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Catalytically-inactive β-amylase BAM4 required for starch breakdown in *Arabidopsis* leaves is a starch-binding-protein

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ABSTRACT

Of the four chloroplast β-amylase (BAM) proteins identified in *Arabidopsis*, BAM3 and BAM4 were previously shown to play the major roles in leaf starch breakdown, although BAM4 apparently lacks key active site residues and β-amylase activity. Here we tested multiple BAM4 proteins with different N-terminal sequences with a range of glucan substrates and assay methods, but detected no α-1,4-glucan hydrolase activity. BAM4 did not affect BAM1, BAM2 or BAM3 activity even when added in 10-fold excess, nor the BAM3-catalysed release of maltose from isolated starch granules in the presence of glucan water dikinase. However, BAM4 binds to amylopectin and to amylose–Sepharose whereas BAM2 has very low β-amylase activity and poor glucan binding. The low activity of BAM2 may be explained by poor glucan binding but absence of BAM4 activity is not. These results suggest that BAM4 facilitates starch breakdown by a mechanism involving direct interaction with starch or other α-1,4-glucan.

Introduction

Starch provides most of the calories in the human diet, is a valuable industrial resource and an increasingly important bioethanol feedstock [1,2]. To the plant, starch provides an energy currency and carbon reserve that is deployed to fuel growth and development. The leaf provides an excellent example of this function since starch synthesised as a product of photosynthesis accumulates during the day and is mobilised at night to support energy metabolism and growth [3]. *Arabidopsis* leaves have provided a good model system to study both synthesis and breakdown of starch because of the genetic resources available [4–11]. Such studies have led to the discovery of new enzymes that participate in starch metabolism including disproportionating enzymes [9,12], glucan water dikinase [13,14], phosphoglucan water dikinase [15,16], a phosphoglucan phosphatase [17,18] and a novel maltose transporter in the chloroplast envelope [19]. Further studies have surprisingly shown that α-amylase and starch phosphorylase are dispensable for starch breakdown [20,21]. Our current understanding of starch breakdown in the leaf at night is that iso-amylase (ISA3)[3] and β-amylases are responsible for hydrolysis of the glucans comprising the starch granule and that maltose is the primary product of this activity [8,10,22–25].

A detailed genetic analysis of β-amylase function revealed four chloroplast localised isoforms, BAM1 to BAM4 [25]. BAM3 and BAM4 play important roles in leaf starch breakdown at night, since mutants lacking either protein breakdown their starch more slowly than wild type. At the end of the night, leaves of either mutant contain at least five times more starch than wild type leaves, and leaves of the *bam3 bam4* double mutant contain at least 15 times more starch than wild type. Starch metabolism returns to the wildtype pattern upon transformation of either mutant with the respective wildtype gene [25]. These results establish that BAM3 and BAM4 are both required for starch breakdown in *Arabidopsis* leaves, and that they might act cooperatively. In contrast, BAM1 is important only in the absence of BAM3 and no function for BAM2 has been identified. Recombinant BAM4 produced in *Escherichia coli* has no detectable β-amylase activity. The lack of BAM4 β-amylase activity is consistent with the absence of key

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[3] *Abbreviations used:* At, *Arabidopsis thaliana*; St, potato; BAM, β-amylase; ISA, isoamylase; GWD, glucan water dikinase; GST, glutathione-S-transferase; PNPG5, p-nitrophenylmaltopentoaside; BSTFA, *N,O*-Bis(trimethylsilyl)trifluoroacetamide; GC–MS, gas chromatography–mass spectrometry.

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active-site amino acid residues [25]. However, these previous experiments employed BAM proteins synthesised in *E. coli* with N-terminal glutathione-S-transferase (GST) tags [25]. We consider it is important to test enzymes without GST tags to confirm previous results. Furthermore, given its apparent absence of β-amylase activity, it is intriguing that BAM4 plays a key role in starch breakdown. This therefore requires further investigation as to the mechanism by which BAM4 is involved in such a process.

One possibility is that BAM4 functions as a polypeptide subunit of an enzyme heteromer. A precedent for an essential role for a catalytically-inactive polypeptide in starch metabolism comes from the iso-amylases involved in starch synthesis. In potato [26], *Arabidopsis* [27] and rice endosperm [28], the active iso-amylase is a multimeric enzyme comprising two closely-related polypeptides, termed ISA1 and ISA2. Although ISA2 lacks key active-site amino acid residues, it retains ability to bind glucan. When ISA2 forms a hetero-oligomer with ISA1, the K_m value of the enzyme complexes for polyglucans increases compared to that of the ISA1 homo-oligomer [28]. Another similar example is ADP-glucose pyrophosphorylase (AGPase), an allosteric enzyme which produces ADP-glucose, a precursor for starch synthesis [29]. AGPase is a heterotetramer comprised of large and small subunit polypeptides. It has been believed that the small subunit has catalytic activity while the large subunit is regulatory. However very recent results indicate that some large subunit types may also have catalytic activity even though amino acid sequences are significantly different to small subunits [30–33]. This observation reaffirms the importance of re-investigating possible catalytic activity of BAM4 and its glucan binding ability.

The aim of the present study was to first investigate the activities, substrate specificities, and binding properties of BAM1 to BAM4 with a range of glucans. The second aim was to examine whether BAM4 can modify the activities of BAMs 1, 2 and 3 in such a way that could explain its function in starch breakdown. We discovered that the low activity of BAM2 could be explained by the loss of glucan binding activity and surprisingly that BAM4 is a starch-binding protein.

