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Note

Cloning of Myrosinase cDNA from *Wasabia japonica* Matsum.

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Myrosinase (β-thioglucoside glucohydrolase, EC 3.2.3.1) is involved in pungency generation in wasabi (*Wasabia japonica* Matsum.). We isolated full-length cDNA (WjMY1) encoding myrosinase from the petiole of wasabi. The deduced polypeptide has 545 amino acids with a molecular weight of 62107. WjMY1 mRNA is accumulated in whole organs of the wasabi plant; the order of expression is petiole≥rhizome>leaf. The order was found to be similar to that of the specific activity of myrosinase. This is the first report describing the isolation of a myrosinase gene from wasabi.

Keywords: myrosinase, wasabi, *Wasabia japonica* Matsum.

Introduction

Wasabi, or Japanese horseradish (*Wasabia japonica* Matsum.), is a perennial herb traditionally used in Japanese food. Demand for wasabi is increasing as Japanese food increases in popularity around the world. Recently, it has been reported that wasabi paste or compounds extracted from wasabi have many health benefits (Kinae *et al.,* 2000). Wasabi showed bactericidal activity against *Helicobacter pylori* by inhibiting bacterial urease activity (Masuda *et al.,* 2004, Shin *et al.,* 2004). The growth of *Vibrio parahaemolyticus* was inhibited by allyl isothiocyanate from wasabi (Hasegawa *et al.,* 1999). 6-Methylsulfinylhexyl isothiocyanate has been found to induce apoptosis in human monoblastic leukemia U937 cells (Watanabe *et al.,* 2003). Antiplatelet and anticancer activities were found in isothiocyanates from wasabi (Morimitsu *et al.,* 2000). A wasabi-derived isothiocyanate inhibited the development of lung tumors in mice (Yano *et al.,* 2000).

Isothiocyanates are generated by a myrosinase-glucosinolate system (Hara *et al.,* 2003a). The system, which is common in Brassicaceae species, consists of two components, the enzyme myrosinase and the substrate glucosinolate. Mechanical disruption of the plant tissue causes an enzymatic reaction, and glucosinolates are converted to the corresponding isothiocyanates. Myrosinase genes have been isolated from some Brassicaceae species (Rask *et al.,* 2000). Although amino acid sequences are conserved in all myrosinases of Brassicaceae, two subfamilies, the Brassicaceae crop type and the *Arabidopsis* type, were classified by their sequence similarity (Hara *et al.,* 2003a). Wasabi-derived myrosinases were purified and characterized by Ohtsuru and Kawatani (1979), and Hara *et al.,* (2001) demonstrated that the myrosinase-glucosinolate system in wasabi exists in the epidermis and vascular cambium of the root. However, there are no reports concerning the myrosinase gene of wasabi. In this paper, we report the sequence of wasabi myrosinase and examine its expression in wasabi plants.

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Materials and Methods

Plant materials Wasabi (*Wasabia japonica* Matsum. cultivar Mazuma) was grown in a field developed in a mountain stream in Amagi, Shizuoka, Japan. One-year-old plants were harvested, separated into leaves, petioles, and rhizomes, frozen immediately with liquid N_2, and kept at −70℃ until use.

cDNA cloning of wasabi myrosinase (WjMY1) Total RNA was extracted from the wasabi petiole with an RNeasy Plant Mini Kit (Qiagen, Tokyo, Japan). The cDNA fragments of wasabi myrosinase were prepared by reverse transcription-polymerase chain reaction (RT-PCR) using an RNA PCR Kit Ver. 2.1 (Takara, Tokyo, Japan). Reverse transcription was carried out at 45℃ for 30 min, and PCR was conducted through 30 cycles of 94℃ for 30 s, 42℃ for 30 s, and 72℃ for 90 s. Degenerated primers were designed based on conserved regions of myrosinases from *Brassica napus* (myrosinase A type, EMBL accession number Z21978; myrosinase B type, EMBL accession number X60214; myrosinase C type, EMBL accession number X79080), *Sinapis alba* (myrosinase B type, EMBL accession number X59879), and *Arabidopsis thaliana* (thioglucoside glucohydrolase 1, EMBL accession number X79194; thioglucoside glucohydrolase 2, EMBL accession number X79195). PCR with the MYR1 primer (5′-TTRYMRAAYTCRTARTTRTCNCC-3′) and the MYR2 primer (5′-AAYTAYTAYGTNACNCARTAYGC-3′) gave a predicted fragment of ca. 420 bp. The annealing sites of the primers are shown in Fig. 1B. The PCR

