Differential Chain-Length Specificities of Two Isoamylase-Type Starch-Debranching Enzymes from Developing Seeds of Kidney Bean

Yoshinori Takashima, Takeshi Senoura, Takayuki Yoshizaki, Shigeki Hamada, Hiroyuki Ito, and Hirokazu Matsui

Division of Applied Bioscience, Graduate School of Agriculture, Hokkaido University, Sapporo 060-8589, Japan

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Plant isoamylase-type starch-debranching enzymes (ISAs) hydrolyze \(\alpha\)-1,6-linkages in \(\alpha\)-1,4/\(\alpha\)-1,6-linked polyglucans. Two ISAs, designated PvISA1/2 and PvISA3, were purified from developing seeds of kidney bean by ammonium sulfate fractionation and several column chromatographic procedures. The enzymes displayed different substrate specificities for polyglucans: PvISA1/2 showed broad chain-length specificities, whereas PvISA3 liberated specific chains with a DP of 2 to 4.

Key words: kidney bean (Phaseolus vulgaris L.); isoamylase; starch-debranching enzyme; amylopectin

Starch-debranching enzymes (DBEs) catalyze the hydrolysis of \(\alpha\)-1,6 glucosidic linkages of polyglucans. In higher plants, two types of DBEs with distinct substrate specificities have been identified: the pullulanase-type (EC 3.2.1.41) and the isoamylase-type (EC 3.2.1.68).1) The isoamylase-type DBEs (ISAs) are subdivided into three isoforms (ISA1, ISA2, and ISA3) based on the primary sequences.2,3) The sugary-1 (su1) mutants of maize and rice show a common phenotype in endosperms, which is characterized by reduced starch content and accumulation of water-soluble and highly branched polysaccharides (phytoglycogen).4–6) Since the su1 loci encode the genes for ISAs,5,7) it is believed that the formation of the highly ordered amylopectin in vivo requires the actions not only of starch synthases and starch-branching enzymes but also of DBEs. Recent studies suggest that ISA2 has no catalytic activity due to a lack of catalytic residues and that ISA1 and ISA2 form a heteromultimeric complex.2,8,9) The formation of the ISA1/2 complex is also supported by the fact that Arabidopsis mutants in ISA1 and ISA2 display identical phenotypes.8) In contrast to the ISA1/2 complex, ISA3 appears to be a monomeric enzyme.21 Arabidopsis mutants deficient in ISA3 show a starch-excess phenotype in leaves and an altered amylopectin structure with increases in very short chains, suggesting that ISA3 plays a role in transitory starch degradation, particularly in the breakdown of \(\beta\)-limit dextrin produced by \(\beta\)-amylase.3,10) We have isolated cDNA clones for ISA1, ISA2, and ISA3 from immature seeds of kidney bean, and these were designated PvISA1, PvISA2, and PvISA3 cDNA respectively (the respective nucleotide sequence data can be accessed from the DDBJ Nucleotide Sequence Database under accession nos. AB300052, AB300053, and AB300054). RNA gel blot analysis using each cDNA fragment as a probe revealed that the three ISA transcripts accumulated both in leaves and in developing seeds (Fig. 1A). In this paper, we describe the purification of two ISAs (designated PvISA1/2 and PvISA3) from the developing seeds of kidney bean and the distinct substrate specificities of the purified ISAs.

The plant materials were prepared as described previously.11) Two ISA proteins were purified from large-sized developing seeds by several successive column chromatographic procedures. The ISA proteins in the purification steps were monitored by native-PAGE/active staining, which was performed on polyacrylamide gels containing 0.3% (w/v) potato amylopectin (Sigma Chemical, St. Louis, MO) according to the method of Fujita et al.12) Approximately 1 kg of large-sized developing seeds was homogenized with Polytron in 5-liter of extraction buffer (40 mM MOPS–NaOH, pH 7.0, containing 4 mM DTT, 5 mM MgCl2, and 1 mM PMSF). The homogenate was filtrated through three layers of gauze and the supernatant (crude extract) was obtained by centrifugation at 13,000 \(\times\) \(g\) for 10 min at 4 °C. Our pilot study showed that PvISA1/2 and PvISA3 are precipitated at ammonium sulfate concentrations of 0 to 25% and 25 to 40% saturation respectively.

