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An n-terminal acidic domain in β-amylase2 is required for k+ regulation.

Jillian Breault
James Madison University

An N-terminal acidic domain in β-amylase2 is required for K^+ regulation.

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by Jillian Simine Breault

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FACULTY COMMITTEE:

Project Advisor: Jonathan Monroe, Ph. D.
Professor, Biology

Reader: Marquis Walker, Ph. D.
Assistant Professor, Biology

Reader: Nathan Wright, Ph. D.
Assistant Professor, Chemistry

HONORS COLLEGE APPROVAL:

Bradley R. Newcomer, Ph.D.,
Dean, Honors College

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Table of Contents

List of Figures	3
Acknowledgements	4
Abstract	5
Introduction	7
Methods	14
Results	19
Discussion	31
Bibliography	35

List of Figures

1	Analysis of BAM-like genes and BAM subfamilies	8
2	BAM2 Tetramer Model	13
3	N-terminal sequence alignment of BAM2 orthologs	20
4	Acidic domain on the BAM2 dimer model	21
5	Sequence alignment of BAM2 acidic domains and percentages of charged residues	24
6	Diagram of N-termini of BAM2 and B2-NDel mutants	25
7	SDS PAGE gel of purified proteins	27
8	Salt curve of wild type BAM2 and B2-NDel1	28
9	Activity of B2-NDels with high and low concentrations of KCl	29
10	SEC-MALS of BAM2 and B2-NDel1	30

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Abstract

In the chloroplasts of leaf mesophyll cells, β-amylase proteins (BAMs) are responsible for breaking down starch into maltose when the plant cannot undergo photosynthesis. BAM2, which was previously considered inactive, was recently shown to be active under stromal-like levels of salt and has optimal activity at 80mM KCl. In addition, BAM2 is active as a tetramer *in vivo* and displays sigmoidal kinetics due to a secondary binding site that is responsible for activating BAM2 when bound to starch. A hypothesized tetramer model was created using a homology model of a BAM2 monomer and the configuration of a crystallized sweet potato BAM5. This model was supported by mutations, made by other members of the lab, in specific interfaces, which disrupted tetramerization and activity. This model along with sequence alignments of BAM2 orthologs revealed a potential acidic domain, containing 2-fold more acidic residues than the catalytic domain and 10-fold more acidic residues than the chloroplast transit peptide. This acidic domain is adjacent to the N-terminal end of the catalytic domain and is just upstream from a short peptide of conserved residues (ERDF). Further, the acidic domain and ERDF peptide are located very close to the starch-binding groove, which is an area of the enzyme where two secondary binding sites face each other. Hypothesizing that this acidic domain and ERDF peptide could interact with KCl and the starch binding groove, we created two mutant BAM2 proteins—one lacking just the acidic domain (B2-NDel1), and one lacking both the acidic domain and the ERDF peptide (B2-NDel2). We hypothesized that by removing the acidic domain and the ERDF peptide, the enzyme would not require salt to function. When

the activity of these enzymes were compared to the activity of wild type BAM2, we found that neither of the mutants required salt to function, while BAM2 had negligible activity without KCl. Additionally, both of these mutants still functioned as a tetramer, even though the activity of B2-NDel2 was about 5-fold lower than NDel1. Together, these data indicate that the acidic domain and ERDF peptide might be of importance to the salt dependency of BAM2.

Introduction

In the leaves of plants, starch is synthesized and stored in the chloroplast stroma when sunlight is available. At night, β-amylases (BAMs) are primarily responsible for hydrolyzing this starch to create maltose by breaking the penultimate α-1,4-glycosidic bond on the non-reducing ends of starch. Maltose is exported from the plastid and further metabolized for energy or biosynthesis (Weise et al., 2004). In the β-amylase gene family, two sub-families have been described based on intron positions and amino acid alignments (Figure 1) (Monroe et. al., 2017). In the first subfamily, a past homolog of both *BAM1* and *-3* is hypothesized to be the most ancestral, which eventually gave rise to *BAM9*, *-1*, and *-3*. Likewise, in the second subfamily, *BAM2* is hypothesized to be the most ancestral, which subsequently gave rise to *BAM4*, *-5*, *-6*, *-7*, and *-8* from gene duplication events (Monroe et al., 2017).

Of the nine β-amylases in *Arabidopsis thaliana*, only BAM1, -2, -3, and -6 are catalytically active and found in the chloroplast stroma (Fulton et al., 2008; Valerio et al., 2011; Monroe et al., 2014; Monroe et al., 2017). BAM1 and -3 are thought to account for the majority of the starch degradation activity due to the accumulation of starch in *bam1* and *bam3* knock-out mutant plants and the low activity in leaf extracts from *bam1* and *bam3* plants (Fulton et al., 2008; Monroe et al., 2014).