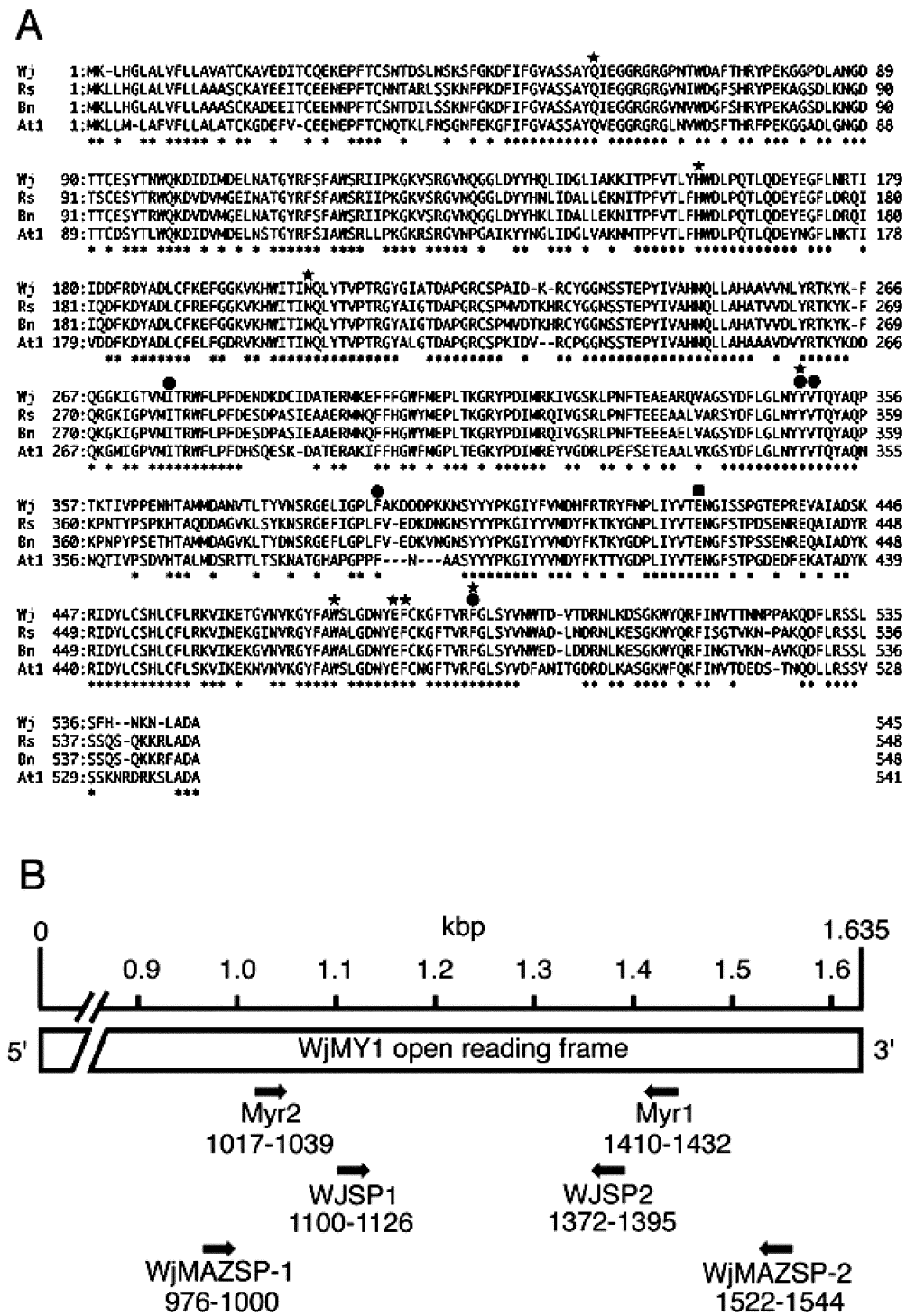

Fig. 1. Comparison of amino acid sequences of wasabi myrosinase (WjMY1) and other myrosinases, and PCR primers used in this study.

