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The purification and characterization of beta-amylase6 in Arabidopsis thaliana

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The Purification and Characterization of

β-amylase6 in *Arabidopsis thaliana*

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Abstract

Experimental evidence indicates that the family of β-amylase (BAM) proteins is largely responsible for the hydrolysis of starch in land plants. In *Arabidopsis thaliana* there are nine BAM genes, six of which are targeted to the chloroplast, but only four of those are presumed to be catalytically active: BAM1, -2, -3, and -6. Currently, little is known about the expression, characterization, or function of BAM6. Our study of starch accumulation in *Arabidopsis* indicates that BAM6 may be playing a role in older plants, although it has a minimal role in young plants. To further investigate the function of BAM6 we over-expressed the BAM6 protein by ligating the mature protein coding sequence in fusion with a His-tag into pETDuet-1, an *E. coli* expression vector and purified the protein by affinity chromatography. Using the purified BAM6 protein, β-amylase activity assays were conducted to begin to characterize BAM6. The effects of pH and temperature on BAM6 activity revealed maximum activity at pH 7.5 and 39 °C, which more closely resembles the profile of BAM1, an enzyme that is known to function primarily during the day. These findings indicate that BAM6 may also contribute to starch metabolism mostly during the day.

Plants reduce carbon during photosynthesis, but only half of the carbon reduced is used by the plant throughout the day to support metabolism (Smith and Stitt 2007). The remaining half is converted to starch, the primary storage carbohydrate in plants, to support metabolism at night (Li et al. 2009; Kotting et al. 2010). Experimental evidence demonstrates that the family of β-amylase (BAM) proteins is one of the primary groups of enzymes responsible for the hydrolysis of starch in *Arabidopsis thaliana* (Fulton et al. 2008). The BAM proteins are exo-amylases that hydrolyze β-1,4 glucosidic bonds in starch to yield maltose (Zeeman et al. 2010). There are nine genes in the *Arabidopsis* BAM family that can be divided into subfamilies based on phylogenetic analysis. Subfamily 1 contains BAM5 and BAM6, subfamily 2 contains BAM1 and BAM3, subfamily three contains BAM4 and BAM9, and lastly, subfamily four contains BAM2, BAM7 and BAM8 (Fulton et al. 2008). BAM5 is located in the cytosol as indicated by the lack of a targeting signal (Lao et al. 1999; Monroe et al. 1991), while BAM7 and -8 reside in the nucleus (Reinhold et al. 2011). Lastly, BAM1, -2, -3, -4, -6, and -9 are targeted to the chloroplast (Fulton et al. 2008; Zeeman, personal communication). Although the BAM family of proteins is important for the metabolism of starch there is still uncertainty in how each enzyme is involved in this process.

In the cytosol, BAM5 is known to be catalytically active and can contribute up to approximately 80% of BAM activity in leaves (Lin et al 1988). However, starch biosynthetic pathways and storage is known to be confined to plastids (Wise and Hoober 2006). Furthermore, *bam5* mutants do not exhibit a noticeable phenotype, making the exact function of BAM5 unclear (Laby et al. 2001). In the nucleus, BAM7 and BAM8 are not catalytically active (Reinhold et al. 2011) and instead act as DNA-binding transcription factors (Soyk et al. 2014).

Of the chloroplast-targeted BAM proteins, BAM1 and BAM3 are known to be catalytically active, contribute significantly to the breakdown of starch, and possess partially overlapping functions (Fulton et al. 2008). Recently it was found that the pH and temperature stability for enzyme activity of these two enzymes differ, which allows them to carry out a similar function in two distinct environments (Monroe et al. 2014). BAM1 resides in guard cells and is involved in the degradation of starch during the day (Valerio et al. 2011). In comparison to BAM3, BAM1 exhibits more activity at high pH and has a temperature optimum about 10 °C higher than the temperature optimum of BAM3 (Monroe et al. 2014). These findings are consistent with BAM1 functioning within the guard cells during the day as the temperature is warmer during the day and the pH is higher due to the transport of protons to support the photosynthetic pathway. BAM3 resides in mesophyll cells and is involved in the degradation of starch at night. During the night, BAM3 operates at cooler temperatures and in the absence of photosynthesis, thus it makes sense that BAM3 would function more at lower temperatures and at a lower pH than BAM1. These environmental adaptions are likely due to variations in the amino acid compositions of BAM1 and BAM3 (Monroe et al. 2014).