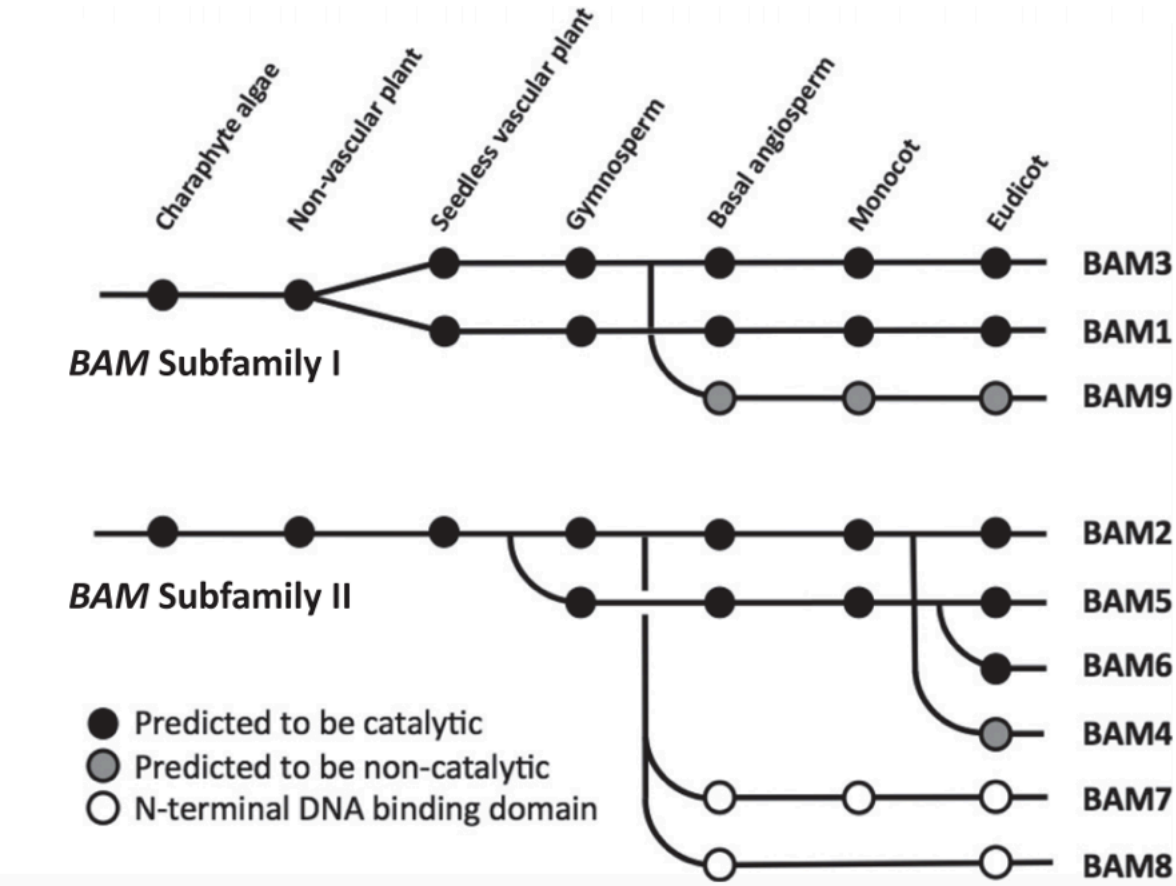

Figure 1. **An analysis of BAM-like genes dating back to charaphyte algae.** The black dots show the genes that are predicted to code for a catalytic enzyme, while grey dots show the genes that are predicted to code for a non-catalytic enzyme. White dots show genes that code for an enzyme with an N-terminal DNA binding domain. The nine *BAM* genes found in Arabidopsis are divided into two subfamilies, one deriving from a *BAM* similar to *BAM1* and -*3*, and the other deriving from *BAM2*. (From Monroe et al., 2017).

BAM4 and -9 are plastid localized, but do not have any observable catalytic activity (Fulton et al., 2008; Li et al., 2009; Monroe and Fedkenheuer, unpublished). Additionally, BAM5 is catalytically active, but is instead found in the cytosol of phloem cells and its function is currently unknown (Laby et al., 2001; Monroe et al., 2014). BAM7 and -8 are both transcription factors found in the nucleus (Reinhold et al., 2011; Soyk et al., 2014).

By identifying and understanding the roles of each β-amylase and how they effect the growth of a plant, we open the door to better understanding the entire pathway of starch degradation. In a time where genetically modified crops are becoming more necessary in the goal to feed our growing population, an increased understanding of β-amylases and related enzymes is required to create crops that grow faster or contain more starch, depending on the needs of a certain population. Additionally, β-amylases are commonly used in the production of glucose syrups and other foods (Silano et al., 2017). By identifying new BAMs, which can be regulated in different ways, companies utilizing BAMs as a production mechanism may find new, cheaper, and more efficient ways of making their product.

While the functions of the enzymes found in the *Arabidopsis* β-amylase family differ, the qualities of most of the catalytic enzymes are similar. BAM1, -3 and -5 all function as monomers, display hyperbolic kinetics, and do not seem to have any requirements for activity other than substrate and a pH of approximately 6 (Monroe et al., 2017). However, BAM2, which was shown to be catalytically active in a recent paper from our lab, has several unique qualities (Monroe et al., 2017). Previous papers concluded that BAM2 was essentially inactive, due to the

extremely low activity in comparison to BAM1 and -3 (Fulton et al., 2008; Li et al., 2009). However, after comparing active site residues and considering the fact that BAM5, a derivative of BAM2, is catalytically active, we predicted that BAM2 should be active (Monroe et al., 2017). This led to the discovery that BAM2 has a requirement for KCl, with the optimal activity at 80 mM KCl (Monroe et al., 2017). Further, the ionic strength of the stroma is consistently at the level required for activation, so it is likely that BAM2 is active at all times *in vivo*. Previous *in vitro* studies concluding that BAM2 was inactive did not include salt when measuring the activity of the enzyme.

Salts are common regulatory effectors of enzymes. Specifically, the dissociated anions and cations will often interact with charged side-chains as well as the protein backbone, changing the function of an enzyme (Okur et al., 2017). Additionally, K^+ can affect enzymes in two ways: it can interact with the substrate directly (Type I interaction), or it can indirectly change the structure of the active site of the enzyme (Type II interaction) (Di Cera et al., 2005). BAM2 is believed to interact with K^+ as a Type II interaction due to data showing BAM2 has activity with a variety of monovalent cations (Monroe et al., 2017).

In addition to this salt requirement, BAM2 displays sigmoidal kinetics, with a Hill Coefficient greater than three (Monroe et al., 2017). Currently, it is hypothesized that the allostery observed in the kinetics of BAM2 is due to a secondary binding site (SBS) for starch (Monroe et al., 2017). This SBS is thought to bind non-catalytically to starch affecting the conformation of the enzyme and turning on the active site, making starch a homotropic effector.

To test this hypothesis, two conserved glycine residues in the SBS were changed in order to prevent substrate binding by introducing methionines with bulky, uncharged side chains. These mutations decreased BAM2's activity by up to 95% (Monroe et al., 2017). Finally, using Multi-Angle Light Scattering we discovered that BAM2 functions as a tetramer and is the only known *Arabidopsis* β-amylase to do so (Monroe et al., 2017).

