

## Wasabi (*Wasabia japonica* Matsum.) Rhizome Possesses Potent Amylolytic Activity

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## Note

# Wasabi (*Wasabia japonica* Matsum.) Rhizome Possesses Potent Amylolytic Activity

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**Wasabi (*Wasabia japonica*) is a traditional condiment used in Japanese dishes. The wasabi rhizome shows potent amylolytic activity. The amylase activities of 12 Brassicaceae vegetables, including wasabi and three non-Brassicaceae root vegetables, were determined and compared. Among the 15 vegetables, wasabi showed the highest amylase activity (203 U g<sup>-1</sup> fresh weight). The second and third highest activities were found in horseradish (14 U g<sup>-1</sup> fresh weight) and sweet potato (8.6 U g<sup>-1</sup> fresh weight), respectively. The 12 other vegetables exhibited activities less than 3 U g<sup>-1</sup> fresh weight. Immunoblot analyses using an anti-radish (*Raphanus sativus*)  $\beta$ -amylase antibody indicated that wasabi contained an abundance of the antigen. Tissue printing indicated that the  $\beta$ -amylase antigen is generally distributed throughout the wasabi rhizome. These results suggest that wasabi contains a large amount of  $\beta$ -amylase, and therefore shows strong amylolytic activity.**

Keywords: amylase, rhizome, starch, wasabi

## Introduction

Wasabi (*Wasabia japonica* Matsum.) is a perennial herb used in Japanese cuisine as a pungent condiment. The consumption of wasabi is spreading globally, as Japanese cuisine has been introduced to many countries. Recent studies have focused on the health-promoting effects of wasabi (Depree *et al.*, 1998; Kinae *et al.*, 2000). 6-Methylsulfinylhexyl isothiocyanate, a component of wasabi, has shown anti-carcinogenic activities in many experimental systems (Fuke *et al.*, 1997; Yano *et al.*, 2000; Nomura *et al.*, 2005) and inhibition of human platelet aggregation (Morimitsu *et al.*, 2000). The wasabi leaf extract has shown an anabolic effect on bone components (Yamaguchi *et al.*, 2003) and anti-influenza virus activity (Mochida and Ogawa, 2008). Wasabi leaf extract also inhibits the differentiation of preadipocytes, suggesting that the extract may be able to prevent obesity and insulin resistance (Ogawa *et al.*, 2010). In addition, wasabi rhizome extract improved the atopic dermatitis-like symptoms of HR-1 hairless mice (Nagai and Okunishi, 2009).

Wasabi root is ground into a paste in practical cooking.

This paste is frequently used with the cooked rice of sushi and bowl dishes. The pungency of wasabi not only stimulates the appetite (Kojima, 1988), but also inhibits microbial growth due to the antimicrobial activities of pungent components such as allyl isothiocyanate and 6-methylsulfinylhexyl isothiocyanate (Hasegawa *et al.*, 1999; Ono *et al.*, 1998). Accordingly, wasabi is used in appetizers and as an antidote. On the other hand, it has been observed that cooked rice in close contact with wasabi paste becomes soft. This phenomenon suggests that wasabi may possess amylolytic activity. A few studies have reported amylase activities of Brassicaceae vegetables such as radish, cabbage, and broccoli (Hara *et al.*, 2009; El-Sayed *et al.*, 1995). However, there is little information regarding wasabi amylase. In this paper, we determined the amylase activity of wasabi, and then compared the amylase activities of wasabi and other vegetables. We used 15 vegetables for comparison purposes, i.e., 12 vegetables of the Brassicaceae family, to which wasabi belongs, and three vegetables that, like wasabi, are primarily root crops.