Unlike BAM1 and BAM3, it is suspected that BAM2 plays a small role in starch degradation as shown through experiments demonstrating that *bam2* mutants do not accumulate excess starch in the leaves of young plants (Kaplan and Guy 2005). Additional experiments on BAM2 propose that its low activity could be a result of poor binding with starch (Li et al. 2009). Recent experiments in our lab have demonstrated that BAM2 is significantly more catalytically active in the presence of KCl but its physiological role remains unclear (Monroe laboratory, unpublished). The exact function of BAM4 is also undetermined. Evidence supports that it is not a catalytically active hydrolase. However, it still possesses the ability to bind to starch and

mutants lacking BAM4 accumulate starch, indicating that BAM4 may function as a regulatory enzyme for starch metabolism (Fulton et al. 2008; Li et al. 2009). Similar to BAM4, BAM9 lacks conserved residues vital for catalytic activity and is not catalytically active (Fulton et al. 2008).

Lastly, the function of BAM6 is also undetermined although our unpublished data suggests BAM6 is catalytically active. Thus, we suspect the only catalytically active BAMs in the chloroplast are BAM1, 2, 3, and 6. Much of the previous research on BAMs has centered on BAM1 and BAM3, therefore little is known about the structure or function of BAM6. However, given that BAM1 and BAM3 appear to be adapted to specific environments, it is probable that BAM6 also has unique properties that suit it for a particular role in starch metabolism. In an attempt to acquire more knowledge on BAM6 we used a combination of plant mutagenesis and characterization of purified BAM6 protein to begin to characterize this catalytic BAM.

Plant Material and Growth

Arabidopsis ecotype Columbia-0 plants were grown five per pot in a 5-inch pot at 22°C on a growth cart using a 12 hour light/12 hour dark photoperiod with 130 μmol m^{-2} s^{-1} illumination. Sunshine Mix#3 (Sun Gro Horticulture) was used as the growth medium and was supplemented with macro and micronutrients (Monroe et al. 2014). Transfer DNA (T-DNA) mutant plants were obtained from the Arabidopsis Biological Resource Center's SALK collection and included *bam1* (Salk_039895), *bam2* (Salk_086084), *bam3* (Salk 041214), *bam5* (Salk_004259), and *bam6* (Salk_023637) (Alonso et al. 2003; Monroe et al. 2014). Double mutant plants were generated by genetic crosses using the T-DNA mutant plants.

Iodine Leaf Staining

Starch accumulation in leaves can be qualitatively observed through iodine staining (Caspar et al. 1991). Leaves were harvested at the end of the 12-hour dark period from wild type plants, single mutant plants including *bam1, bam2, bam3,* and *bam6,* and double mutant plants including *bam3/*2 and *bam3/6.* Each extracted leaf was decolorized with hot 80% ethanol and stained with iodine (5.7 mM I_2, 43.4 mM KI, 0.2 N HCl) to visualize starch accumulation (Caspar et al. 1985).

BAM6 cDNA Acquisition

Full length BAM6 cDNA in a modified bluescript 2 vector was purchased from Riken BioResource Center Experimental Plant Division Ibaraki, Japan, clone R24930. The BAM6 bluescript 2 vector was transformed into *E. coli* by incubating the DNA with *E. coli* competent cells on ice for 10 minutes, heat shocking the cells at 42 °C for 90 seconds, returning the cells to

ice for 1 minute and then incubating the cells with Luria-Bertani media supplemented with 50 μg mL^{-1} Ampicillin at 37 °C for 1 hour. The transformed cells were then plated on an agar plate supplemented with Ampicillin and incubated overnight at 37 °C. Two colonies from this plate were selected for a DNA mini prep and double digested with EcoRI and BamHI, as well as, EcoRI and PstI to confirm their identity by visualization on an agarose gel. The mini prepped BAM6 bluescript 2 DNA was then used in a standard PCR amplification involving primers specific for BAM6, 5'- GGCATATGACAAGCGTATTAGGAATGATGAACC-3' and 5'-TTCTCGAGGGACTTCTTCTCAAAATGTG-3', and Deep Vent Polymerase. Using an annealing temperature of 55°C and extension temperature of 59°C for 2.5 minutes. The resulting PCR was run on an agarose gel and the BAM6 band (~1.6 KB) was gel purified using GE Healthcare Illustra GFX PCR DNA and Gel Band Purification Kit and protocol.