A. Alignment of amino acid sequences. Wj: wasabi myrosinase WjMY1 (DDBJ accession number AB194903, this study); Rs: radish (*Raphanus sativus*) myrosinase (DDBJ accession number AB042186); Bn: rape (*Brassica napus*) myrosinase (EMBL accession number X60214); At1: *Arabidopsis thaliana* myrosinase (thioglucoside glucohydrolase 1, EMBL accession number X79194). Asterisks indicate identical residues among the four sequences. Glucose ring recognition sites (★), residues involved in an aglycon pocket (●), and a residue forming a glucosidic bond (■) are shown.

B. Design of PCR primers. Arrows indicate the direction from 5′ to 3′ in the primers. Numbers under the names of primers are the positions of the corresponding primers in the nucleic acid sequence of WjMY1 cDNA.

products were cloned into the pDrive Cloning Vector of a PCR Cloning Kit (Qiagen) and sequenced. Nucleotide sequencing was performed using a SequiTherm EXCEL II Long-Read Premix DNA Sequencing Kit-LC (Epicenter Technologies, WI, USA) on a DNA sequencer (Model 4000 L, Aloka, Tokyo, Japan). The 3′-rapid amplification of

cDNA ends (RACE) method and 5′-RACE method were applied to isolate full-length clones. The primer sequences were as follows: WJSP1 primer (5′-CTGCCATGATGGACGCAAACGTAACGC-3′) for 3′-RACE and WJSP 2 primer (5′-CGTCTCCTTGATGACCTTACGGAG-3′) for 5′-RACE. The annealing sites of the primers are shown in Fig. 1B.

Quantification of myrosinase transcripts Total RNA was extracted from the leaves, petioles, and rhizomes of wasabi with the RNeasy Plant Mini Kit (Qiagen). One microgram of RNA was subjected to semiquantitative RT-PCR (Goidin *et al.,* 2001) using the QuantumRNA 18S Internal Standards Kit (Ambion, TX, USA). In this assay, the target RNA can be quantified as a relative amount using endogenous rRNA as an internal standard. Reverse transcription was performed at 45℃ for 30 min, and PCR proceeded through 30 cycles of 94℃ for 30 s, 55℃ for 30 s, and 72℃ for 90 s. Specific primers for wasabi myrosinase, designed from sequences revealed in this study, were 5′-CTTCCCAACTTCACGGAAGCAGAAG-3′ (WjMAZSP-1 primer) and 5′-GTTGGTGGTAACGTTAATGAACC-3′ (WjMAZSP-2 primer). The annealing sites of the primers are shown in Fig. 1B. The ratio of 18S primer to competimer was 3: 7. The PCR reactions were stopped at 12, 18, 24, and 30 cycles and the products were analyzed by 1.5% agarose gel electrophoresis with ethidium bromide staining. After determining band intensity using NIH-Image software, the relative mRNA contents were deduced from the intensities of the target PCR products and the rRNA PCR products according to the instruction manual for the QuantumRNA Kit. A logarithmic increase in band intensity was observed from 12 cycles to 24 cycles. Thus, the values at 18 cycles were used to determine the relative mRNA content.

Myrosinase activity Myrosinase activity was measured by the method of Palmieri *et al.,* (1982) with slight modifications. Fresh tissues (1 g) were ground in a mortar with 5 ml of reaction buffer (33 mM potassium phosphate buffer pH 7.0) containing 1 mM dithiothreitol on ice. The homogenate was centrifuged at 12,000×*g* for 15 min at 4℃. The supernatant was applied to a PD-10 gel-filtration column (Amersham Pharmacia Biotech, Tokyo, Japan) equilibrated with the reaction buffer. The dialyzed sample was used as an enzyme solution. The substrate solution (1 ml of total volume) contained 33 mM potassium phosphate buffer adjusted to pH 7.0 at 30℃, 500 μM sodium ascorbate, and 150 μM sinigrin (Sigma, Tokyo, Japan). The enzyme solution (10 μl, 5 μg protein) was added to the substrate solution and incubated at 30℃. The decrease in UV absorption at 227 nm (molecular extinction coefficient for sinigrin: 6784 $mol^{-1} cm^{-1}$) was monitored for 15 min by a photometer (UV-240, Shimadzu, Kyoto, Japan). An initial velocity was calculated for enzymatic activity: one unit (U) of myrosinase activity was defined as the enzymatic hydrolysis of 1 nmol glucosinolate min^{-1}. The protein amount was determined by the Bradford method (Bradford, 1976).