## Materials and Methods

**Plant materials** We used 15 vegetables. Watercress (*Nasturtium officinale*), rocket salad (*Eruca vesicaria* subsp.

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sativa), komatsuna (*Brassica rapa* var. *perviridis*), chinese cabbage (*B. rapa* subsp. *pekinensis*), cabbage (*B. oleracea* var. *capitata*), broccoli (*B. oleracea* var. *italica*), cauliflower (*B. oleracea* var. *botrytis*), wasabi (*Wasabia japonica*), radish (*Raphanus sativus*), turnip (*B. rapa* subsp. *rapifera*), carrot (*Daucus carota*), sweet potato (*Ipomoea batatas*), and potato (*Solanum tuberosum*) were purchased from local markets in Shizuoka, Japan. Horseradish (*Armoracia rusticana*) was a gift from S&B Foods Inc. (Tokyo, Japan). Kohlrabi (*B. oleracea* var. *gongylodes*) was grown in a plastic pot containing Peatban (Sakata Seed, Yokohama, Japan) in a naturally illuminated greenhouse, at uncontrolled temperatures, at Shizuoka University, Japan. The seeds were purchased from Takii Seed (Kyoto, Japan). The plants were watered every week with Hyponex solution (500 times dilution; Hyponex, Tokyo, Japan).

Tested organs comprised leaf blades and petioles of leafy vegetables, flower heads of flowering vegetables, rhizomes (or swollen hypocotyls) of Brassicaceae root vegetables, and tubers of non-Brassicaceae root vegetables.

**Crude protein extraction** Crude protein was extracted according to the previously described method (Hara *et al.*, 2009) with slight modifications. An organ (5 g fresh weight), i.e., leaf, flower head, rhizome, or tuber, was cut into small pieces for preparation of the crude protein extract. Organ pieces were homogenized on ice twice in a volume of 10 mM Tris-HCl (pH 7.5) using a mortar and pestle. The homogenate was centrifuged at  $10,000 \times g$  for 15 min at 4°C. The supernatant was a crude protein extract that was used for both the amylase assay and the immunoblot analysis. The pellet was resuspended, and its volume was adjusted to the same volume as the supernatant with 10 mM Tris-HCl (pH 7.5). This pellet fraction was also used for the immunoblot analysis.

The protein concentrations of the supernatant and pellet fractions were determined spectrophotometrically at 595 nm according to a previously described method (Bradford, 1976). Bovine  $\gamma$ -globulin was used as the standard protein.

**Amylase assay** The amylase assay was carried out by measuring the release of reducing sugars from soluble starch (Hara *et al.*, 2009). The crude enzyme solution (4  $\mu$ L) was combined with a substrate solution (36  $\mu$ L) consisting of 20  $\mu$ L of 1% soluble starch and 16  $\mu$ L of 100 mM sodium acetate buffer (pH 4.8). The reaction mixture was incubated at 37°C for 5 min. For optimum pH analysis, 100 mM sodium acetate buffer (pH 4, 4.5, 5, and 5.5) and 100 mM sodium phosphate buffer (pH 5.5, 6.5, and 7.5) were used.

Under the present incubation condition, the enzyme solutions were diluted to maintain linearity during the incubation periods. The final concentration of 0.5% soluble starch was

determined by producing the  $v$ -[S] plot of Michaelis-Menten kinetics. Before analyzing one plant species, one enzyme sample was randomly selected out of the 5 samples, and then the dilution rate was determined under the 0.5% substrate to obtain accurate initial velocities.

Immediately, 40  $\mu$ L of the 3,5-dinitrosalicylic acid reagent containing 44 mM 3,5-dinitrosalicylic acid (Sigma, Tokyo, Japan), 1 M sodium potassium tartrate, and 0.4 M sodium hydroxide was added to the reaction mixture. The solution was heated at 100°C for 5 min. After the addition of distilled water (360  $\mu$ L), the absorbance at 540 nm was measured. Calibration curves were made using maltose. One unit (U) represented the formation of 1  $\mu$ mol maltose per min. Finally, the amylase activities were expressed as U g<sup>-1</sup> fresh weight and U mg<sup>-1</sup> protein.

**Immunoblot analysis** Protein samples were separated using sodium dodecyl sulfate-polyacrylamide gel electrophoresis [12.5% (w/v) acrylamide] and transferred to a nitrocellulose membrane filter (Hybond-ECL; GE Healthcare, Tokyo, Japan) with a Mini Trans-Blot (Bio-Rad). A filter blocked with skimmed milk (Wako, Tokyo, Japan) was probed with a RsBAMY1-specific antibody (Hara *et al.*, 2009), used at a 1:5000 dilution. After washing, the filter was reacted with horseradish peroxidase-conjugated anti-rabbit immunoglobulin G (GE Healthcare; 1:5000 dilution) as a secondary antibody. Labeled proteins were detected by a chemiluminescence technique with the ECL Western Blotting Analysis System (GE Healthcare). The signals were detected by an LAS-4000 Image Analyzer (Fujifilm, Tokyo, Japan). A radish  $\beta$ -amylase purified by the method (Hara *et al.*, 2009) was loaded as a control.

