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A major β-amylase expressed in radish taproots

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ABSTRACT

Radish (*Raphanus sativus* L.) taproots display high amylolytic activity. Amylase zymograms using gels containing soluble starch and β-limit dextrin have indicated that β-amylase is a major amylolytic enzyme. We purified β-amylase with anion-exchange chromatography followed by glycogen precipitation. From the deduced amino acid sequences, a cDNA clone designated *Raphanus sativus* β-amylase 1 (RsBAMY1) was isolated. RsBAMY1 closely resembled *Arabidopsis thaliana* major β-amylase (At4g15210). Immunoblot analyses performed using an anti-RsBAMY1 antibody that was raised against a peptide sequence found in RsBAMY1 showed that the RsBAMY1 protein accumulated in the taproot. In addition, the transcriptional level and the protein accumulation of RsBAMY1 were enhanced during the taproot growth. These results suggest that RsBAMY1 is a major starch-digestive enzyme in the radish taproot.

1. Introduction

The radish (*Raphanus sativus* L.) is a Brassicaceae root vegetable. Although the radish is grown and consumed throughout the world, Far East Asian countries, such as Japan, Korea, and China, are the major consuming regions. In Japan, 1.65 million tons of radishes were harvested in 2006 (Preliminary Statistical Report on Agriculture, Forestry and Fisheries of Japan, 2007), and people in Japan consume on average 20 kg of radish per year (Talalay & Fahey, 2001). Radish taproots are eaten raw, pickled, and boiled. Radish sprouts are also consumed as herbs in Japanese dishes. Recently, researchers have become interested in the radish because extracts and compounds made from radishes have shown many health benefits. Glucoraphasatin, a major glucosinolate in the radish, quenches hydrogen peroxide and can be efficiently oxidised to glucoraphenin, which is a minor glucosinolate in the radish (Barillari et al., 2005). Antioxidative effects of radish sprouts have been reported in rats (Ippoushi, Takeuchi, Ito, Horie, & Azuma, 2007).

Glucosinolates are substrates of myrosinases, which convert the glucosinolates to the corresponding isothiocyanates when plant tissues are mechanically disrupted. Glucoraphasatin- and glucoraphenin-derived isothiocyanates induced apoptosis in cancer cells (Papi et al., 2008). A glucoraphasatin-derived isothiocyanate is a potent inducer of detoxification enzymes in the HepG2 cell line (Hanlon, Webber, & Barnes, 2007). Radish myrosinases were purified (Shikita, Fahey, Golden, Holtzclaw, & Talalay, 1999), and their cDNAs were isolated (Hara, Fujii, Sasada, & Kuboi, 2000). These results suggest that the radish produces health benefits through the antioxidative and anticarcinogenic activities of glucosinolates and isothiocyanates.

In Japan, people use a grated raw radish, known as daikon oroshi, which is a common garnish in Japanese dishes. They prefer the pungency of isothiocyanates generated by daikon oroshi because the pungency promotes their appetite. In addition, they add daikon oroshi to boiled rice, rice cakes, and noodles, because it is believed to help in the digestion of starch-containing foods. Indeed, amylolytic activity has been recorded in the radish taproot. The existence of potent diastase activity in the radish was first described in the early 19th century (Aoki, 2007).

In general, plant starch-hydrolysing enzymes include α-amylase, β-amylase, isoamylase, and limit dextrinase (Smith, Zeeman, & Smith, 2005). α-Amylase is an endoamylase which hydrolyses internal α-1,4 linkages of linear or branched glucans. β-Amylase is an exoamylase which releases β-maltose from the non-reducing ends of α-1,4-linked glucans. β-Amylase cannot pass α-1,6 linkages, so it does not digest a β-limit dextrin. Isoamylase and limit dextrinase hydrolyse the α-1,6 linkages of amylopectin. A β-amylase was purified from an Egyptian radish (Rashad, Jwanny, El-Sayed, Mahmoud, & Abdallah, 1995), suggesting that the β-amylase is related to at least a portion of the amylolytic activity in radish taproots. In order to study the starch-hydrolysing activity in the radish, it is necessary to show that the starch-hydrolysing activity in radish taproots depends on β-amylase, and to obtain information about the primary structure of the β-amylase. In this paper, we report a cDNA for a major β-amylase expressed in the radish taproot. We also discuss the role of β-amylase in the radish as a functional vegetable.

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2. Materials and methods

2.1. Plant materials

Radishes were grown in a greenhouse located at Shizuoka University, Japan. We cultivated radishes three times from 2006 to 2007 for this study. Radish seeds (*Raphanus sativus* L. cv. Comet, Takii, Kyoto, Japan) were sown in a plastic planter containing vermiculite. The plants were watered with Hyponex solution (500 times dilution) (Hyponex, Tokyo, Japan) every week, and harvested on the eighth day after sowing (DAS) (stage I), the 34th DAS (stage II), and the 44th DAS (stage III). The harvest periods were slightly different amongst cultivations, because the growth depended on the climate. The plants were used to extract crude enzyme, starch, and total RNA. For purification of β-amylase, European red radish was purchased at a local market in Japan.

