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Abstract

The radish (*Raphanus sativus*) is a root vegetable of the Brassicaceae family which shows amylolytic activity in the taproot. However, there is little information about differences in these amylolytic activities among radish cultivars. We analyzed the amylase activities and starch contents of 7 kinds of radish cultivars. The Koshin cultivar showed the highest amylase activity, with a level approximately 6 times higher than that of the Sobutori cultivar, which had the lowest. Cultivars with higher amylase activities showed higher starch contents. These results suggest that there are intraspecies variations in amylolytic activities in radishes, and positive correlations between amylase activity and starch content.

Keywords separated by ' - '
β-amylase - Radish - *Raphanus sativus* - Starch - Taproot

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ORIGINAL PAPER

Variation in Amylase Activities in Radish (*Raphanus sativus*) Cultivars

Masakazu Hara · Fumio Ito · Tatsuo Asai · Toru Kuboi

Abstract The radish (*Raphanus sativus*) is a root vegetable of the Brassicaceae family which shows amylolytic activity in the taproot. However, there is little information about differences in these amylolytic activities among radish cultivars. We analyzed the amylase activities and starch contents of 7 kinds of radish cultivars. The Koshin cultivar showed the highest amylase activity, with a level approximately 6 times higher than that of the Sobutori cultivar, which had the lowest. Cultivars with higher amylase activities showed higher starch contents. These results suggest that there are intraspecies variations in amylolytic activities in radishes, and positive correlations between amylase activity and starch content.

Keywords β-amylase · Radish · *Raphanus sativus* · Starch · Taproot

Abbreviations

DNSA	3,5-dinitrosalicylic acid
IgG	immunoglobulin G
RsBAMY1	*Raphanus sativus* β-amylase 1
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis

Introduction

The radish (*Raphanus sativus*) is a root vegetable of the Brassicaceae family which is consumed primarily in the Far East Asian countries, such as Japan, Korea and China. In Japan, the country with the highest radish consumption, 1.65 million tons of radishes were harvested in 2006 (Preliminary Statistical Report on Agriculture, Forestry and Fisheries of Japan, 2007), and consumption averages 20 kg per person per year [1].

Many recent studies have examined the health benefits of radishes. Radishes contain a large amount of soluble dietary fibers [2]. Tunisian radish extract was found to be effective in protecting against zearalenone-induced immunological disorders in mice [3], and radish root extracts show inhibitory effects on lipid peroxidation in rats [4]. Additionally, glucoraphasatin, a major radish glucosinolate, quenches hydrogen peroxide [5]. The antioxidative effects of radish sprouts have been reported in rats [6], and isothiocyanates, which are derived from glucoraphasatin and glucoraphanin, induce apoptosis in cancer cells [7]. A glucoraphasatin-derived isothiocyanate was found to be a potent inducer of detoxification enzymes in the HepG2 cell line [8]. And finally, radish crude extract and its isothiocyanates inhibit the abnormal growth of vascular smooth muscle cells, which is a prominent feature of vascular disease, including atherosclerosis, and restenosis after angioplasty [9]. These reports suggest that the health benefits of radishes are due to the antioxidative and anticarcinogenic activities of glucosinolates and isothiocyanates.

In Japan, grated raw radish (daikon oroshi) is frequently used as a garnish. Because daikon oroshi is believed to show amylolytic activity, it is added to starch-containing foods, such as boiled rice, rice cakes and noodles, to aid digestion. We recently reported that the amylolytic activity of the radish (Comet cultivar) is due to the accumulation of β-amylase in the taproot [10]. There are many radish cultivars; however, there is little information on differences in their amylase activities. In this paper, we report

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that 7 radish cultivars show different degrees of amylase activity. Correlations between amylase activity and starch content in the different cultivars were also investigated.