Knowing that BAM2 functions as a tetramer, but without any resolved crystal structure for BAM2, Dr. Chris Berndsen (JMU Dept. of Chemistry and Biochemistry) created a hypothesized tetramer model. To do this, he created a monomeric BAM2 homology model using I-TASSER and a resolved crystal structure from a soybean β-amylase5. Then, using YASARA, he placed four monomers in the same orientations as the four subunits in a previously crystallized sweet potato β-amylase. Although this sweet potato BAM is a monomer in its native state, it crystallized as a tetramer (Cheong et al., 1995). Dr. Berndsen then allowed the model to find the most stable conformation on YASARA (Figure 2). Using this hypothesized tetramer model, mutations were made in each of the two resulting interfaces to test the validity of the model. When mutations were made in the hypothesized interface A, which binds two monomers together, the resulting protein was monomeric and inactive (Pope, 2017). When mutations were made in the hypothesized interface B, which binds two dimers together, the resulting protein dimer had similar activity to the wild-type enzyme (Pope, 2017). Using these biochemical data as a guide, the homology model was further modified to better explain the results from these

mutations. Because the biochemical data supported the tetramer model, we used this model to help formulate additional hypotheses about the structure of BAM2.

This study focused on the salt requirement of BAM2. We identified an N-terminal domain containing a higher percentage of acidic residues in comparison to the catalytic domain of the enzyme, which we hypothesized might be involved in the sensitivity of BAM2 to salt. This acidic N-terminal domain was only found in BAM2 and not in the other *Arabidopsis* β-amylases. To determine the role of this domain, we created mutant BAM2 enzymes lacking this acidic N-terminus. By observing the activity of these mutant enzymes under varying conditions in comparison to the wild-type BAM2, we were able to observe possible interactions of the acidic domain with salt.

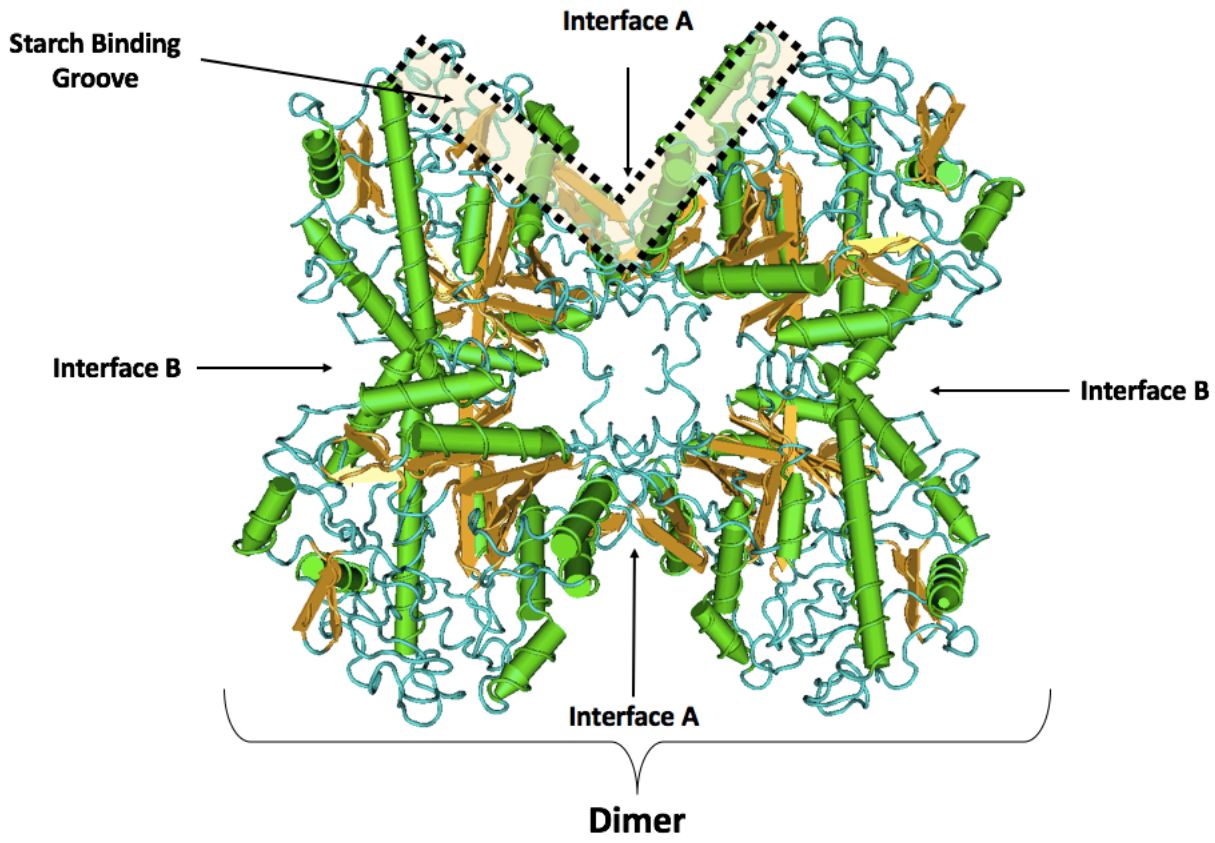

Figure 2. A tetramer model of BAM2 from Arabidopsis. This structure shows two interfaces that might play a role in tetramerization. Interface A binds two monomers together to form a dimer, and interface B binds two of those dimers together (Based on a figure from Pope, 2018).

Materials and Methods

Production and Purification of Recombinant Proteins

According to Monroe et al. (2017), which described the cloning of the BAM2 gene, a cDNA clone of the BAM2 gene was isolated from mRNA from an *Arabidopsis* plant. The primers used to isolate the gene in a polymerase chain reaction (PCR) were 5'-GGGAATTCAT-GGCGATTAGGTTGAATCATAGTG-3' and 5'-ATGGATCCATCTCGGGGTTGGTCTCTT-GTGT-3'. The product of this PCR was then cloned into pMOSBlue (GE Life Sciences) and subsequently digested using *Bam*HI and *Sal*I. The resulting DNA was then ligated into a pETDuet-1 vector, which attached an N-terminal 6-His tag to the resulting protein. This plasmid was then sequenced to verify that it matched the BAM2 reference sequence NM_116273.5.