2.2. Crude enzyme extract

Radish taproots (5 g fresh weight) were ground by a steel musher on ice until they became a paste. The paste was centrifuged at 10,000g for 15 min at 4 °C. The supernatant was a crude enzyme extract from the taproots. Leaves (1 g fresh weight) were homogenised in 2 ml of deionised water using a mortar and pestle. The supernatant after centrifugation (10,000g, 15 min, 4 °C) was a leaf crude enzyme extract. As an extraction from leaves, roots were homogenised and centrifuged to obtain a root crude enzyme extract. The extracts were kept at −20 °C until use. The amylolytic activity and antigenicity for an anti-RsBAMY1 antibody in the extracts did not change during storage at −20 °C for 6 months.

2.3. Determination of amylase activity

Glucan hydrolysing activity was measured by the 3,5-dinitrosalicylic acid (DNSA) method described previously (Lizotte, Henson, & Duke, 1990) with slight modifications. 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. The mixture was incubated at 37 °C for 5 min. Immediately, 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. After cooling to room temperature, 360 μl of deionised water was added. Then the absorbance was read at 540 nm. For the blank reaction, the DNSA reagent was combined with the substrate solution before the enzyme solution was added. Calibration curves were produced by reacting different concentrations of maltose with the DNSA reagent.

2.4. Protein quantification

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

2.5. Amylase zymography

Native polyacrylamide gel electrophoresis (native PAGE) and an amylase activity stain were done by the method of the previous report (Lin, Spilatro, & Preiss, 1988) with modifications. Proteins in the samples were separated by the native PAGE (7% polyacrylamide gel) containing 0.1% soluble starch. After electrophoresis, the gel was soaked in 100 mM sodium acetate buffer pH 4.8 containing 0.2% soluble starch at 37 °C for 30 min. The starch in the gel was stained by immersing the gel in 1% acetic acid containing 10 mM I_2 and 14 mM KI for 3 min at room temperature. The presence of amylolytic activity was represented as a lytic band. For a β-limit dextrin-hydrolysing activity, β-limit dextrin was substituted for soluble starch.

2.6. Sodium dodecyl sulfate–PAGE (SDS–PAGE)

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

2.7. β-Amylase purification

Crude enzyme extract (50 ml) was obtained from radish taproots (70 g fresh weight) by the method described above. The extract was dialysed for 24 h against deionised water with SnakeSkin Pleated Dialysis Tubing (10,000 MWCO, PIERCE, IL, USA). The dialysed sample was loaded onto the TOYOPEARL DEAE-650M (TOSOH, Tokyo, Japan) column (15 × 200 mm), equilibrated with deionised water. After the column was washed with three volumes of 10 mM Tris–HCl buffer pH 7.5 containing 1 mM dithiothreitol, bound protein was eluted by a 130-ml linear gradient of NaCl (0–500 mM) in 10 mM Tris–HCl buffer pH 7.5 containing 1 mM dithiothreitol. Fractions of 5 ml each were collected. The fractions showing amylase activity were combined. Affinity precipitation was done on ice according to the previous method (Lizotte et al., 1990) with slight modifications. Ethanol was added to the active fraction until the final concentration of ethanol reached 40%. After centrifugation at 10,000g for 20 min at 4 °C, the supernatant (30 ml) was used for the affinity precipitation. The following reagents were added to the supernatant in order, with gentle stirring: 1.5 ml of 0.2 M sodium phosphate buffer pH 7.9, 2.1 ml of 2% oyster glycogen solution, and 3 ml ethanol. The suspension was agitated for 5 min, and then centrifuged at 2000g for 6 min at 4 °C. The pellet was washed twice with 10 mM sodium phosphate buffer pH 7.9 containing 40% ethanol. The washed pellet was suspended in 1 ml of 100 mM sodium acetate buffer pH 4.8 and kept at 37 °C for 1 h to digest glycogen completely. After centrifugation (10,000g, 5 min, 4 °C), the supernatant was subjected to the NAP-5 column (a Sephadex G-25 disposable column, GE Healthcare, Tokyo, Japan), which was equilibrated with 100 mM sodium acetate buffer pH 4.8. The purified β-amylase was stable at −20 °C for 3 months.

2.8. Protein mass spectrometry

A purified protein, which was treated with 10 mM dithiothreitol and 55 mM iodoacetamide, was digested by trypsin. The fragments were analysed by nanoflow liquid chromatography coupled to electrospray ionisation quadrupole time-of-flight tandem mass spectrometry (nanoLC-ESI-Q-TOF/MS/MS). A database search was performed with the MASCOT MS/MS Ion Search against the NCBInr database in the taxonomy of *Arabidopsis thaliana*.

2.9. 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 raised against a synthetic peptide (FKEAAAKAGHPEWDLPEDAGE), which is found in the sequence of RsBAMY1. Horseradish peroxidase-conjugated anti-rabbit IgG (GE Healthcare) was a secondary