PvISA1/2 proteins were purified from the crude extract by the following procedure: The precipitate

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1 To whom correspondence should be addressed. Fax: +81-11-706-2508; E-mail: otih@chem.agr.hokudai.ac.jp

Abbreviations: \(\beta\)-CD, \(\beta\)-cycloextrin; DBE, starch-debranching enzyme; DP, degree of polymerization; ISA, isoamylase-type DBE; PMSF, phenylmethylsulfonyl fluoride; PvISA, ISA from kidney bean (Phaseolus vulgaris L.)
obtained from a fraction of 25% ammonium sulfate was dissolved in a small amount of buffer A (40 mM MOPS–NaOH, pH 7.0, containing 2 mM DTT, 5 mM MgCl₂, and 10% v/v glycerol), and then dialyzed against buffer A. The dialyzed sample was passed through a Q-Sepharose FF column (GE Healthcare Bio-Sciences, Piscataway, NJ) equilibrated with buffer A. The column was washed with buffer A, followed by a gradient of 0 to 1 M NaCl. The fractions with ISA activity were dialyzed against buffer B (5 mM sodium phosphate buffer, pH 7.0, containing 2 mM DTT and 5 mM MgCl₂) and resolved on a Gigapite column (Seikagaku, Tokyo) equilibrated with buffer B. The enzyme solution was eluted with a 5 to 100 mM gradient of sodium phosphate buffer. The active fractions were pooled, concentrated, and purified on a Superdex 200 HR 10/30 column (GE Healthcare Bio-Sciences) equilibrated with buffer A containing sodium phosphate buffer. The active fractions were pooled, concentrated, and purified on a Sephacryl S-300 column (Pharmacia, Uppsala, Sweden) equilibrated with buffer A containing sodium phosphate buffer. The purified enzyme solution yielded two polypeptide bands, with molecular masses of approximately 87 and 93 kDa on an SDS–PAGE gel (Fig. 1B). The N-terminal sequences were determined to be AARNG and SIEETEQVE. They were detected in the amino acid sequences deduced from PvISA1 and PvISA2 cDNA respectively. Hence the purified enzyme was a complex of PvISA1 and PvISA2. It was designated PvISA1/2. This indicates that PvISA1 and PvISA2 are synthesized as preproteins with signal sequences of 50 and 62 residues respectively, and that mature PvISA1 and PvISA2 correspond to 741 and 803 amino acids with predicted molecular masses of 83.6 and 89.2 kDa respectively. The molecular mass of PvISA1/2 was estimated to be approximately 370 kDa by gel permeation chromatography (data not shown). Considering the stained intensities of the two bands on the gel, PvISA1/2 might be a heterotetramer. The final preparation was determined to be 0.15 mg by the method of Bradford. 13) PvISA3 proteins were purified from the crude extract by the following procedure: The precipitate from the fraction of 25–40% ammonium sulfate was dissolved in a small amount of buffer A and dialyzed against buffer A. The dialyzed sample was subjected to Q-Sepharose FF column chromatography as well as the case of the PvISA1/2 purification. Ammonium sulfate was added to the ISA-active fractions to facilitate 30% saturation. The solution was loaded on a Toyopearl-Butyl 650M column (Tosoh Bioscience, Tokyo) equilibrated with buffer C (40 mM MOPS–NaOH, pH 7.0, containing 4 mM DTT, 5 mM MgCl₂, and 10% v/v glycerol), containing ammonium sulfate at 30% saturation. The column was washed with buffer C containing 30% ammonium sulfate, followed by a reverse gradient of 30% to 0% ammonium sulfate. The active fractions were dialyzed against buffer A and resolved on a Sephacryl S-300 column equilibrated with buffer A. The column was prepared by coupling Sephacryl S-300 (Pharmacia, Uppsala, Sweden) to CNBr-activated Sepharose 4B (GE Healthcare Bio-Sciences) according to instructions. After the column was washed with buffer A, the proteins were eluted with a linear gradient of 0 to 2 M NaCl. The purified enzyme solution migrated as a single polypeptide band with a molecular mass of approximately 75 kDa on an SDS–PAGE gel (Fig. 1B). The N-terminal sequence was determined to be AYGRRAQEGV, which was included in the sequence predicted from PvISA3 cDNA, indicating that PvISA3 is synthesized as a preprotein with a signal sequence of 77 residues and that the mature form corresponds to 706 amino acids with predicted molecu-
lar mass of 80.1 kDa. The molecular mass of PvISA3 was estimated to be 50–60 kDa by gel permeation chromatography (data not shown), suggesting that PvISA3 is a monomeric enzyme. The final preparation was determined to be 0.2 mg from 1 kg of immature seeds.