Materials and Methods

Plant Materials

Seeds of 7 radish cultivars (Icicle, Sobutori, Kouto, Shinhasshu, Kuromaru, Shogoin and Koshin) were purchased from seed companies: Icicle, Sobutori and Shinhasshu were obtained from Takii (Kyoto, Japan), Kouto and Koshin from Sakata (Yokohama, Japan), and Kuromaru and Shogoin from Fujita (Osaka, Japan) and Takayama (Kyoto, Japan), respectively. Icicle was grown in a plastic planter containing vermiculite at a greenhouse at Shizuoka University, Japan, in 2007. The plants were watered every week with Hyponex solution (500 times dilution; Hyponex, Tokyo, Japan), and were harvested on the 6th week after sowing. The other 6 cultivars were cultivated in a field at Fujieda, Shizuoka, Japan from September 2007 to January 2008. A fertilizer was applied to the soil before planting. The plants were harvested when they matured (at 14 to 18 weeks). After harvest, the taproot was weighed and maintained at −70 °C until use.

Extraction of Crude Enzyme

Radish taproots (20 g fresh weight) were ground by a steel musher on ice until they became a paste. The paste was centrifuged at 10,000 g for 15 min at 4 °C. The supernatant was a crude enzyme extract that was maintained at −20 °C until use. Neither the amylolytic activity nor the antigenicity for an anti-*Raphanus sativus* β-amylase 1 (RsBAMY1) antibody in the extracts changed during storage at −20 °C for 6 months.

Amylase Activity

Glucan hydrolyzing activities in the radish extracts were measured by the 3,5-dinitrosalicylic acid (DNSA) method described previously [10]. Briefly, an enzyme solution (4 μl) was combined with a substrate solution (36 μl) consisting of 20 μl of 1% soluble starch and 16 μl of 100 mM sodium acetate buffer (pH 4.8). After incubation at 37 °C for 5 min, 40 μl of the DNSA reagent containing 44 mM DNSA, 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 and the absorbance was read at 540 nm. Calibration curves were produced using maltose solutions.

Protein Determination

The amount of protein was determined by Quick Start Bradford Protein Assay (Bio-Rad, Tokyo, Japan) with bovine γ-globulin as a standard. Assays were performed according to the manufacturer's instructions.

Amylase Purification

Amylase from each cultivar was purified following the method previously described [10]. Briefly, crude enzyme solution (35 ml) derived from fresh taproots (50 g) was dialyzed for 24 h against deionized water. The dialyzed sample was subjected to Toyopearl DEAE-650 M (Tosoh, Tokyo, Japan) column chromatography. The fractions showing amylase activity were then subjected to an affinity precipitation technique which is used to purify glycogen-binding proteins, including amylases. The active fraction was further purified by a NAP-5 column (Sephadex G-25 disposable column, GE Healthcare, Tokyo, Japan) equilibrated with 100 mM sodium acetate buffer (pH 4.8). The purified β-amylase remained stable at −20 °C for 3 months.

Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Protein samples were fully denatured by boiling with 2-mercaptoethanol and SDS, and separated in a 12.5% polyacrylamide gel using the Mini-Protean III electrophoresis system (Bio-Rad). Gels were stained with colloidal Coomassie Blue (Bio-Safe; Bio-Rad).

Immunoblot Analysis

Protein samples were resolved by 12% SDS-PAGE as described above. After electrophoresis, the proteins were blotted onto a nitrocellulose membrane filter (Hybond-ECL; GE Healthcare) with a Mini Trans-Blot (Bio-Rad). A blocked filter was incubated with a primary antibody, i.e., a rabbit polyclonal anti-RsBAMY1 antibody [10]. Horseradish peroxidase-conjugated anti-rabbit immunoglobulin G (IgG; GE Healthcare) was used as a secondary antibody. Positive signals were detected by a chemiluminescence technique with the ECL Western Blotting Detection System (GE Healthcare). The signals were detected by an LAS-4000 Image Analyzer (Fujifilm, Tokyo, Japan).

Starch Analysis

Starch content was measured as described previously [10]. Radish taproots (10 g fresh weight) were extracted twice by refluxing with 100 ml of 80% (*v*/*v*) ethanol for 20 min. Insoluble material was hydrated in 10 ml deionized water.

The hydrated material was incubated with 0.4 M potassium hydroxide (10 ml) at 95 °C for 1 h to solubilize starch. After neutralizing with 2 M acetic acid, the starch was digested to glucose by α-amylase (10 U) and amyloglucosidase (7 U) at 37 °C for 18 h. Glucose formation was determined by the hexokinase and glucose-6-phosphate dehydrogenase system described previously [11].