The resulting plasmid was then transformed into BL21+ *E. coli*. These cells were then grown at 37°C in a total volume of 500 mL of Luria-Bertani broth and 100 mg/mL carbenicillin until it reached an optical density of 0.5 at 600 nm. A final concentration of 1 mM isopropylthio-β-galactosidase was added in order to initiate translation of the BAM2 gene, and the flasks were shaken at 20°C overnight. After growth, the cells were centrifuged at 6,000 xg for 5 minutes, frozen at -20°C for 24 hours, and resuspended in 30 mL of buffer containing 50 mM NaH_2PO_4 pH 8, 10 mM imidazole, and 0.3 mM NaCl. This mixture was then sonicated on ice at 50 amps for bursts of 5 seconds, with 20 seconds breaks in between the bursts, repeated 18 times, and then centrifuged again at 9,072 xg for 15 minutes at 4°C. The resulting pelleted cell debris was

discarded and the protein-containing supernatant was bound to nickel-nitrilotriacetic acid agarose His-Bind resin (QIAGEN) and gently shaken for 1.5 hours at 4°C. The resulting slurry was centrifuged at 1,150 xg for 5 minutes at 4°C. The beads were then transferred to a 5mL column (Agilent) and washed with wash buffer containing 50 mM NaH_2PO_4 pH 8, 40 mM imidazole, and 0.3 mM NaCl at a volume of 20x the bead volume. Once the wash buffer had completely run through the column, the protein was eluted by adding 10x the bead volume of elution buffer containing 50 mM NaH_2PO_4 pH 8, 200 mM imidazole, and 0.3 mM NaCl. The eluate was dialyzed for a minimum of 6 hours against 20 mM MOPS pH 7.0, 0.2 M NaCl, and 0.5 mM TCEP, replenishing the buffer after 3 hours. The diazylate was then transferred into a 5 mL concentrating column (Agilent) and centrifuged at 3,500 xg for 15 minutes at 4°C, mixed, and centrifuged again at 3,000 xg for 10 minutes at 4°C or until the volume of the diazylate reached 250 μL. The concentrated protein was then transferred to a new 1.5 mL tube and centrifuged at 3,200 xg for 5 minutes and then aliquoted into new tubes in 25 μL aliquots. The Bio-Rad Assay Kit was used to determine the concentration of the resulting protein and compared to a BSA standard.

To create the first of the N-terminal deletion mutants lacking the acidic domain (B2-NDel1), we introduced a *Bam*HI site between the acidic domain and the catalytic domain. The primers used for PCR were 5'-TGATGAAGAAATTGTGCAGGATCCAGAGCGTGATT-TTGCTGGC-3' and 3'-GCCAGCAAAATCACGCTCTGGATCCTGCACAATTTCTTCATCA-5'. Then, to create the second N-terminal deletion mutant lacking both the acidic domain and the

ERDF sequence (B2-NDel2), the primers used for PCR were 5'-TCGAAGAGCGTGATTTTG-CGGATCCAGCGTGTGTTCCTGTATAT-3' and 3'-ATATACAGGAACACACGCTGGATC-CGCAAAATCACGCTCTTCGA-5'. The protocol for the PCR to isolate the entire plasmid was as follows: 95°C for 5 minutes; cycle of 95°C for 30 seconds, 60°C for 30 seconds, and 72°C for 4 minutes, repeated 20 times; followed by 72°C for 10 minutes. The resulting DNA was then digested with *Bam*HI for 4 hours at 37°C, and then *Dpn*I for 1.5 hours at 37°C. After digestion, this DNA did not contain the DNA coding for the acidic domain, and was subsequently ligated using the Rapid DNA Ligation Kit from Roche Diagnostics for a total of 5 minutes. The resulting plasmid was transformed into DH5α E. coli cells, grown overnight, and miniprepped to isolate the DNA. The DNA was sequenced to ensure the BAM2 gene was cut as desired, and the plasmid was transformed into BL21+ cells and the proteins were expressed and purified as per the protocol used for wild-type BAM2.

Enzyme Activity Assays

Purified BAM enzymes were assayed for activity using the Somogyi-Nelson method of measuring reducing sugars (Nelson, 1944). First, the enzyme was diluted in buffer containing 50 mM MOPS pH, 7.0, and 1 mg/mL BSA or porcine gelatin. The total reaction volume was 0.5 mL, which contained 50 mM MES, pH 6.0, variable concentrations of KCl and either 80 mg/mL or 100 mg/mL soluble starch (Arcos Organics). Reactions proceeded at 25°C for 20

minutes and were stopped by placing reactions in boiling water for 3 minutes. Each reaction product was then diluted to 2.5 mL with diH_2O and vortexed. From that, 0.5 mL was transferred to a new, clean tube. The reducing sugar content of this 0.5 mL diluted product was analyzed as per the instructions in Nelson (1944). The final product was then pipetted into a well in a 96-well plate and read using a spectrophotometer at a wavelength of 660 nm. The optical density of each of the samples was then compared to maltose standards in order to determine how many moles of maltose were produced in each sample. The corresponding V_{max}, $K_{M,}$ and Hill Coefficients were generated using Microsoft Excel Solver (Gadagkar and Call, 2015).