BAM6 pMOS Blue Clone

The BAM6 PCR product was ligated into the pMOS Blue vector and transformed into *E. coli* according to the protocols outlined by GE Healthcare pMOSBlue Blunt Ended Cloning Kit product booklet in which a phosphorylation (pk) reaction proceeded the ligation. For the pk reaction a vector-to-insert ratio of 1:2.5 was used to calculate the appropriate amount of BAM6 insert to add to 10 x pk buffer, 100 mM DTT, and 1 μL pk enzyme mix in a 10 μL reaction that was incubated at room temperature for 5 minutes, heat inactivated at 75 °C for 10 minutes, cooled on ice for 2 minutes, and then centrifuged briefly. For the ligation, 1 μL pMOSBlue vector (50 ng/μL) and 1 μL T4 DNA ligase were added to the 10 μL of the pk reaction and incubated overnight at room temperature. This ligation was then transformed into *E. coli* cells by adding 1 μL of the ligation mix to 20 μL competent cells on ice and incubating the mixture on

ice for 30 minutes before heat shocking at 42 °C for 40 seconds. The mixture was placed on ice for 2 minutes before being incubated with 80 μL SOC medium for 1 hour at 37 °C. The solution was then plated and incubated at 37 °C overnight on agar plates containing 50 μg/ml ampicillin, 15 μg/ml tetracycline, and 20 mg/mL X-gal and IPTG for blue and white colony screening. Several white colonies were selected for colony PCR in which Go-Taq polymerase was used with a colony from the agar plate as the template DNA to confirm their identity. The PCR protocol used for BAM6 cDNA acquisition was also used here. A colony that had an amplified BAM6 product was then mini-prepped.

BAM6 pET Duet-1 Clone

BAM6 pMOS Blue and pET Duet-1 with a N-terminal His-tag were double digested with BamHI and SalI and then gel purified using GE Healthcare Illustra GFX PCR DNA and Gel Band Purification Kit according to the manufacturer's protocol. The purified BAM6 insert and pET Duet-1 were then ligated together and transformed into *E. coli* cells following the same protocols used in the BAM6 cDNA acquisition methods section. Several colonies were selected for colony PCR to confirm their identity, and then a colony that had an amplified BAM6 product was mini-prepped. The plasmid DNA was digested with BamHI and SalI to further confirm the cells' identity by visualization on an agarose gel. Next, a rapid colony transformation of BAM6 pET Duet-1 into BL21+ *E. coli* cells was performed by transferring and resuspending one to two large *E. coli* colonies in 250 μL $CaCl_2$ and then adding 0.005 μg/ μL BAM6 pET Duet-1 plasmid DNA. This homogeneous mixture was then incubated on ice for 15 minutes, heat shocked at 42 °C for 90 seconds, and returned to ice for 1 minute before incubating with Luria-Bertani media at 37 °C for 30 minutes. Lastly, the suspension was spread on an agar plate supplemented with

ampicilin and incubated overnight at 37 °C (Micklos and Freyer 1990). Several colonies from the agar plate were then selected and mini-prepped.

