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Research article

Cloning of the full-length isoamylase3 gene from cassava *Manihot esculenta* Crantz 'KU50' and its heterologous expression in *E. coli*

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ABSTRACT

Isoamylase (EC.3.2.1.68), an essential enzyme in starch metabolism, catalyses the cleavage of α-1,6 glucosidic linkages of branched α-polyglucans such as beta-limit dextrin and amylopectin, but not pullulan. Three different isoamylase isoforms have been reported in plants and algae. We herein report on the first success in preparation of full-length isoamylase3 gene (*MeISA3*) of cassava *Manihot esculenta* Crantz 'KU50' from 5′ Rapid Amplification of cDNA Ends (5′ RACE). The *MeISA3* was cloned to pET21b and expressed in *E. coli*. The Histrap™-purified rMeISA3 appeared as a single band protein with approximate molecular size of 75 kDa on SDS-PAGE and Western blot, while 80 kDa was shown by gel filtration chromatography. This indicated the existence of a monomeric enzyme. Biochemical characterisation of rMeISA3 showed that the enzyme was specific towards beta-limit dextrin, with optimal activity at 37 °C pH 6.0. Activity of rMeISA3 could be significantly promoted by Mg^{2+} and Co^{2+}. rMeISA3 debranched glucan chains of amylopectin were confirmed by HPAEC-PAD analysis.

1. Introduction

Starch, a major carbohydrate reserve in plants (Ball and Morell, 2003; Fresco, 2005) and algae (Busi et al., 2014), is synthesised in the plastids, including chloroplasts in leaves and amyloplasts in tubers and grains (Ball et al., 1996). Starch comprises two types of α-linked polyglucans: amylose and amylopectin. Amylose, a linear α-1,4 linked polyglucan with less than 1% α-1,6 branch points (Ball and Morell, 2003), is synthesised by ADP-glucose pyrophosphorylase (AGPase) and granule-bound starch synthase I (GBSSI). Amylopectin, a highly branched α-polyglucan that is composed of α-1,4 and α-1,6 linkages, is synthesised by the concerted action of AGPase, soluble starch synthases (SSs), starch branching enzymes (SBEs) and starch debranching enzymes (DBEs).

DBEs, members of glycoside hydrolase family 13 (GH13) found across the plants and algae, can be classified as isoamylase-type (EC 3.2.1.68; ISA) and pullulanase-type (EC 3.2.1.41; PUL), according to peptide sequence similarity and substrate preferences. Three conserved isoamylases, ISA1 to ISA3, have been reported in higher plants (Hussain et al., 2003; Facon et al., 2013; Deschamps et al., 2008). Several studies show that ISA1 (a catalytic isoform) and ISA2 (a non-catalytic protein) exist as homo/heteromultimeric enzymes (Hussain et al., 2003; Facon et al., 2013; Fujita et al., 1999; Streb and Zeeman, 2014; Sundberg et al., 2013; Utsumi and Nakamura, 2006; Sim et al., 2014) and are implicated in starch biosynthesis via mature amylopectin synthesis (Hussain et al., 2003; Bustos et al., 2004). Phenotypic changes, such as alteration of amylopectin structure and increasing amount of water soluble polysaccharide (WSP), were observed in ISA1/ISA2 mutants (Fujita et al., 1999, 2003; James et al., 1995; Burton et al., 2002; Kubo et al., 1999). ISA3 mutants showed a starch-excess phenotype and starch mobilisation in leaves was also interfered with (Wattebled et al., 2005, 2008; Delatte et al., 2006; Streb et al., 2008), indicating an essential role for ISA3 in starch degradation (Wattebled et al., 2005).