SEC-MALS

The molecular weight of the proteins was determined using Size Exclusion Chromatography Multi Angle Light Scattering using an HPLC (High Performance Liquid Chromatography). Purified proteins were diluted to approximately 1.5 mg/mL and the molecular weights and extinction coefficients were calculated using ExPASy (https://web.expasy.org/protparam/). To prepare the column, a 10% methanol solution that was sterilized with a 0.2 μm filter was run through the 4.6 x 300 mm column for 12 hours at 0.1 mL/min. The column had a pore size of 300 Å and a particle size of 5 μm. Then, diH_2O was run through the column for another 12 hours at 0.1 mL/min. Then, we ran our buffer containing 10 mM MOPS pH 7.0, and either 250 mM or 10 mM KCl, depending on the experiment. This

buffer was first run at 0.1 mL/min for the first 12 hours, and finally at 0.5 mL/min for the next 3 hours. A total of 20 μL of sample was injected a minimum of three times per sample. The absorbance was measured at a wavelength of 280 nm and 212 nm with an Agilent G1315B Diode Array detector. To collect the samples, we used miniDAWN-TREOS (Wyatt 571 Technologies) and analyzed them with ASTRA version 6.1.5.22.

Results

To gain further insight into why BAM2 requires KCl to function, we aligned sequences from 24 BAM2 orthologs in other land plants (Figure 3). Each sequence contained a chloroplast transit peptide, which was not conserved, and a catalytic domain, which showed areas of high conservation. However, unlike other BAMs, the 30 residues between the chloroplast transit peptide and the catalytic domain appeared to contain a high percentage of acidic residues (Figure 3). While the positions of these residues are not conserved, the high percentage of acidic residues of the acidic domain in comparison to the catalytic domain is conserved among BAM2 orthologs from all of the major groups of land plants dating back to green alga (*Klebsormidium)*, which is the likely the last universal common ancestor of all land plants (Figure 3). Additionally, right after this observed "acidic domain", a highly conserved four-residue peptide is found, containing a glutamic acid, arginine, aspartic acid, and a phenylalanine. This peptide is not found in other catalytically active BAMs (Monroe, unpublished). We refer to this sequence as the "ERDF" sequence. In the homology model, this acidic domain and ERDF sequence can be seen in interface A of the tetrameric model (Figure 4).

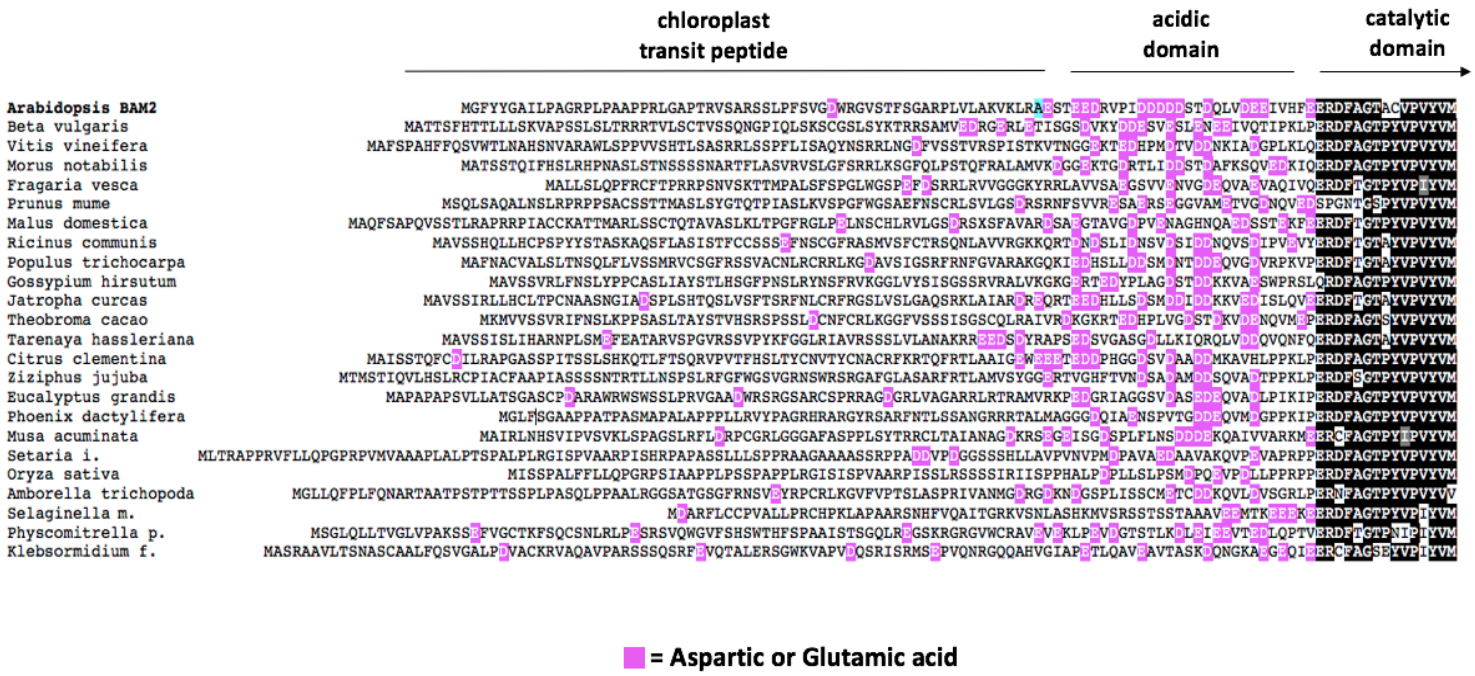

Figure 3. **N-terminal region of a sequence alignment of 24 BAM2 orthologs from different land plants.** The chloroplast transit peptide is the most N-terminal, followed by the hypothesized acidic domain, and then the catalytic domain (from left to right). The ERDF peptide is found C-terminal to the acidic domain and is the first part of the conserved catalytic domain, highlighted in black with white writing.