**Determination of soluble sugars and starch** Fresh organs (3 g) were used. Soluble sugars were extracted twice using 10 volumes of 80% (v/v) ethanol at 80°C for 20 min. Ethanol-insoluble materials were saved for starch analysis. The supernatant was dried, and the dried residue was dissolved in deionized water. The sample was passed through a Sep-Pak C18 cartridge (Millipore, MA, USA) that had been activated with ethanol and then equilibrated with water. The contents of glucose, fructose, and sucrose were determined using high-performance liquid chromatography (HPLC; LC-2000; Jasco, Tokyo, Japan) as described previously (Hara *et al.*, 2009). Soluble sugars were separated at 30°C on an Asahipak NH2P-50 4E column (4.6 mm internal diameter  $\times$  250 mm long; Showa Denko, Tokyo, Japan), and the refractive index (RI) was detected using an RI detector (RI-2031; Jasco). Acetonitrile in water (75:25) was used as the mobile phase. The flow rate was 1 ml min<sup>-1</sup>. For determination of starch content, the ethanol-insoluble residue, after extracting the soluble sugars, was extracted by an equal volume

of 0.4 M potassium hydroxide at 80°C for 60 min. After neutralizing the extract, soluble starch was digested by 10 U  $\alpha$ -amylase (Wako) and 7 U amyloglucosidase (Wako, one unit representing a release of 1  $\mu$ mol glucose from starch per min) at 37°C for 20 h. Glucose formation was determined by the glucose oxidase- and peroxidase-based enzyme assay (Papadopoulos and Hess, 1960). Starch content was calculated based on the released glucose with adjustment of free glucose to starch.

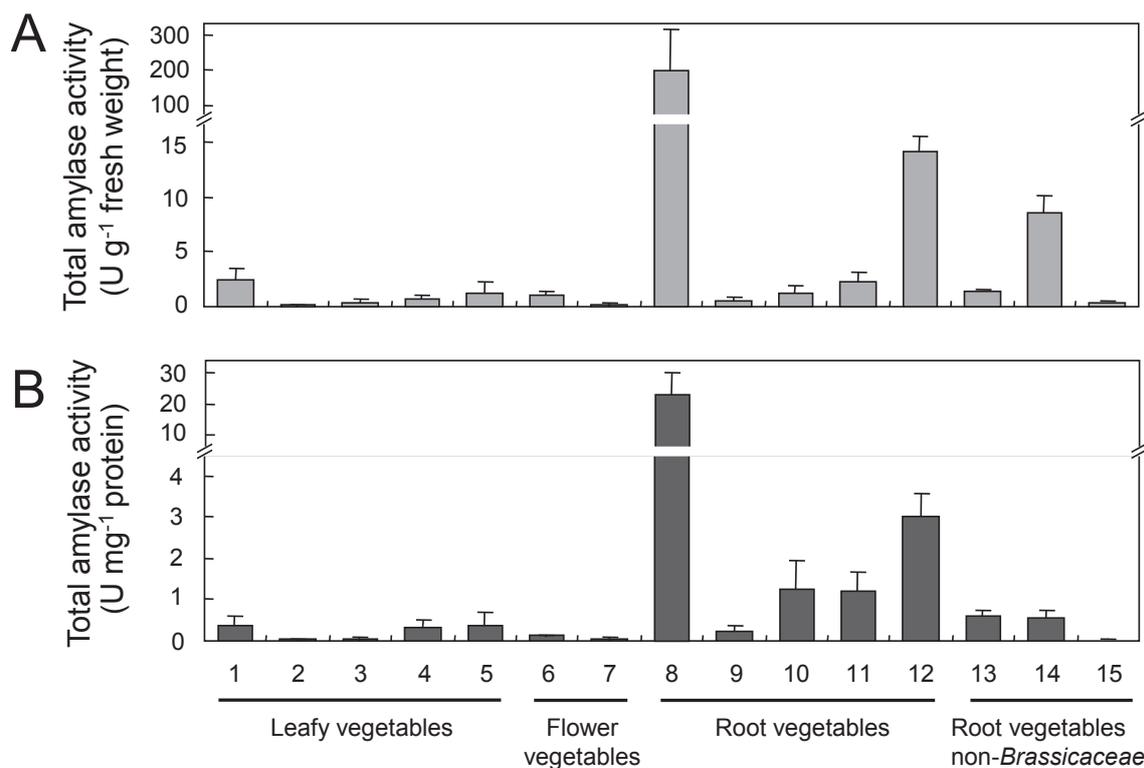
**Tissue printing and starch staining** Tissue printing was performed as described previously (Hara *et al.*, 2000) with modifications. Transverse and vertical cut ends of the wasabi rhizome were printed onto a nitrocellulose membrane filter (Hybond-ECL; GE Healthcare) for 15 s. The filters were blocked with 5% (w/v) skimmed milk protein in PBST [phosphate-buffered saline (10 mM potassium phosphate buffer pH 7.4 and 137 mM NaCl) with 0.05% (v/v) Tween-20] and then probed with a RsBAMY1-specific antibody at a 1:1000 dilution. Following washing, the membranes were incubated with the secondary antibody, anti-rabbit IgG (FC)-alkaline phosphatase conjugate antibody (Promega, WI, USA) at a 1:5000 dilution in PBST. The color reaction was developed by adding NBT/BCIP (nitroblue