Materials and methods

Expression of recombinant proteins in E. coli

DNA sequences encoding the four BAM proteins were cloned into the *E. coli* expression vector pGEX-4T-1 for BAM1 and pGEX-2T for the others (Amersham Biosciences, NJ, USA). In each case, the sequence encoding the predicted transit peptide was removed at amino acid residues (AA) 41, 55, 85, and 62 for BAM1, BAM2, BAM3 and BAM4, respectively. In the case of BAM4, extra constructs were made including N-terminal AA sequences starting from positions 1, 48, or 91. The constructs encoded the respective BAM with glutathione-S-transferase (GST) fused to the carboxyl terminus. Proteins expressed in *E. coli* BL21 or in the case of BAM3, BL21Codon-Plus (DE3)-RIL (Stratagene, La Jolla, CA, USA) were extracted and affinity purified to near homogeneity using Glutathione Sepharose 4B as described previously [25]. The GST tags of BAM1, BAM2, BAM3 and BAM4 were cleaved by thrombin protease according to the manufacturer's protocol (Amersham Biosciences). Recombinant GWD of potato (StGWD) was prepared as described previously [34].

Assays of β-amylase and α-amylase

Recombinant BAMs both with or without GST-tag were assayed for β-amylase activities using Betamyl kits (Megazyme, Bray, Ireland) according to manufacturer's instructions [25]. For kinetics, the substrate containing p-nitrophenylmaltopentoaside (PNPG5) was diluted to a range of concentrations from 1 mM to 8 mM. Enzymes were added to the substrate solution in 0.1 M maleic acid buffer, pH 6.2, incubated for 10 min at 40 °C. Under these conditions, the reaction rates were linear. Kinetic Parameters were obtained by Lineweaver Burk regression analysis based on the Michaelis–Menten equation using SigmaPlot. Recombinant proteins were assayed for α-amylase activity using an ALPHA-AMYLASE kit (Megazyme, Bray, Ireland) according to manufacturer's instructions. Pancreatic α-amylase (Roche, 10102814001) was used as a positive control. The amount of enzyme in the experiments was 1 μg per assay except α-amylase where 0.025 μg was used. Activity is measured as μmol p-nitrophenol/mg protein/min released from BPNPG7 in the presence of excess α-glucosidase.

Amylolytic activity of BAMs on amylopectin, starch and glycogen detected by GC–MS

The β-amylase activities of recombinant BAMs were assayed using potato amylopectin (Fluka, 10118), maize starch (Sigma, S4180) and oyster glycogen type II (Sigma, G8751). The assay mixture of 100 μl containing 10 mg/ml of each glucan, 0.1–0.3 μg of each enzyme was incubated at 37 °C for 30 min in 50 mM sodium acetate buffer pH 6.0, and the reactions were terminated at 65 °C with addition of 500 μl of methanol, and sucrose was added as an internal standard prior to GC–MS analysis of maltose content. For amyloglucosidase activity, the assay mixture of 100 μl containing 10 mg/ml potato amylopectin (Fluka, 10118) and 0.1–0.3 μg of each enzyme was incubated at 37 °C for 2 h in 100 mM sodium acetate buffer pH 4.8. α-Amyloglucosidase (Roche, 11202332001) was used as a positive control. The reaction was terminated at 65 °C with addition of 500 μl of methanol, and sucrose was added as an internal standard prior to GC–MS analysis of glucose, maltose and maltotriose content. For GC–MS analysis 100 μl of the mixture was brought to dryness under vaccum and sugars in the residue were converted to their trimethylsilyl ester using BSTFA [*N*,*O*-Bis(trimethylsilyl)trifluoroacetamide] at 80 °C for 30 min. Sugar derivatives were subjected to GC–MS analysis and maltose was quantified based on its TIC (total ion current) peak comparing to that of internal standard. GC–MS conditions are the following: Agilent 6890GC coupled with 5975 N mass selective detector. GC column was Varian Factor 4 VF-5 ms capillary column and the oven was programmed with initial temperature at 200 °C and held for 1 min, the temperature was then ramped to 340 °C at 10 °C/min and held for 5 min. Helium was the carrier gas and held constant flow at 1 ml/min.

BAM activity on Arabidopsis starch granules in vitro

Starch granules were prepared from *Arabidopsis* At*sex1–3* leaves as described previously [16,35]. Starch granules (2.5 mg) were mixed with the appropriate recombinant proteins: 1.25 μg BAM4 (GST-fusion), 2 μg BAM3 (GST-fusion), 2 μg GWD. Total volume of 105 μl of assay mixture containing 30 mM HEPES–KOH, pH 7.5, 5 mM $MgCl_2$, 5 mM $CaCl_2$, 1 mg/ml BSA, and 0.25 mM ATP was incubated at 25 °C for 45 min with shaking, the reaction was terminated by centrifugation twice (1 min at 20,000*g*), and the total solubilised sugar or glucan was determined after hydrolysis of the supernatant solution with HCl [35].

Starch binding assay

Amylopectin (potato starch, Fluka, 10118), and amylose immobilized on agarose resin (New England Biolabs, E8022S) were used as the substrates. Starch or amylose beads were pre-incubated with 1% (w/v) bovine serum albumin (BSA) at room temperature