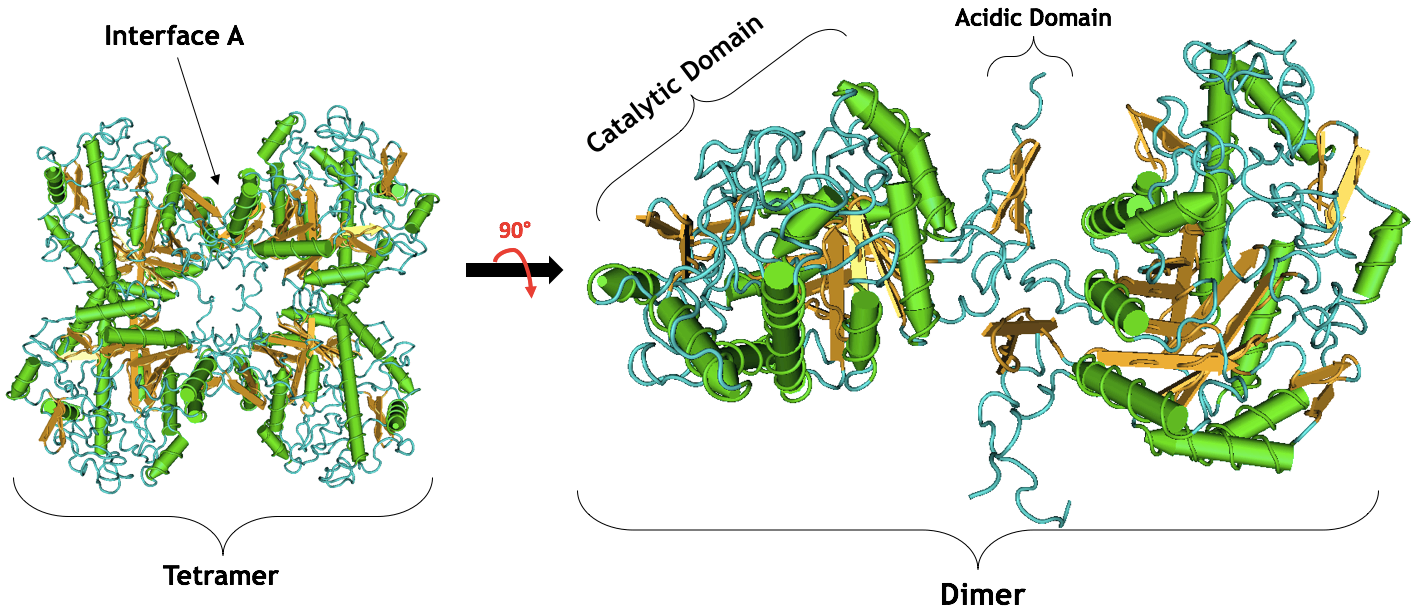

Figure 4. A tetramer model (left) and a dimer model (right) depicting the hypothesized structure of BAM2 and the potential acidic domain. The tetramer model was rotated 90° on the x-axis out of the paper in order to get to the dimer model depicted on the right. The observer is looking down into interface A and the starch-binding groove. At the bottom of this starch-binding groove are the flexible acidic domains of each monomer.

To quantify the percentage of acidic and basic residues in the target peptide, the acidic domain, and the catalytic domain, we aligned 14 BAM2 proteins from other eudicots (Figure 5). Because each protein had a different length transit peptide, we considered the 30 residues upstream from the catalytic domain as the acidic domain. While each domain has a similar proportion of basic residues, the acidic domain has about 2-fold more acidic residues than the catalytic domain, and about 10-fold more acidic residues than the chloroplast transit peptide.

The pH of the chloroplast stroma is approximately 8 during the day resulting from protons being pumped into the thylakoid lumen, and then drops to about 7 during the night (Werdan et al., 1975). Therefore, it is likely that most of these acidic residues, which have a pKa of approximately 4, will be negatively charged. To test this, we estimated the pKa's of each acidic residue from the model and found that none had an unusually high pKa near the N-terminus (cospi.iiserpune.ac.in/depth/htdocs/index.html). Under this assumption, we hypothesized that the negatively charged residues in the acidic domain might interact with the positively charged K^+ from the salt. Interestingly, on the tetramer model this acidic domain is located near the starch-binding groove. Therefore, it was hypothesized that this seemingly flexible acidic domain might be interfering with starch binding, preventing BAM2 from being activated when lacking salt. By this logic, when salt is added, the positively charged cation could bind to the acidic domain, thus allowing starch to bind to the starch-binding groove. To test this hypothesis we created two mutant BAM2 enzymes lacking the acidic domain. To do this, we inserted a *BamHI* restriction enzyme site between the acidic domain and the catalytic domain,

just before the perfectly conserved ERDF sequence (Figure 6). Additionally, we created a mutant enzyme containing a *BamHI* site directly after the same ERDF sequence to determine if this highly conserved and highly negative peptide played an additional role in activation. Once those sites were confirmed in each mutant, we subjected the respective plasmid to *BamHI* digestion to create mutants lacking the acidic domain, and both the acidic domain and the perfectly conserved ERDF sequence. These two mutant BAM2 enzymes are referred to as B2-NDel1 and B2-NDel2, respectively.