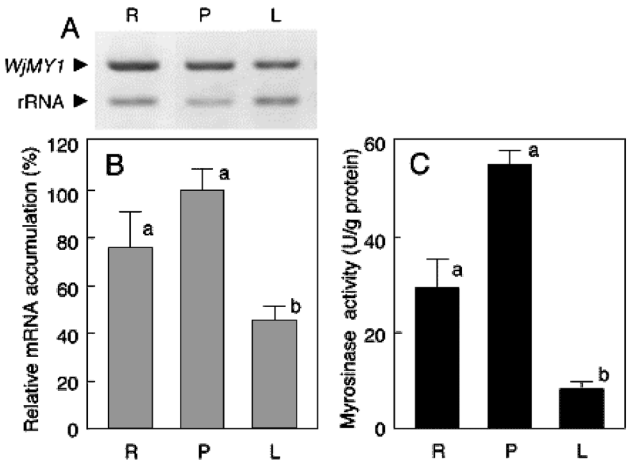

Fig. 2. Distribution of mRNA accumulation and enzymatic activity of myrosinase in wasabi plants.
A. Agarose gel electrophoresis during semiquantitative RT-PCR (18 cycles). RT-PCR products from wasabi myrosinase (WjMY1) mRNA and rRNA (an internal standard) are shown. Details are described in Materials and Methods. R, P, and L represent rhizome, petiole, and leaf, respectively.
B. Relative amounts of WjMY1 mRNA in different organs of wasabi. Values were calculated from the band intensities shown in Fig. 2A. The PCR product of rRNA was used as the internal standard. R, P, and L represent rhizome, petiole, and leaf, respectively. Values and bars indicate means and differences, respectively. Experiments were repeated twice. [a] No significant difference; [b] significant difference at $P<0.05$. To determine [a] or [b], the value of column P was standardized.
C. Specific activity of myrosinase in different organs of wasabi. R, P, and L represent rhizome, petiole, and leaf, respectively. Values and bars indicate means and differences, respectively. Experiments were repeated twice. [a] No significant difference; [b] significant difference at $P<0.05$. To determine [a] or [b], the value of column P was standardized. Protein contents of rhizome, petiole, and leaf were 12.9±0.5 mg/g fresh weight, 5.5±0.1 mg/g fresh weight, and 22.1±2.5 mg/g fresh weight, respectively. The values indicate mean±difference (n=2).

Results and Discussion

As the sequences of the PCR clones were similar to those of myrosinases of other species published previously (Rask *et al.,* 2000), it was concluded that the products derived from RT-PCR using degenerated primers (MYR1 and MYR2) corresponded to regions of the wasabi myrosinase gene. Six full-length wasabi myrosinase clones were obtained by the RACE method. Because the partial sequences from the 5′ ends to the positions at 350 bp were identical, it was suggested that the six clones were identical. We determined the whole sequence of one of the six clones, designated as WjMY1 (*Wasabia japonica* myrosinase 1). The open reading frame (1635 nucleic acids) encoded polypeptides with 545 amino acids, and the predicted molecular weight was 62107. The amino acid sequence is shown in Fig. 1A. The WjMY1

myrosinase showed high similarity to the myrosinases of radish, rape, and *Arabidopsis*. The identities at amino acid level were 78.6% (wasabi vs radish), 80.0% (wasabi vs rape), and 70.7% (wasabi vs *Arabidopsis*). Glucose ring recognition sites, residues of an aglycon pocket, and a residue forming a glucosidic bond, all of which were identified by X-ray structural analysis (Burmeister *et al.,* 1997), were conserved in WjMY1.

Semiquantitative RT-PCR analysis showed that WjMY 1 mRNA accumulated in all organs tested (Figs. 2A, B). The order of mRNA accumulation was petiole≥rhizome >leaf; as indicated in Fig. 2C, the order of myrosinase-specific activity was petiole>rhizome>leaf. The results shown in Fig. 2C do not contradict the report that myrosinase activity in petioles and rhizomes was higher than in leaves during the cultivation of wasabi (Hara *et al.,* 2003b). These results suggest that high myrosinase activity results from the high expression of myrosinase gene(s) in petioles and rhizomes.

This study examined the amino acid sequence of wasabi myrosinase. The molecular mass predicted from the sequence was 62.1 kDa; however, Ohtsuru and Kawatani (1979) reported that the molecular mass of the subunit in wasabi myrosinase was about 45 kDa. Mature enzymes may be generated from the proprotein by processing. Further analysis will elucidate the processing mechanism of wasabi myrosinase.