Structurally, the GH13 enzymes generally harbour multiple domains: a catalytic $(\beta/\alpha)_8$ barrel (domain A), with a small protruding loop between β3/α3 (domain B) (Janecek et al., 1997), and the C-terminal domain C. Moreover, an extra N-terminal domain preceding the catalytic domain, so-called domain N, possessing a Carbohydrate Binding Module 48 (CBM48) sequence, was also observed in isoamylases (Sim et al., 2014; Katsuya et al., 1998). Although several plant ISA3s, such as from kidney bean (Takashima et al., 2007), common wheat (Kang et al., 2013), potato (Hussain et al., 2003) and *Arabidopsis* (Wattebled et al., 2005; Streb et al., 2008), have been reported, only the full-length potato recombinant ISA3 (rStISA3) was fully characterised *in vitro* (Hussain et al., 2003). On the other hand, we recently found that MeISA1 (Panpetch et al., 2018) absolutely required MeISA2 for debranching activity, in contrast to StISA1 which can function on its own. To date, several enzymes in cassava starch metabolism have been

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reported: the granule-bound starch synthase (Salehuzzaman et al., 1992), catalysing the elongation of α-1,4-linked linear polyglucan, three isoforms of starch branching enzymes (Yaiyen, 2009), involved in formation of α-1,6 linkages in amylose and especially in amylopectin. D-enzyme or 4-α-glucanotransferase, catalysing the transfer of maltosyl unit of (1,4)-α-D-glucose to produce longer chain oligosaccharides, has also been reported (Tantanarat et al., 2014). We recently reported the coexpression of cassava rMeISA1 and rMeISA2 (Panpetch et al., 2018). To advance knowledge of starch metabolism in cassava tubers, we here report on the study of cassava ISA3, which is generally assumed to function in starch breakdown, by cloning the isoamylase3 gene, its heterologous expression in *E. coli* and biochemical characterisation.

2. Materials and methods

2.1. Plant materials

Cassava tubers (*Manihot esculenta* Crantz cultivar 'KU50') were harvested from The National Research Centre of Millet and Corn, Thailand.

2.2. Full length MeISA3 construction by 5′ Rapid Amplification of cDNA ends (5′ RACE)

Based on the Phytozome and NCBI databases, only partial sequences of cassava *MeISA3* have been reported to date. Thus, 5′ RACE was performed to obtain complete sequence. Total RNA was isolated from nine-month-old cassava by using PureLink™ Plant RNA Reagent (Invitrogen) following the manufacturer's manual. Construction of full-length cDNA was conducted by using SMARTer® RACE 5′/3′ Kit (Clontech). Approximately 1 μg of total RNA was mixed with 1 μl of 5′-CDS Primer A, 1 μl of SMARTer II A Oligonucleotide and nuclease-free ultra-pure water to the final volume of 11 μl as 5′-RACE-Ready cDNA mixture, and then placed on ice. After that the Master Mix was prepared as follows; 4 μl of 5X First Strand Buffer was mixed with 0.5 μl of 100 mM Dithiothreitol (DTT), 1 μl of 20 mM dNTP mix, 20 U of RNase Inhibitor and 200 U of SMARTScribe Reverse Transcriptase (RT). The Master Mix was then added to the 5′-RACE-Ready cDNA mixture. Reverse transcription was conducted by incubating at 42 °C for 90 min, and then RT was inactivated at 70 °C for 10 min. The cDNA was stored at -80 °C.

2.3. Cloning of putative isoamylase3 gene (MeISA3)

Full length *MeISA3* was amplified by using PrimeSTAR™ HS DNA Polymerase (Clontech), using the 5′ RACE cDNA as template. Forward primer; 10X Universal Primer A Mix (UPM) provided in the RACE kit, and gene specific reverse primer; R1_pET21b (5′—CCTGGTCGACTGTC AGTTTGCGTTCAAG-3′) were used. The temperature profile was as follows; initial denaturation at 98 °C for 10 s, 30 cycles of 98 °C for 10 s, 60 °C for 10 s, 72 °C for 3 min, and final extension at 72 °C for 7 min. The PCR product was analysed, cloned into pJET1.2/blunt (Thermo Fisher Scientific™) and sequenced. A new gene specific forward primer, F1full_pET21b (5′-GGAGTCCATATGCTAATGTCGGAAACCAGCCC-3′), was designed to amplify the mature *MeISA3*, excluding the signal peptide predicted by ChloroP1.1.

The PCR product of mature *MeISA3* was amplified by using the F1full_pET21b and R1_pET21b, and then cloned into pET21b fused with C-terminal 6xHis (pET21bMeISA3). The pET21bMeISA3 was multiplied by *E. coli* TOP10 (Invitrogen).