Results and Discussion

We obtained 7 kinds of radish cultivars, Icicle, Sobutori, Kouto, Shinhasshu, Kuromaru, Shogoin and Koshin, by growing them as described in Materials and Methods above. Pictures of the cultivars are shown in Fig. 1. Icicle (Fig. 1a) is a small white radish cultivated through the world. Sobutori (Fig. 1b) is a white radish most commonly cultivated in Japan. Kouto (Fig. 1c) is a green radish whose origin is China. Shinhasshu (Fig. 1d) is a white Japanese radish produced for pickled vegetables. Kuromaru (Fig. 1e) is a black radish. Shogoin (Fig. 1f) is a white radish which is eaten pickled and boiled in Japanese dishes. Koshin (Fig. 1g) is a Chinese radish whose outside is white but inside is red. Table 1 shows the amylase activities, fresh weights, and protein and starch contents of the 7 radish cultivars. The 7 cultivars exhibited different amylase activities, with the Koshin cultivar showing the highest activity (approximately 4 U/g fresh weight), and the Sobutori showing the lowest (approximately 0.7 U/g fresh weight). The order of averages of amylase activities was

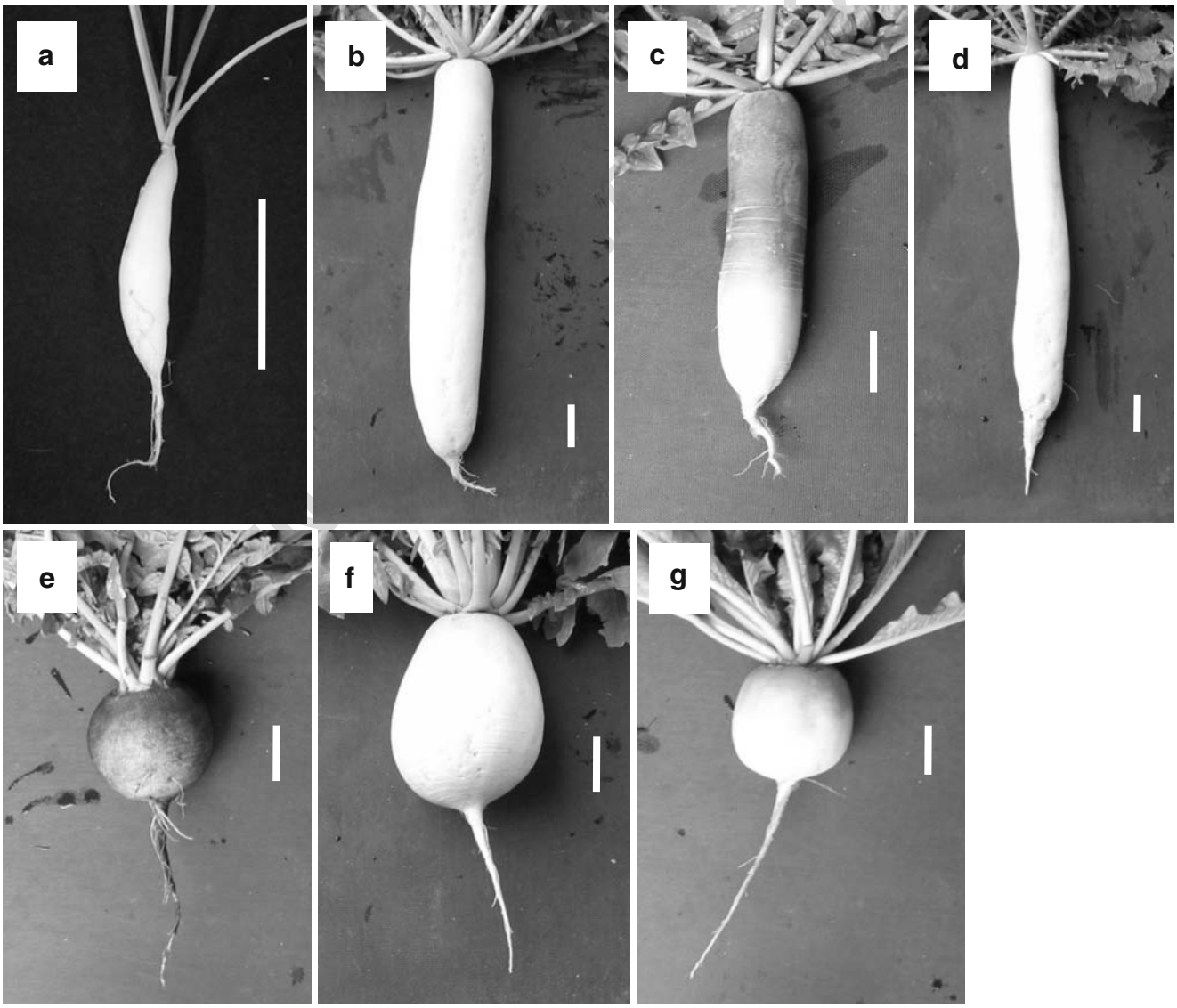

Fig. 1 Photographs of the 7 radish cultivars: a, Icicle; b, Sobutori; c, Kouto; d, Shinhasshu; e, Kuromaru; f, Shogoin; g, Koshin. Bars represent 5 cm

t1.1 **Table 1** Amylase activities, fresh weights, protein contents, and starch contents in 7 kinds of radish cultivars. Values represent means ± SD of 4 plants

t1.2 Cultivar	Amylase activity (U/g fresh weight)	Fresh weight (g)	Protein content (mg/g fresh weight)	Starch content (mg/g fresh weight)
t1.3 Icicle	1.35±0.278	8.90±1.20	4.10±0.175	0.228±0.165
t1.4 Sobutori	0.696±0.546	1560±119	1.43±0.343	1.44±0.388
t1.5 Kouto	2.93±1.01	418±19.2	3.54±0.624	6.00±4.34
t1.6 Shinhasshu	2.69±0.514	1050±182	1.66±0.171	9.76±5.14
t1.7 Kuromaru	2.43±1.69	374±44.9	4.42±0.274	6.64±1.32