tetrazolium chloride/5-bromo-4-chloro-3-indolyl-phosphate) Stock Solution (Roche Diagnostics, Mannheim, Germany). A control membrane was incubated with pre-immune serum instead of the RsBAMY1 antibody. The total protein on the membrane was stained with Coomassie Brilliant Blue (Sigma). To visualize the histochemical position of the signals in the tissue printings, crude slices were stained with 0.05% Toluidine Blue O (Sigma). Starch localization was analyzed by incubating sections in a solution containing 10 mM I<sub>2</sub> and 14 mM KI for 20 min at room temperature.

**Statistical analysis** Data for P values were analyzed by Student's t-test at a significance level of 0.05.

## Results and Discussion

First, we measured the amylase activities of the extracts from the 15 species (Fig. 1). When the activities were expressed as U g<sup>-1</sup> fresh weight, wasabi showed the highest activity (203 U g<sup>-1</sup> fresh weight) (Fig. 1A). The second-highest activity was detected in horseradish (14 U g<sup>-1</sup> fresh weight), which had approximately one-fifteenth the activity of wasabi. The ten other Brassicaceae vegetables showed lower amylase activities (less than 3 U g<sup>-1</sup> fresh weight). Sweet potato, which is known to accumulate  $\beta$ -amylase (Hagenimana *et*



**Fig. 1.** Amylase activities of vegetables. Amylase activities are expressed in two ways: U g<sup>-1</sup> fresh weight (A) and U mg<sup>-1</sup> protein (B). 1, watercress (*Nasturtium officinale*); 2, rocket salad (*Eruca vesicaria* subsp. *sativa*); 3, komatsuna (*Brassica rapa* var. *perviridis*); 4, Chinese cabbage (*B. rapa* subsp. *pekinensis*); 5, cabbage (*B. oleracea* var. *capitata*); 6, broccoli (*B. oleracea* var. *italica*); 7, cauliflower (*B. oleracea* var. *botrytis*); 8, wasabi (*Wasabia japonica*); 9, radish (*Raphanus sativus*); 10, turnip (*B. rapa* subsp. *rapifera*); 11, kohlrabi (*B. oleracea* var. *gongylodes*); 12, horseradish (*Armoracia rusticana*); 13, carrot (*Daucus carota*); 14, sweet potato (*Ipomoea batatas*); and 15, potato (*Solanum tuberosum*). Values represent means  $\pm$  SD ( $n = 5$ ).