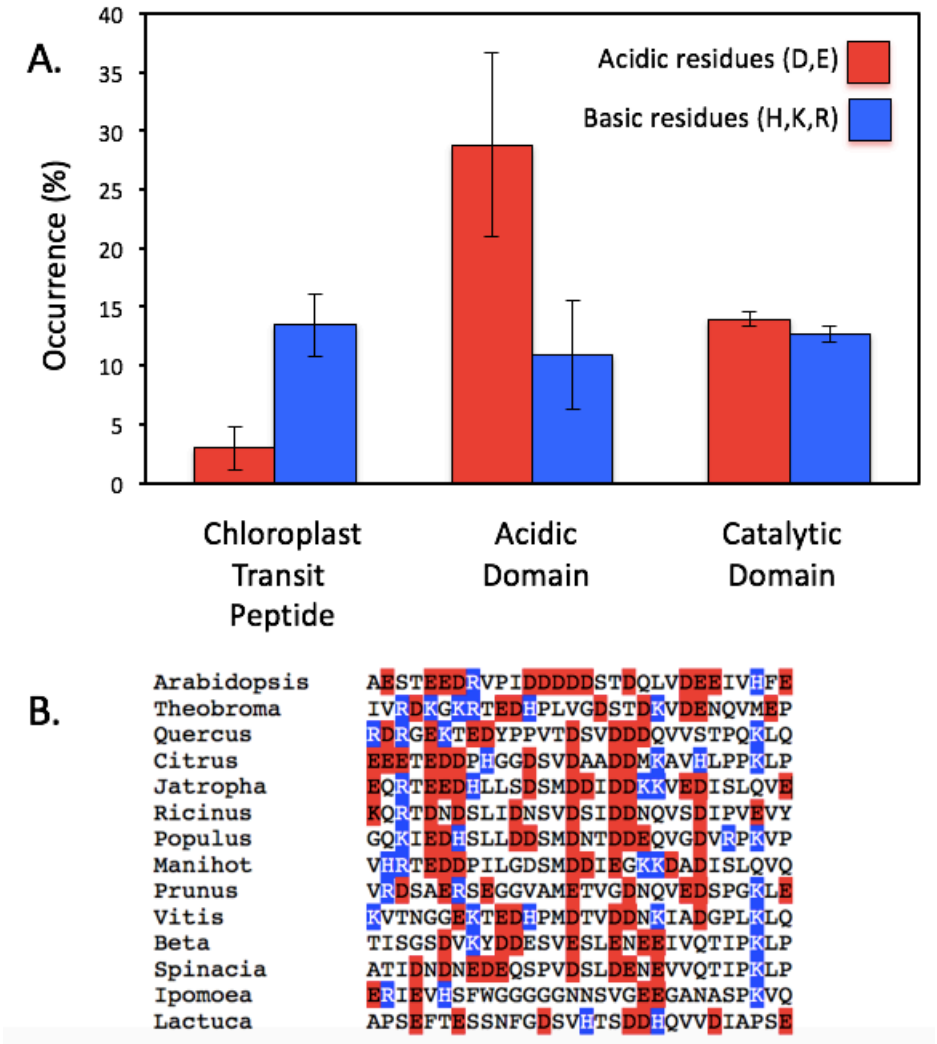

Figure 5. **Analysis of acidic and basic residues in BAM2 orthologs from 14 eudicots**. **A.** Proportion of acidic (D, E) and basic (H, K, R) residues in the chloroplast transit peptides, acidic domains and catalytic domains, means +/- standard deviation, n=14. **B.** Charged residues (acidic=red, basic=blue) in the acidic domains only. These sequences were not aligned. Species used for both A and B were *Arabidopsis thaliana*, *Beta vulgaris*, *Citrus clementina*, *Ipomoea nil*, *Jatropha curcas*, *Lactuca sativa*, *Manihot esculenta*, *Populus trichocarpa*, *Prunus persica*, *Quercus suber*, *Ricinus communis*, *Spinacia oleracea*, *Theobroma cacao*, and *Vitis vinifera*.

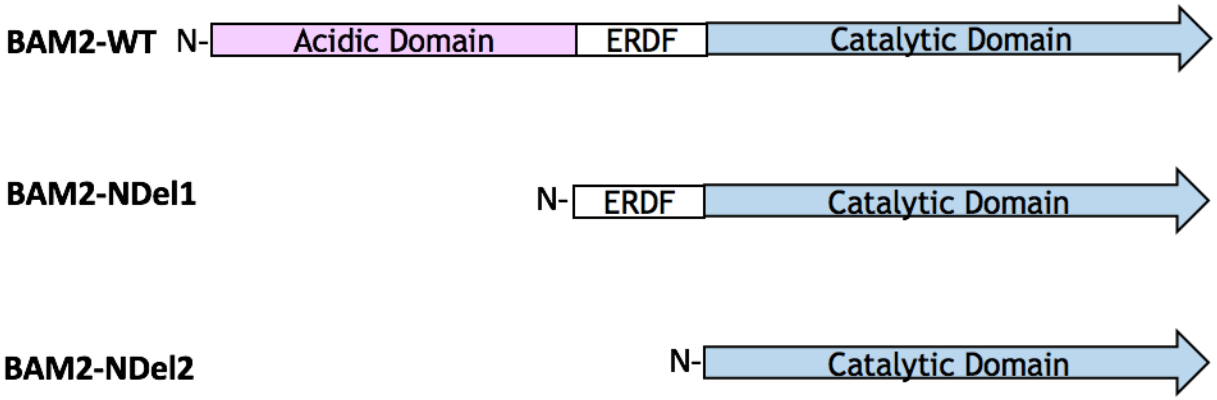

Figure 6. A diagram showing the domains of each enzyme used to analyze the dependence of BAM2 activity on salt. Wild type BAM2 contains the acidic domain, the conserved ERDF sequence, and the catalytic domain. B2-NDel1 contains the conserved ERDF sequence and the catalytic domain, and B2-NDel2 contains only the catalytic domain.

To purify these enzymes, each mutated gene generated from the *BAM2* gene isolated in a pET-DUET plasmid which contained both an upstream IPTG sensitive promoter and a carbenicillin resistance gene. Proteins were purified as described in the methods section, and analyzed using an SDS-PAGE gel to ensure each protein was at least 90% pure (Figure 7).

Once enzymes were purified, the activity of each enzyme was analyzed at varying levels of KCl using the Somogyi-Nelson method of measuring reducing sugars. First, when activity was measured at increasing concentrations of KCl, wild type BAM2 displayed a sigmoidal-like curve, while B2-NDel1 was unaffected (Figure 8). Additionally, both B2-NDel1 and B2-NDel2 were unaffected by salt when comparing activity at 5 mM KCl and 100 mM KCl (Figure 9)

Finally, to determine whether the removal of the acidic domain affected tetramerization, we used SEC-MALS to measure the molecular weight of both wild type BAM2 and B2-NDel1. BAM2 had an average molecular weight of 214.9 ± 9.1 kDaltons (n=3), and B2-NDel1 had an average molecular weight of 221.7 ± 16.3 kDaltons (n=3) (Figure 10). B2-NDel2 was not purified enough to utilize SEC-MALS.