The ISA activities of purified enzymes were assayed using potato amylopectin (Sigma), rabbit liver glycogen (Sigma), pullulan (Hayashibara), and β-limit dextrin as substrates. The reaction mixture (0.5 ml) contained 50 mM MES–NaOH (pH 6.0), 0.1 mg/ml BSA, 10 mg/ml of each polyglucan, and 50 μl of enzyme solution (0.1–0.3 μg), and was incubated at 30°C for 10 min. The activities were measured based on the increase in reducing power by the methods of Somogyi and Nelson,14,15) using maltotriose as a standard, and were expressed in terms of 1 μmol of maltotriose equivalent/min/mg protein. We prepared β-limit dextrin from potato starch (Sigma) according to the method of Doehlert and Knutson,1) As shown in Table 1, purified PvISA1/2 and PvISA3 displayed distinct reactivities for several polyglucans. PvISA1/2 showed high activity for amylopectin, whereas PvISA3 preferred β-limit dextrin and glycogen to amylopectin. The two enzymes barely reacted to pullulan. The substrate specificities of PvISA1/2 and PvISA3 were consistent with those of potato ISAs, Stisa1, and Stisa3 respectively.2)

To determine the chain-length specificities of PvISA1/2 and PvISA3, glucan chains liberated by enzyme reactions were analyzed using amylopectin, glycogen, and β-limit dextrin as substrates (Fig. 2). The reaction was done under the same conditions as the assay of ISA activity. After the reaction was terminated by boiling for 3 min, the sample (50 μl) was labeled with 0.2 M 8-aminopyrene-1,3,6-trisulfonate, as described by O’Shea et al.16) The labeled products were subjected to capillary electrophoresis (310 Genetic Analyzer; Applied Biosystems, Foster City, CA). The chain-length distribution profiles of PvISA1/2 and PvISA3 were compared with that of isomylase from Pseudomonas (Nacali Tesque, Kyoto, Japan), which has broad chain-length specificity and is used routinely in structural analysis of polysaccharides. The products obtained by PvISA1/2 from each polyglucan were highly similar to those obtained by Pseudomonas isoamylase. In contrast, the products obtained by PvISA3 from amylopectin and glycogen predominantly had DPs of 3 and 4, although branch chains of various lengths were included in these substrates. The products obtained with these enzymes from β-limit dextrin were limited to DPs of 2 and 3. These results suggest that PvISA1/2 has broad chain-length specificity for amylopectin and glycogen, while PvISA3 has high specificity for a chain-length of DP 2 to 4. Hence the high specific activity of PvISA3 for β-limit dextrin (Table 1) was attributed to the elevated concentration of chains (DPs 2 and 3) that can be reacted. The chain-length specificity of PvISA3 was identical to those of GlgX proteins from Escherichia coli17) and cyanobacteria.18)

Currently, the ISA1/2 complex is assumed to play a role in the biosynthesis of starch because a mutation in the ISA1 or ISA2 gene causes an accumulation of phytoglycogen in the plastids of both photosynthetic and storage organs.4–6) Although the role of the ISA3 isoform in starch biosynthesis is not yet established, its occurrence in storage organs such as immature seeds and tubers2) suggests that the isoform is also involved in starch synthesis. Considering the enzymatic properties of the isoform, its presence in storage organs might contribute to the maturation of starch granules by the removal of short chains on the surface of growing granules.19)

Plant ISAs belong to the α-amylase family and contain a catalytic (β/α)8 barrel structure.20–22) In addition to the barrel domain, ISAs have N-terminal and C-terminal domains whose functions remain unclear. Our preliminary experiments using recombinant PvISA proteins suggest that PvISA1 alone displays ISA activity and has the same chain-length specificity as purified PvISA1/2. PvISA1 and PvISA3 share a significant similarity (63%), and probably a similar conformation. Indeed, no large difference was observed in the overall structural models of PvISA1 and PvISA3 constructed from the structure of Pseudomonas isoamylase.23) but both enzymes have distinct differences in substrate specificity, indicating that minor structural differences in the active clefts exert a major effect on enzyme properties. Probably, PvISA3 has active clefts which branch chains with a DP of greater than 5 are unable to access. Identification of the residues or regions determining chain-length specificity is current underway.

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