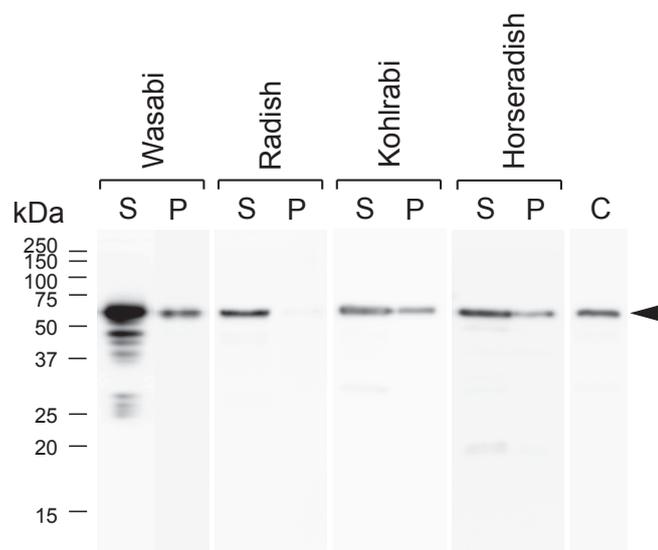
*al.*, 1992), showed amylase activity at  $8.6 \text{ U g}^{-1}$  fresh weight. The predominance of amylase activity in wasabi was also found when the activities were expressed as  $\text{U mg}^{-1}$  protein (Fig. 1B). Because it is known that  $\beta$ -amylases frequently accumulate in vegetative organs that show amylolytic activities (Ziegler, 1999), we predicted that wasabi also contains  $\beta$ -amylase. To confirm this hypothesis, immunoblots using anti-radish  $\beta$ -amylase antibody were performed to detect the  $\beta$ -amylase proteins in the extracts of four Brassicaceae root vegetables: wasabi, radish, kohlrabi, and horseradish. Figure 2 indicates that all four species possessed antigens against the antibody. Positive signals were detected at 57 kDa, which was close to the size where the purified radish  $\beta$ -amylase was detected (Fig. 2, lane C). Although the signals were mainly detected in the centrifugation supernatants (Fig. 2, lanes S), the pellet fractions of the extracts of wasabi, kohlrabi, and horseradish also gave bands (Fig. 2, lanes P) albeit at weaker intensities than the bands of the supernatants. This suggests that the antigens were mainly free-form proteins. In the case of wasabi, many minor signals whose mobilities on the gel electrophoresis were larger than the major signal were detected. These minor bands may be fragments of the major band and/or isoforms whose sequences are similar to the major antigen's sequence.

We also determined the contents of starch and soluble sugars in wasabi, radish, kohlrabi, and horseradish (Fig. 3). Wasabi and horseradish contained more starch than radish and kohlrabi (Fig. 3A). Conversely, wasabi and horseradish

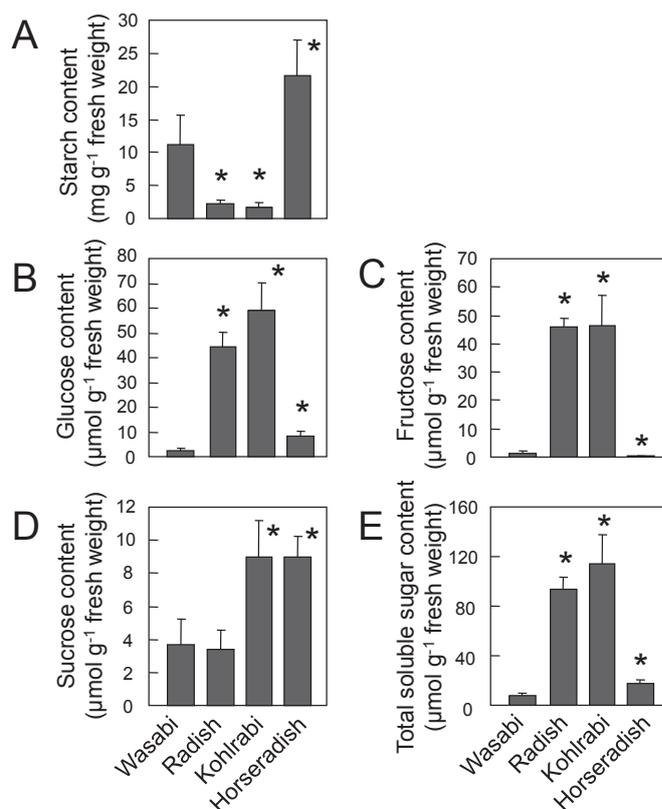
showed lower total soluble sugar contents (sum of glucose, fructose, and sucrose contents) than radish and kohlrabi (Fig. 3E). In radish and kohlrabi, the major soluble sugars are glucose and fructose (Figs. 3B-D). Wasabi and horseradish manifested higher abundance ratios of sucrose to the total soluble sugar content than radish and kohlrabi.