BAM6 Protein Purification

The BAM6 pET Duet-1 plasmid in BL21+ *E. coli* cells was used for BAM6 protein over expression and purification. Cells were grown to an optical density (OD_{600}) of 0.4 in 1L of Luria-Bertani media with 50 μg mL^{-1} kanamycin at 37 °C. Then 0.75 mM IPTG was added and the media was incubated at 20 °C while shaking at 250 rpm overnight. The cells were centrifuged at 4 °C using 8000 rpm for 15 minutes. The supernatant was discarded and the resulting pellet was frozen overnight. Next the pellet was suspended in 30 mL binding buffer (50 mM pH8 NaH_2PO_4, 0.3 M NaCl, and 10 mM imidazole) and the cells were lysed via sonication (5 seconds on, 20 seconds off for 4 minutes). The sonicated solution was centrifuged at 4 °C using 10,000 x g for 15 minutes. The sonicated supernatant was incubated with 2 mL of Ni-NTA His-Bind Resin beads at 4 °C for 1.5 hours inverting at 5 rpm and then centrifuged at 1000 x g for 1 minute. The supernatant was carefully removed and the remaining resin beads were resuspended in 30 mL binding buffer and then loaded into a purification column. The column was then washed with 20 mL wash buffer (50 mM pH8 NaH_2PO_4, 0.3 M NaCl, and 40 mM imidazole) followed by 10 mL elution buffer (50 mM pH8 NaH_2PO_4, 0.3 M NaCl, and 200 mM imidazole). The eluted solution was then dialyzed in 20 mM MOPS, 0.1 M NaCl, and 0.2 mM TCEP prior to being concentrated with an Amicon Ultra-4 10K filter and stored at -80 °C. Coomassie Blue-stained SDS-PAGE and Western blot probed with anti-His tag antibodies were used to visualize the purified protein.

To optimize the purification procedure, a range of induction conditions were tested, including inducing cells at different optical densities (0.2, 0.4, 0.6 and 0.8), varying IPTG

concentrations (0.5 mM, 0.75 mM, 1 mM, and 1.25 mM), and induction temperatures (15 °C, 20 °C, 30 °C, and 37 °C). Additionally, the binding and wash buffers during affinity purification were used with increased levels of imidazole, 20 mM and 60 mM, respectively, in an attempt to reduce contaminants. Ultimately, it was determined that the optimal conditions for BAM6 protein purification were inducing at an OD of 0.4 using 0.75 mM IPTG at an induction temperature of 20 °C in conjunction with binding and wash buffers containing concentrations of 10 mM and 40 mM imidazole, respectively.

β-amylase Activity Assays

Assays were performed in 0.5 mL of 50 mM MES (pH 6.0) and 5 mg of Lintner soluble starch (Pfansteihl Laboratories) using purified protein suspended in a dilution buffer (50 mM MOPS pH 7.0 and 1 mg/ml bovine serum albumin). A typical assay was conducted for 30 minutes to an hour at room temperature. However, reaction time, temperature, and pH were manipulated depending on the experiment. Reactions were stopped by immersion in boiling water for 5 minutes. Production of maltose was detected by quantifying reducing sugars using the Somogyi-Nelson assay (Nelson 1944) and maltose standards. Protein concentrations were quantified with the Bio-Rad Protein Assay Kit with bovine serum albumin as standards.

BAM6 Contributes to Starch Metabolism

To gain a better understanding of starch metabolism in *Arabidopsis thaliana,* we used a combination of plant mutagenesis and characterization of purified BAM6 protein to begin to comprehend the function of this plastid localized, catalytic BAM. Iodine leaf staining of various *bam* mutants were conducted in order to observe the effects of a BAM deficiency on starch accumulation. Elevated starch levels were only observed for *bam3* mutants in five-week old leaves (Figure 1). Furthermore, at five weeks old, the *bam32* and *bam36* double mutants displayed a similar starch accumulation to the *bam3* single mutant. However, in eight-week old flowering plants *bam1, bam2,* and *bam6* also displayed starch accumulation similar to that of *bam3* and the double mutants *bam32* and *bam 36* had greater starch accumulation than *bam3* (Figure 1). The iodine staining results in conjunction with Fulton et al. (2008) indicate that prior to flowering it is probable BAM3 is primarily responsible for starch degradation, with BAM1 contributing to a smaller extent. Whereas after flowering, each of the plastid-localized, active BAMs, BAM1, -2, -3, and -6 appear to be contributing to starch degradation. Overall, this reflects a change in starch metabolism after flowering in which the plastid-localized active BAMs may be playing unique roles in starch degradation.