t1.8 Shogoin	2.54±0.796	1180±180	2.02±0.192	14.3±1.45
t1.9 Koshin	3.98±0.452	457±131	3.79±0.637	18.0±4.78

Koshin > Kouto > Shinhasshu > Shogoin > Kuromaru > Icicle > Sobutori. When amylase activities were plotted against fresh weights for the 7 cultivars, no correlation was found (data not shown). Similarly, there was no correlation between amylase activities and protein contents (data not shown). However, when amylase activities were plotted against starch contents, there was a positive correlation (Fig. 2; $R^2 = 0.72$).

Recently, a β-amylase designated RsBAMY1 was purified as a major amylolytic enzyme from the taproots of a Comet radish cultivar by a three-step system including dialysis, anion exchange chromatography and glycogen affinity precipitation [10]. A polyclonal antibody that recognizes RsBAMY1 was also prepared [10]. In order to investigate whether the 7 cultivars tested in the present study possess the β-amylase, we purified β-amylases from the 7 cultivars. The case of the Koshin cultivar is shown in Fig. 3 as an example. When we applied the purification procedure described above to the purification of β-amylase from Koshin, we obtained a homogeneous protein whose molecular mass was approximately 57 kDa (Fig. 3, lane 4, arrowhead). Since the molecular mass of the Comet β-amylase is 57 kDa, the protein is likely a Koshin β-amylase. The purified protein could digest soluble starch but could not digest β-limit dextrin. Through purification from the crude extract to the affinity precipitation step, purification magnifications were 153 and the yield was 52%. Immunoblot analysis showed that the anti-RsBAMY1 antibody recognized the purified protein (Fig. 3, lane 5, arrowhead), suggesting that the purified protein from Koshin is a β-amylase that closely resembles the Comet RsBAMY1. Following the same procedure, we were able to