Finally, we determined the rough distributions of the antigens for the anti-radish  $\beta$ -amylase antibody in the wasabi rhizome using a tissue-printing technique. The obtained tissue printings suggested that the specific antigens were ubiquitously present throughout the rhizome (Figs. 4A, B). Proteins were detected in the whole rhizome (Fig. 4C). Internal anatomies are shown by pictures of crude tissues (Fig. 4E) and slices stained with Toluidine Blue O (Fig. 4D). In addition, starch was generally present in the rhizome (Fig. 5). These results indicate that the antigens (likely  $\beta$ -amylases) and starch accumulate throughout the whole rhizome.

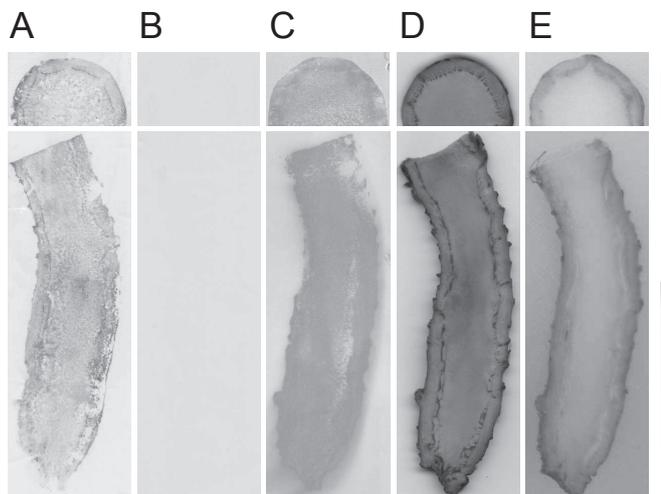
In this study, we found that wasabi shows potent amylolytic activity. When 15 vegetables including wasabi were examined, the amylolytic activity of wasabi ( $203 \text{ U g}^{-1}$  fresh weight) greatly exceeded that of the other 14 vegetables ( $0.1 - 14 \text{ U g}^{-1}$  fresh weight) (Fig. 1). The immunoblot analysis



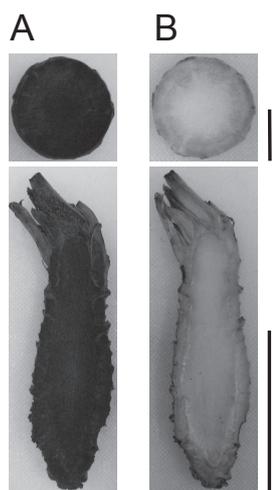
**Fig. 2.** Immunoblot analyses using anti-RsBAMY1 antibody. Protein samples from wasabi, radish, kohlrabi, and horseradish were examined. Lanes S and P represent the supernatant and pellet fractions, respectively, that were formed by centrifugation. Protein amounts loaded were  $3 \mu\text{g}$  for wasabi and  $20 \mu\text{g}$  for radish, kohlrabi, and horseradish. Lane C is a control  $\beta$ -amylase ( $150 \text{ ng}$ ) purified from radish taproots by the method of Hara *et al.* (2009). The arrowhead indicates the position of the radish  $\beta$ -amylase.



**Fig. 3.** Contents of starch and soluble sugars in Brassicaceae vegetables. Wasabi, radish, kohlrabi, and horseradish were analyzed. Contents of starch (A), glucose (B), fructose (C), sucrose (D), and total soluble sugars (E) are shown. Values represent means  $\pm$  SD ( $n = 5$ ). \*Significant difference ( $p < 0.05$ ) in comparison to wasabi was determined by Student's *t*-test.



**Fig. 4.** Localization of  $\beta$ -amylase in wasabi rhizome. Transverse (upper pictures) and vertical (lower pictures) sections were analyzed. Tissue printings with anti-RsBAMY1 antibody (A, apparent signals) and pre-immune antiserum (B, no apparent signals). The protein on the filter was stained with Coomassie Brilliant Blue (C). Crude slices (E) were stained with Toluidine Blue O (D). Bars represent 1 cm (transverse sections) and 5 cm (vertical sections).