BAM6 Cloning and Purification

The BAM6 cDNA was amplified by PCR and gel purified using the BAM6 modified bluescript 2 construct. The BAM6 cDNA was then ligated and transformed with pMOS Blue resulting in a BAM6 pMOS Blue construct. The pMOS Blue vector was used because of its ability to ligate with blunt ended DNA, such as PCR products, and its ability to undergo blue-

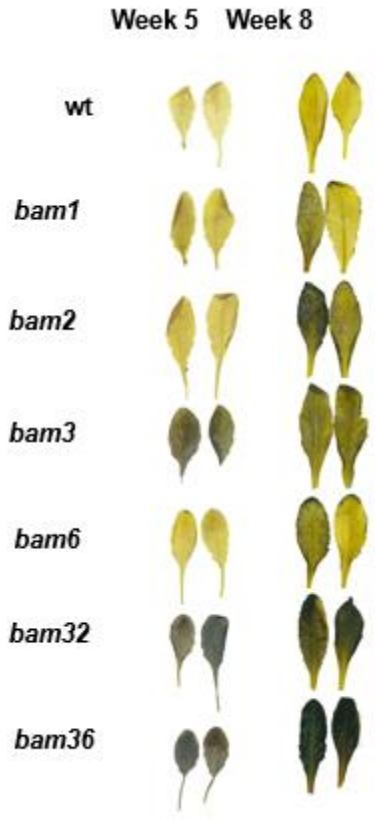

Figure 1. Iodine leaf staining of wild type (wt) and mutant Arabidopsis plants at 5 and 8 weeks old. Plants were grown under a 12-hour light/ 12 hour dark photoperiod. Leaves were harvested at the end of the 12-hour dark period, decolorized with hot 80% ethanol, and stained with iodine (Monroe et al. 2014). The darker the shade of green is representative of a greater amount of starch accumulation.

white screening for easy identification of transformed plasmids. The BAM6 pMOS Blue construct was digested and ligated into pET Duet-1 yielding a BAM6 pET DUET-1 construct. The pET DUET-1 vector was used at this stage because it contains an N-terminal His-Tag that we were then able to use for purification with Ni-NTA His-Bind Resin beads through affinity purification. This final construct was digested with BamHI and SalI to confirm its identity as a pET DUET-1 vector (5.4 KB) with a BAM6 insert (1.6 KB) (Figure 2).

Once the BAM6 pET DUET-1 construct was transformed into BL21+ *E. coli* cells, BAM6 was over-expressed in *E. coli* and purified. The Coomassie Blue-stained SDS-PAGE revealed the *E. coli* cells were over expressing BAM6 (Figure 3A). The elution from the affinity purification contained some proteins other than BAM6, which has a molecular weight of 62.3 kDa. However, the Western blot probed with anti-His tag antibodies makes it apparent that within the concentrated elution BAM6 is the only protein that possesses a His-Tag (Figure 3B). Despite efforts to optimize the purification process by testing a range of induction conditions including different optical densities, IPTG concentrations, and induction temperatures, as well as altering the concentrations of imidazole used in the binding and wash buffers for purification, the contaminants observed in the Coomassie Blue-stained SDS-PAGE continued to reappear in each purification of BAM6. Therefore, the contaminated BAM6 protein was used in β-amylase activity assays with the reasoning that, as the contaminants did not possess His-tags, they were proteins innate to *E. coli* and since *E. coli* is not known to have any BAM proteins to metabolize starch, it is highly unlikely that these proteins would interfere with β-amylase activity assays.

Further confirmation of successful BAM6 purification was seen in the successive purification fractions through the increase of BAM activity in β-amylase activity assays. While the BAM6 sonicated supernatant displayed low specific activity, the BAM6 elution had

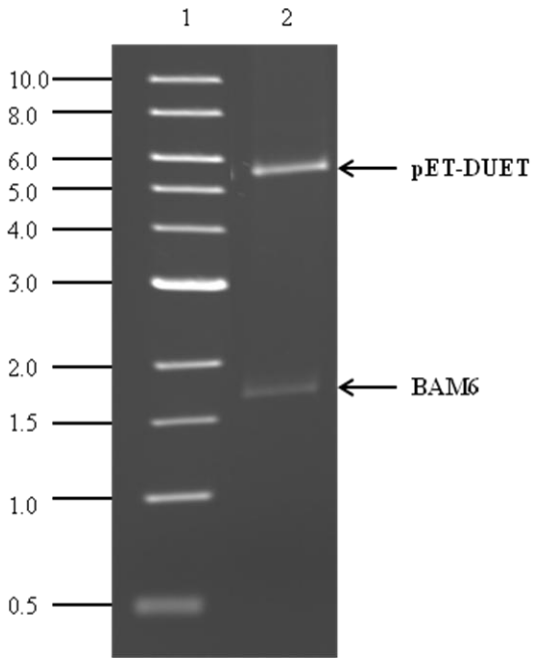

Figure 2. BAM6 pET DUET-1 double digest with BamHI and SalI. Lane 1 contains a 1 KB ladder and lane 2 contains the double digested BAM6 pET DUET construct.