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References

Bradford, M.M. (1976). A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72**, 248–254.

Burmeister, W.P., Cottaz, S., Driguez, H., Iori, R., Palmieri, S. and Henrissat, B. (1997). The crystal structures of *Sinapis alba* myrosinase and a covalent glycosyl-enzyme intermediate provide insights into the substrate recognition and active-site machinery of an S-glycosidase. *Structure,* **5**, 663–675.

Goidin, D., Mamessier, A., Staquet, M.-J., Schmitt, D. and Berthier-Vergnes, O. (2001). Ribosomal 18S RNA prevails over glyceraldehyde-3-phosphate dehydrogenase and β-actin genes as internal standard for quantitative comparison of mRNA levels in invasive and noninvasive human melanoma cell subpopulations. *Anal. Biochem.,* **295**, 17–21.

Hara, M., Etoh, H. and Kuboi, T. (2001). Tissue printing for myrosinase activity in roots of turnip and Japanese radish and horseradish: a technique for localizing myrosinases. *Plant Sci.,* **160**, 425–431.

Hara, M., Kuboi, T. and Etoh, H. (2003a). Regulation of secondary metabolism in Cruciferous crops. *FFI Journal,* **208**, 594–600 (in Japanese).

Hara, M., Mochizuki, K., Kaneko, S., Iiyama, T., Ina, T., Etoh, H. and Kuboi, T. (2003b). Changes in pungent components of two *Wasabia japonica* MATSUM. cultivars during the cultivation period. *Food Sci. Technol. Res.,* **9**, 288–291.

Hasegawa, N., Matsumoto, Y., Hoshino, A. and Iwashita, K. (1999). Comparison of effects of *Wasabia japonica* and allyl isothiocyanate on the growth of four strains of *Vibrio parahaemolyticus* in lean and fatty tuna meat suspensions. *Int. J. Food Microbiol.,* **49**, 27–34.

Kinae, N., Masuda, H., Shin, I.-S., Furugori, M. and Shimoi, K. (2000). Functional properties of wasabi and horseradish. *BioFactors,* **13**, 265–269.

Masuda, H., Kinae, N., Woo, G.-J. and Shin, I.-S. (2004). Inhibitory effects of Gochoonangi (*Wasabia japonica*) against *Helicobacter pylori* and its urease activity. *Food Sci. Biotechnol.,* **13**, 191–196.

Morimitsu, Y., Hayashi, K., Nakagawa, Y., Horio, F., Uchida, K. and Osawa, T. (2000). Antiplatelet and anticancer isothiocyanates in Japanese domestic horseradish, wasabi. *Biofactors,* **13**, 271–276.

Ohtsuru, M. and Kawatani, H. (1979). Studies on the myrosinase from *Wasabia japonica*: Purification and some properties of wasabi myrosinase. *Agric. Biol. Chem.,* **43**, 2249–2255.

Palmieri, S., Leoni, O. and Iori, R. (1982). A steady-state kinetics study of myrosinase with direct ultraviolet spectrophotometric assay. *Anal. Biochem.,* **123**, 320–324.

Rask, L., Andreasson, E., Ekbom, B., Eriksson, S., Pontoppidan, B. and Meijer, J. (2000). Myrosinase: gene family evolution and herbivore defense in Brassicaceae. *Plant Mol Biol.,* **42**, 93–113.

Shin, I.-S., Masuda, H. and Kinae, N. (2004). Bactericidal activity of wasabi (*Wasabia japonica*) against *Helicobacter pylori*. *Int. J. Food Microbiol.,* **94**, 255–261.

Watanabe, M., Ohata, M., Hayakawa, S., Isemura, M., Kumazawa, S., Nakayama, T., Furugori, M. and Kinae, N. (2003). Identification of 6-methylsulfinylhexyl isothiocyanate as an apoptosis-inducing component in wasabi. *Phytochemistry,* **62**, 733–739.

Yano, T., Yajima, S., Virgona, N., Yano, Y., Otani, S., Kumagai, H., Sakurai, H., Kishimoto, M. and Ichikawa, T. (2000). The effect of 6-methylthiohexyl isothiocyanate isolated from *Wasabia japonica* (wasabi) on 4-(methylnitrosamino)-1-(3-pyridyl)-1-buatnone-induced lung tumorigenesis in mice. *Cancer Lett.,* **155**, 115–120.