**Fig. 5.** Localization of starch in wasabi rhizome. Transverse (upper pictures) and vertical (lower pictures) sections were analyzed. Starch staining with iodine solution (A). Black region indicates starch accumulation. Control treated with deionized water (B). Bars represent 1 cm (transverse sections) and 5 cm (vertical sections).

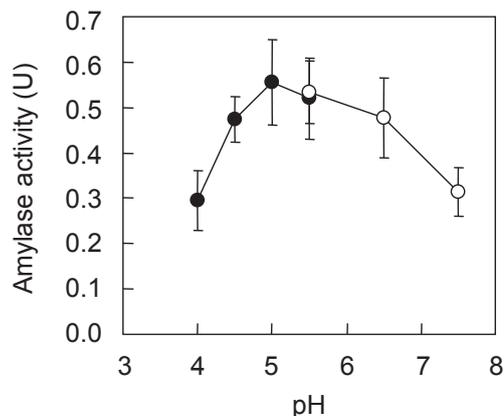
using the anti-radish  $\beta$ -amylase antibody indicated that the wasabi rhizome contained abundant amounts of the antigen (Fig. 2). The size of the major band in wasabi was approximately 57 kDa, which was similar to the size of the radish  $\beta$ -amylase. In addition, maltose was detected when the wasabi extract was incubated with the soluble starch (data not shown). These data suggest that  $\beta$ -amylase contributes to the amylolytic activity of wasabi, although contributions of other amylases are likely.

Wasabi showed a high starch content (approximately 11 mg  $g^{-1}$  fresh weight), which was concomitant with low

soluble sugar contents (less than 10  $\mu\text{mol } g^{-1}$  fresh weight), despite the strong amylolytic activity (Fig. 3). The  $\beta$ -amylase and starch generally accumulated throughout the whole rhizome (Figs. 4, 5). Similarly, sweet potato accumulates both  $\beta$ -amylase and starch throughout the whole tuber (Hagenimana *et al.*, 1992). This suggests that the  $\beta$ -amylase may have little relation to the digestion of endogenous starch in the wasabi rhizome and sweet potato tuber. Researchers have postulated that plant  $\beta$ -amylases that are not involved in the starch degradation of tissues may serve as vegetative storage proteins (VSPs) (Gana *et al.*, 1998; Ziegler, 1999). Although wasabi may accumulate  $\beta$ -amylase as a VSP, its precise role is unknown.

Wasabi is most popularly used as a condiment paste. In Japanese cuisine, wasabi paste is frequently used to garnish starchy foods, such as the cooked rice of sushi, bowls of rice, buckwheat noodles, etc. Although it has been revealed that wasabi paste functions as an appetizer and antidote, we here propose an additional function of wasabi, as a digestive aid. Regarding sushi, the cooked rice is treated with vinegar and, generally, its pH is kept at 4.6 or less (Food safety guidelines for sushi, Australia New Zealand Food Standards Code 2007). Optimum pH analysis indicated that wasabi showed strong amylolytic activity at pH 4.5 (Fig. 6). This suggests that wasabi  $\beta$ -amylase can exhibit its activity under the acidic condition of cooked sushi rice. Indeed, it is sometimes observed that the cooked rice that is in contact with the wasabi paste becomes soft.

In today's market for wasabi-related products, commercial wasabi-flavored pastes are produced mainly from horseradish (Sultana and Savage, 2008). In the future, however, commercial pastes of real wasabi might be more widely used throughout the world, if the production yield and processing techniques for wasabi were improved. When wasabi and



**Fig. 6.** Effect of pH on amylolytic activity of wasabi. Closed and open circles indicate sodium acetate buffer and sodium phosphate buffer, respectively. The quantity of enzyme sample used in each test was 0.55 U at pH 5. Values and bars represent means  $\pm$  SD ( $n = 3$ ).

its paste are used with foods containing gelatinized starch in cooking and food processing, caution should be taken to protect the starchy foods from the wasabi amylase. Storing the starchy foods with wasabi under low temperature and physically separating the starchy foods from wasabi are recommended to avoid starch degradation.

Beta-amylases are used in the production of maltose syrup (Teotia *et al.*, 2001) or added to starchy foods to inhibit starch retrogradation (Yao *et al.*, 2003). The present results suggest that the wasabi amylase may be used for such purposes.

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