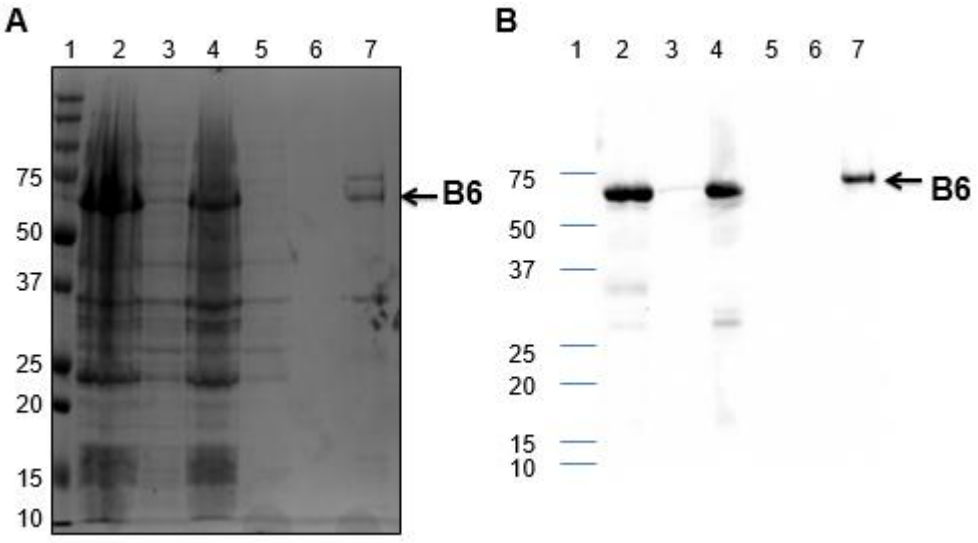

Figure 3. BAM6 protein purification visualization via Coomassie Blue-stained SDS-PAGE (A) and Western blot probed with anti-His tag antibodies (B). Lanes correspond to 1. Protein standards 2. Over expressed pre-sonication sample 3. Soluble supernatant post-sonication 4. Insoluble pellet post-sonication 5. Ni resin unbound supernatant 6. Imidazole wash 7. Concentrated elution. BAM6 bands appear at approximately 62.3 kDa.

significantly higher specific activity indicating that the purification process was successful in decreasing the total proteins while concentrating BAM activity (Figure 4). There was a minor loss of BAM6 protein that did not bind to the resin (Figure 3A) as indicated by a small amount of BAM activity in the Ni resin unbound solution (Figure 4). Additionaly, some BAM6 protein was lost during the imidazole wash, but the stringency of the wash was not reduced due to the presence of contaminants within the elution and concentrated protein.

BAM Activity

The activity of the active chloroplast targeted BAMs, BAM1, -2, -3, and -6 were compared using purified BAM proteins in β-amylase activity assays. Similar methods used to purify BAM6 were used for the purification of BAM1, -2, and -3 by other individuals within the Monroe laboratory. The comparison indicated that under the tested conditions, BAM1 is the most active of the four active chloroplast BAMs at 4414 U/mg, followed by BAM3 at 1263 U/mg, BAM6 at 169 U/mg and lastly, BAM2 at 0.82 U/mg (Figure 5). For this particular experiment, each of the BAMs were tested under identical conditions, and thus BAM2 was not assayed with the presence of KCl, which may have significantly altered the activity of this protein.