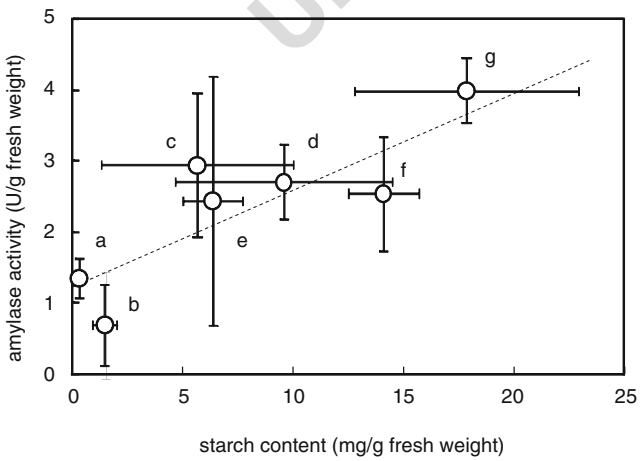

Fig. 2 Relationship between amylase activities and starch contents in 7 radish cultivars. a, Icicle; b, Sobutori; c, Kouto; d, Shinhasshu; e, Kuromaru; f, Shogoin; g, Koshin. Values and bars represent means ± SD of 4 plants. The dotted line is the regression line

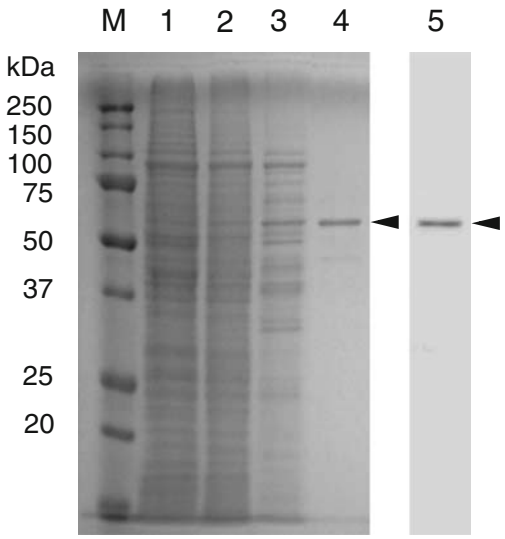

Fig. 3 Purification of β-amylase from the taproots of the Koshin radish. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis analyses of active fractions was performed in purification steps. Lane M, markers; lane 1, crude extract (50 μg protein); lane 2, dialysis (10 μg protein); lane 3, Toyopearl DEAE-650 M (10 μg protein); lane 4, glycogen affinity precipitation (5 μg protein). The gel was stained with colloidal Coomassie Blue. Lane 5, immunoblot analysis with an anti-RsBAMY1 antibody. The affinity-purified protein (20 ng) was analyzed. Arrowheads indicate the position of purified β-amylase

purify β-amylases from the other 6 cultivars, all of which were recognized by the anti-RsBAMY1 antibody (data not shown). These results demonstrate that the 7 cultivars used in the present study possess β-amylases closely related to the Comet RsBAMY1.

In this paper, we first demonstrate variation in the amylase activities of radish cultivars. It is known that there are intraspecies variations in β-amylase activities in some crops, such as barley [12], rice [13] and soybeans [14]. Because amylase is a starch-degrading enzyme, it is curious that starch contents are positively correlated with amylase activities in radish cultivars (Fig. 2). The β-amylase may not be able to access starch in the radish taproot. Similarly, it has been reported that alfalfa β-amylase accumulates in the taproot, where starch content increases [15]. These results suggest that β-amylases may be storage proteins in plant taproots.

In the Japanese market, consumers tend to prefer sweet radishes. When radishes are cooked, the amylase digests endogenous starch. Cultivars showing both high amylase activity and high starch content are therefore attractive to consumers. The present study thus provides information that may be used to select cultivars that increase sweetness when cooked.

The present study found considerable intraspecies variations in radish amylase activities. Intraspecies variations have also been observed in the isothiocyanate-generating activities of Japanese radishes [16]. Radishes may show high intraspecies variations in many respects. In order to use radishes effectively, it is necessary to investigate the intraspecies variations in beneficial components in radish cultivars.

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