroside (**11**) and four kinds of *p*NP β-monoglycosides (**12** ~ **15**) were examined for preliminary characterization of the enzyme extract. The crude enzyme (0.18 unit/ml) was incubated with each glycoside (10 mM) in 20 mM citrate buffer (pH 6) at 37 °C. The liberated 2-phenylethanol was analyzed by HPLC (ODS; 33% acetonitrile) and the *p*-nitrophenol was determined at 405 nm. The activity was shown 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 2-phenylethyl β-primeveroside (**1**: 100%), although the hydrolytic activities on **14** and **15** were very low. The crude enzyme showed 5 ~ 250-fold higher activities on β-primeverosides (**1** and **11**) than on monoglycoside (**10** and **12** ~ **15**). The activity on **1** was about 2-fold higher than that on **11**. The β-glucosidase activity (11%) was almost the same level as the β-galactosidase activity (12%). Activities of these monoglycosidases have been reported in tea leaves, although the enzymatic characterization was still limited.[9,10]

Next, the relative activities of β-primeverosidase (0.22 unit/ml) purified from tea leaves were determined on the same substrates under the same conditions as that of the crude enzyme extract. The purified enzyme showed highly selective activities toward diglycosidic substrate, β-primeverosides (**1** and **11**). The hydrolysis rate of **1** was about 2-fold higher than that of **11**, which was almost identical to the results with the crude enzyme extract. However, in contrast with the activities of the crude enzyme, most of the monoglycoside (**13**, **14** and **15**) were not hydrolyzed at all, while *p*NP β-D-glucopyranoside (**12**) was hydrolyzed only to an appreciable extent. These results indicate that β-primeverosidase is a major glycosidase in tea leaves, although other monoglycosidases such as β-glucosidase and β-galactosidase are present to some extent.[5,8] The 2-phenylethyl group seem to be more preferred aglycon moiety for β-primeverosidase than *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 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 (Table 2). The crude enzyme showed 2.42, 0.60, 0.34 and 0.05% of hydrolytic activities toward **2**, **3**, **4** and **5** relative to the activity of **1**, respectively, but **6** ~ **9** were not hydrolyzed at all. Activities of the crude enzyme toward 2-phenylethyl diglycosides were most likely to 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. No unnatural diglycosides (**6** ~ **9**) with a β (1→4) or an α (1→4) glycosidic linkage were hydrolyzed.

Table 2. Substrate specificity of the crude Enzyme extract and the purified β-primeverosidase from tea leaves toward 2-phenylethyl diglycosides

Substrates	Crude enzyme extract		Purified β-primeverosidase	
	Specific activity (μmol/min/mg protein)	Relative activity (%)	Specific activity (μmol/min/mg protein)	Relative activity (%)
2-Phenylethyl β-primeveroside (**1**)	2.33	100	66.7	100
2-Phenylethyl β-vicianoside (**2**)	0.056	2.42	1.98	2.97
2-Phenylethyl β-acuminoside (**3**)	0.014	0.60	0.511	0.77
2-Phenylethyl β-gentiobioside (**4**)	0.008	0.34	0.168	0.25
2-Phenylethyl6-O-α-L-arabino-furanosyl-β-D-glucopyranoside (**5**)	0.001	0.05	0.047	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
The activity was measured by the liberation of 2-phenyethanol from their glycosides. The liberated 2-phenyethanol was analyzed by HPLC. Reaction mixtures contained 10 mM substrate, the enzyme (crude enzyme extract, 0.18 unit/ml; purified β-primeverosidase, 0.22 unit/ml) and 20 mM citrate buffer (pH 6), and the mixtures were incubated at 37℃.

To define the substrate specificity with respect to the glycon moiety more quantitatively, kinetic parameters were determined for two kinds of 2-phenylethyl diglycosides (**1** and **2**), on which the enzyme showed relatively high activities. 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 constants for **1** (k_{cat}/K_m = 21.9 $s^{-1}mM^{-1}$) was 56-fold higher than that for **2** (k_{cat}/K_m = 0.391 $s^{-1}mM^{-1}$). 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-acuminosides only in some extent. The hydrolysis of β-primeveroside as well as β-vicianoside and β-acuminosides is likely to occur during the fermentation process in manufacturing of oolong tea and black tea. At the fermentation process, β-primeveroside is expected to be hydrolyzed mainly, because the relative activity toward β-primeveroside is much higher than that toward other glycosides, and β-primeverosides contents are higher than others in tea leaves.[10] This is supported by the report that the amount of β-primeverosides decreased greatly during the manufacturing process of black tea.[11] 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 floral aroma in oolong tea and black tea. Investigation of aglycon specificity of this enzyme will give further understanding of this aspect.

Hydrolysis Mode of diglycosides by β-primeverosidase

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) when the reaction completed. This confirmed 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 is a key enzyme responsible for the floral aroma formation during the manufacturing process of oolong tea and black tea.

Acknowledgments - The authors thank Nihon Shokuhin Kako Co. Ltd. for their generous gift of *p*NP β-primeveroside, and Dr. Z. Günata of Institute des Products de la Vigne, France for peracetylated 2-phenylethyl β-acuminoside.

Reference

1. Sakata, K., Watanabe, N., and Usui, T.: Molecular basis of alcoholic aroma formation during tea processing. In "Food for Health in the Pacific Rim," Proceedings of the 3rd International Conference of Food Science and Technology, ed. by J. R. Whitaker, *et al.*, Davis, California, Foods Nutrition Press, Inc., Trumbull, Connecticut, p. 93-105 (1999).
2. Sakata, K., Watanabe, N.: *J. Appl. Glycosci.* **45**, 123-129 (1998).
3. 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).
4. Sakata, K.: β-Primeverosidase relationship with floral tea aroma formation during processing of oolong tea and black tea. In "Caffeinated Beverages. Health Benefits, Physiological Effects, and Chemistry, ACS Symposium Series 754," ed. by Parliament, T. H., Ho, C. and Schieberie, P., American Chemical Society, Washington, DC, 2000, p. 327-335.
5. 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).
6. 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).
7. 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 this proceedings.
8. 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).
9. Ma, S.-J., Mizutani, M., Hiratake, J., Hayashi, K., Yagi, K, Watanabe, N. and Sakata, K.: Substrate specificity of β-primeverosidase, a key enzyme in aroma formation during oolong tea and black tea manufacturing. *Biosci. Biotechnol. Biochem.*, in press.
10. Halder, J., and Bhaduri, A.: Glycosidases from tea-leaf (*Camellia sinensis*) and characterization of β-galactosidase. *Nutritional Biochemistry*, 8, 378-384 (1997).
11. 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).
12. 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).
13. 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).