Upon observing differences in β-amylase activity between the chloroplast targeted BAMs, BAM6 was further characterized by comparing the effects of pH and temperature on its activity to BAM1 and BAM3 since more is currently known about those two proteins. BAM6 exhibited optimal activity at a pH of 7.5 (Figure 6). The pH of the stroma during the day when BAM1 is active is approximately 8, while the pH at night when BAM3 is active is approximately 7 (Werdan et al. 1975). Monroe et al. (2014) concluded that at pHs greater than 6.5 BAM1 was

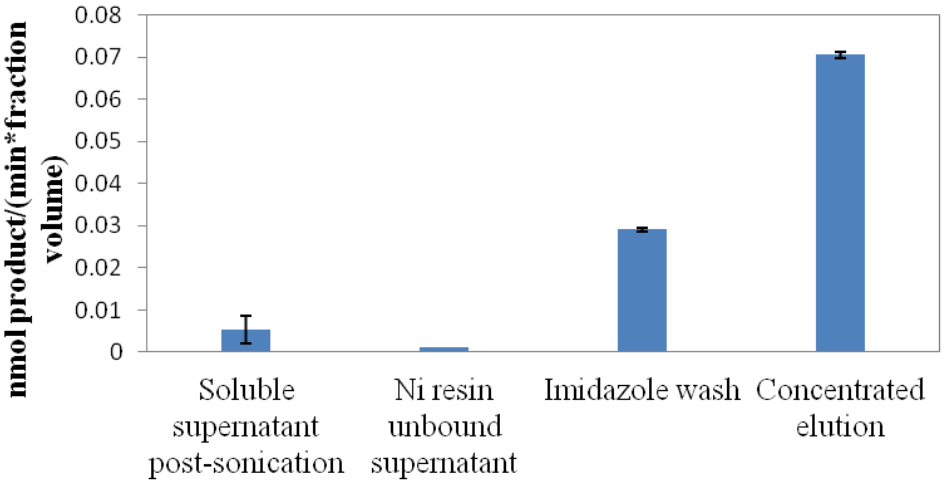

Figure 4. Levels of BAM activity throughout purification. Soluble supernatant post-sonication, Ni resin unbound supernatant, Imidazole wash, and concentrated elution were assayed for β-amylase activity at pH 6 and 22 °C for 30 minutes with soluble starch as the substrate. Error bars represent +/- 1 standard deviation, n=2.

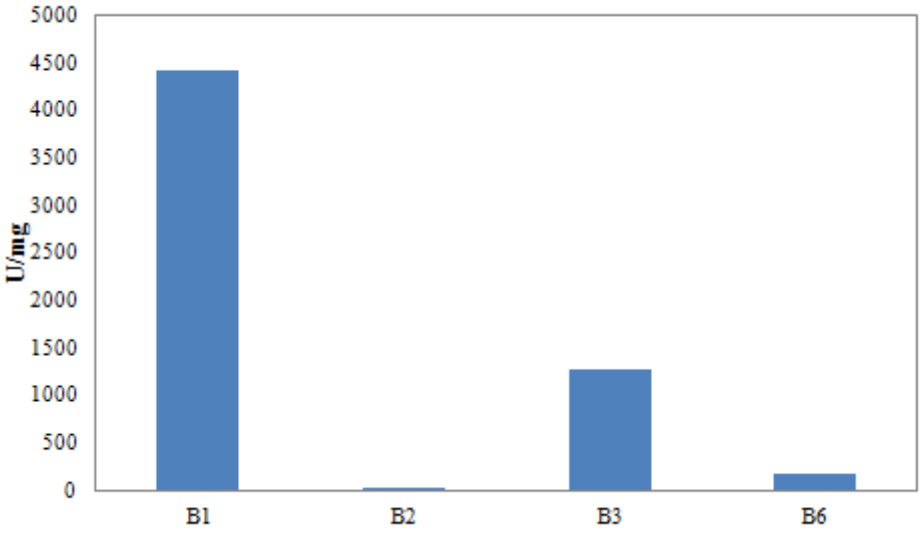

Figure 5. β-amylase activity assays using chloroplast targeted BAMs. Purified proteins, BAM1, 2, 3, and -6 were assayed at pH 6 and 22 °C for 30 minutes with soluble starch as the substrate to determine the amount of activity each protein displayed.