2.4. Gene expression and purification of rMeISA3

The pET21bMeISA3 was transformed into *E. coli* SoluBL21 (DE3) expression host (Amsbio). Cells were grown in ampicillin-containing LB broth at 37 °C with shaking. Cells were continuously grown under induction by 0.4 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) at 16 °C with shaking for 21 h. Cells were collected by centrifugation and suspended in binding buffer (25 mM potassium phosphate buffer, 0.1 M NaCl and 40 mM imidazole, pH 7.2). After cell lysis by ultrasonication and collection of the lysate by centrifugation, the crude extract was primarily analysed on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot (6x-His Epitope Tag Antibody, Thermo Fisher Scientific™). Enzyme activity was measured by a modified DNS assay (Miller, 1959). The soluble proteins were purified by Histrap™ column as previously described (Panpetch et al., 2018). Debranching activity was monitored on nondenaturing-polyacrylamide gel electrophoresis (native-PAGE) containing 0.15% (w/v) beta-limit dextrin. In brief, both crude ΔN-rMeISA3 and rMeISA3 were run in parallel on two separate beta-limit dextrin-containing gels. Subsequently, the first gel was incubated in freshly prepared 50 mM sodium acetate buffer pH 6.0 at 37 °C overnight and ISA debranching activity was visualised by I_2 staining. The second gel was subjected to Western blot for proteins visualisation.

2.5. Characterisation of rMeISA3

ISA3 enzymatic reactions were performed by incubating 50 mU of rMeISA3 with 0.75% (w/v) beta-limit dextrin in 50 mM sodium acetate buffer pH 6.0 at 37 °C for 15 min and analysed by DNS assay. One unit of ISA activity was recorded as the amount of enzyme that produced 1 μmole of reducing sugar in 1 min at 37 °C pH 6.0 with beta-limit dextrin as substrate.

For the effect of temperature, rMeISA3 was incubated in 50 mM sodium acetate buffer pH 6.0 at different temperatures (25–60 °C). For optimum pH, the enzyme was incubated at 37 °C in various pHs [50 mM sodium acetate buffer (pH 4.0, 5.0, 6.0) and 50 mM potassium phosphate buffer (pH 6.0, 7.0, 8.0)]. In addition, the enzyme was incubated in 50 mM sodium acetate buffer pH 6.0 at 37 °C containing metal ions or some chemicals (1 mM of Ca^{2+}, Co^{2+}, Cu^{2+}, Fe^{3+}, Mg^{2+}, Mn^{2+}, Zn^{2+}, EDTA, and 0.04% (w/v) SDS). For substrate specificity of rMeISA3, the enzyme was incubated with 0.75% (w/v) of various types of branched substrate (maize amylopectin, potato amylopectin, potato starch, beta-limit dextrin, glycogen and pullulan) in 50 mM sodium acetate buffer pH 6.0 at 37 °C.

The molecular size of rMeISA3 was analysed by gel filtration chromatography on a Sephacryl S-300 HR column (GE Healthcare Life Sciences™). The molecular mass of rMeISA3 in eluted fractions with enzyme activity was calculated compared to the Gel Filtration Standards (Bio-Rad).

2.6. Determination of debranching activity of rMeISA3 by chain length distribution (CLD) analysis

rMeISA3 (10 mU) was incubated with 0.75% (w/v) maize amylopectin in 50 mM sodium acetate buffer pH 6.0 at 37 °C for 21 h. The reaction was terminated by adding 15 μl of 0.1 N NaOH and filtered prior to analysis on a CarboPac PA100 column on an HPAEC-PAD (Dionex). CLD analysis was performed compared with debranching activity of PaISA as a positive control.

3. Results and discussion

3.1. Cloning of MeISA3 gene

In our initial attempt to clone the *MeISA3* gene of around 1.6 kb using sequences from NCBI and the Phytozome database, we found that almost 700 nucleotides of the 5′ region were missing compared to ISA3s of various plant species available in NCBI: potato (*Solanum tuberosum*), *Arabidopsis* (*Arabidopsis thaliana*), pea (*Pisum sativum*), rice (*Oryza sativa*) and soybean (*Glycine max*). Active MeISA3 was not detectable in recombinant form, either as an intact protein band or enzymatic