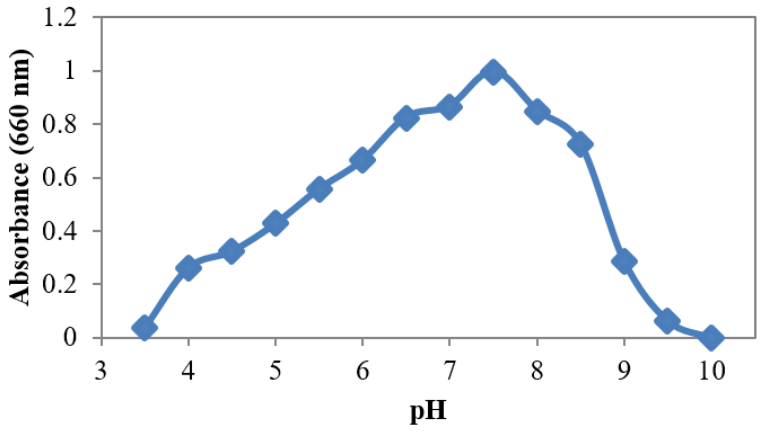

Figure 6. Effect of pH on BAM6 expressed as absorbance at 660 nm. A 50 mM citrate, 50 mM phosphate buffer was used to manipulate the pH using a range of 3.5-10 while all assays were conducted at 22 °C using soluble starch as the substrate.

more active than BAM3. In fact, BAM1 maintained approximately 80% of its maximum activity at pH 8 while the activity the activity of BAM3 was less than 50% of its maximum activity at pH 8, indicating that the BAM1 protein is more active in basic conditions than the BAM3 protein (Monroe et al. 2014). These results indicate that the effects of pH on the activity of BAM6 more closely resembles that of BAM1 however, with a maximum activity displayed at pH 7.5 it is difficult to make any inferences on whether BAM6 is active during the day or night.

Similarly, the effects of temperature on the activity of BAM6 also more closely resembled that of BAM1. The maximum activity observed for BAM6 was 39 °C (Figure 7) while the maximum activities reported for BAM1 and BAM3 were approximately 40 °C and 30 °C, respectively (Monroe et al. 2014). With both the effects of pH and temperature on BAM6 activity resembling that of BAM1 it is possible that BAM6 is adapted to function during the day.

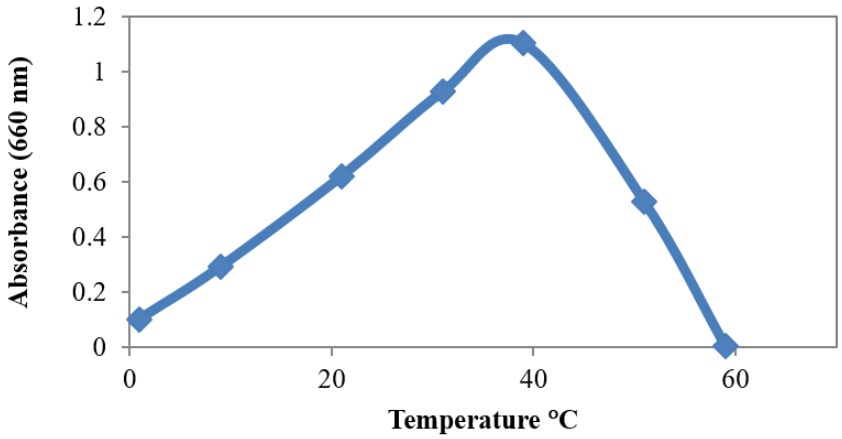

Figure 7. The effects of temperature on BAM6 expressed as absorbance at 660 nm. All assays were conducted at pH 6 using soluble starch, while the temperature was adjusted through the use of water baths with a range of 0 °C- 59 °C.

Conclusion

There are nine genes in the *Arabidopsis* BAM family, four of which encode active chloroplast targeted enzymes. Presently, BAM1 and BAM3 have been studied the most and are the primary contributors to starch metabolism in plants up to 5-weeks old. We found that BAM2 and BAM6 contribute more to starch metabolism after the plants flower. The exact functions of BAM2 and BAM6 in starch metabolism remain unclear as both of these proteins exhibit significantly less β-amylase activity than BAM1 and BAM3. However, the effects of pH and temperature on BAM6 activity more closely resemble the profile of BAM1 indicating that BAM6 may contribute to starch metabolism mostly during the day.

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