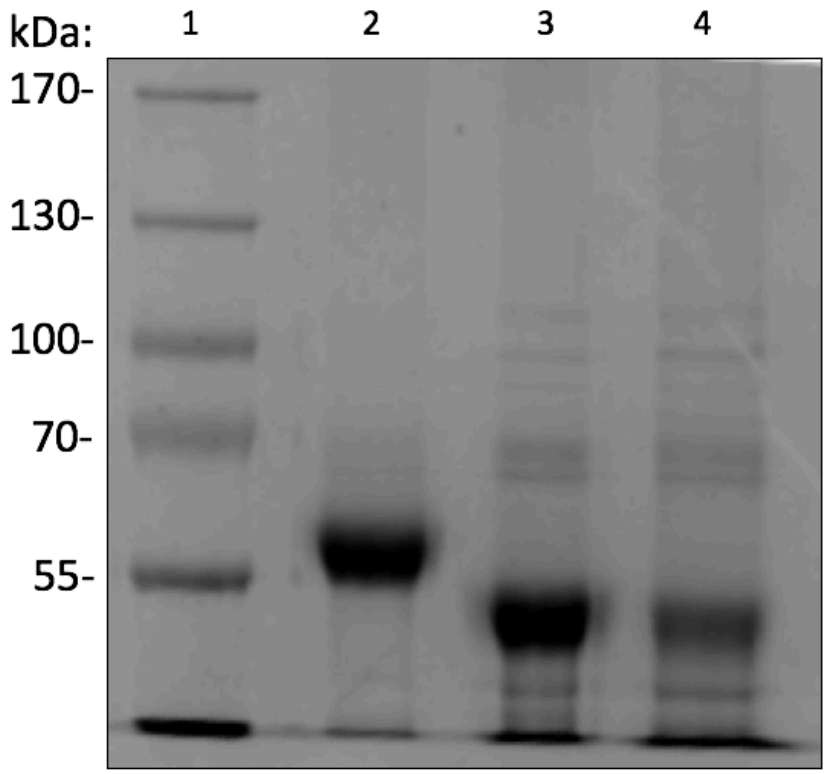

Figure 7. An SDS-PAGE gel analyzing the purity of wild type BAM2, B2-NDel1, and B2-NDel2. Lane 1 contains the marker, lane 2 contains 0.6 μg of wild-type BAM2, lane 3 contains 0.6 μg of B2-NDel1, and lane 4 contains 0.1 μg of B2-NDel2.

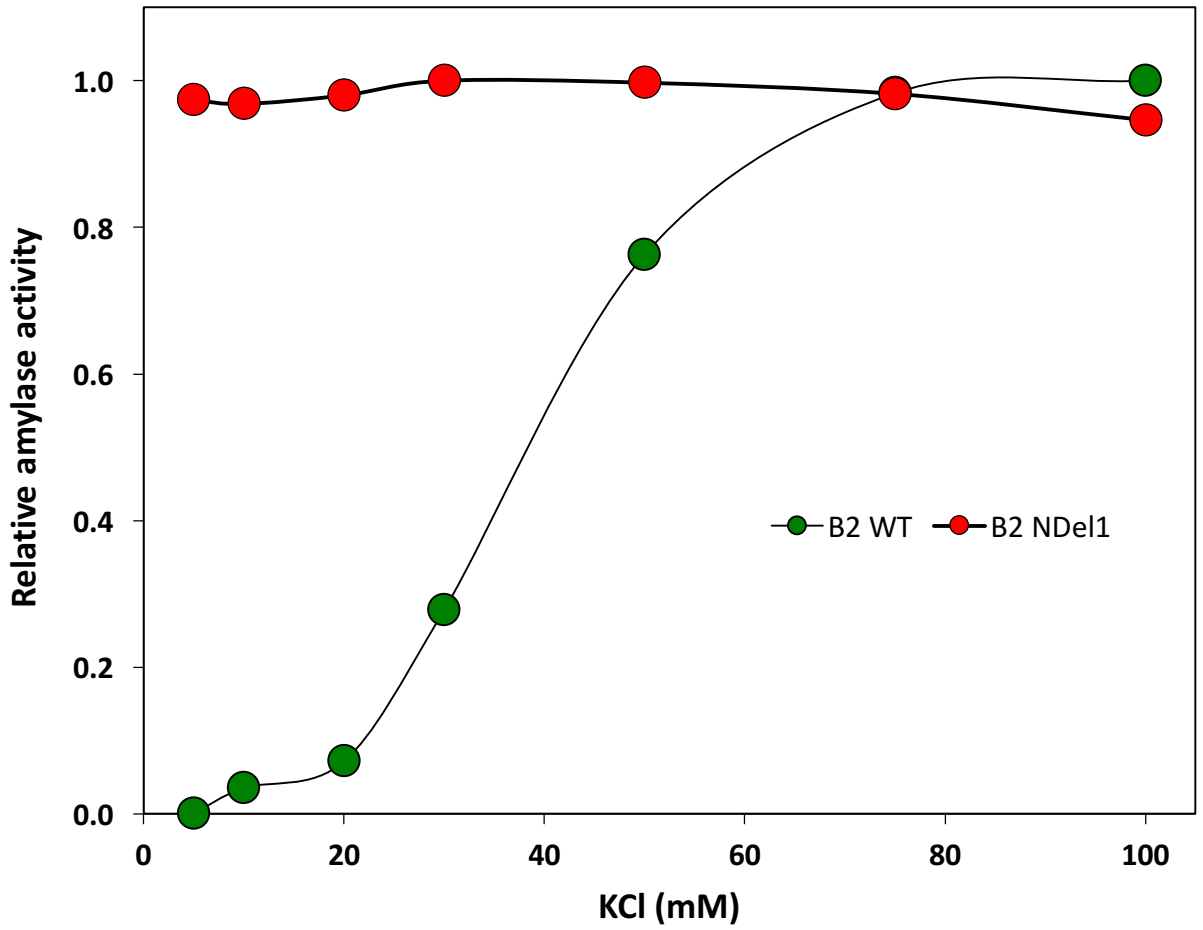

Figure 8. Activity of B2-WT and B2-NDel1 with increasing concentrations of KCl. Activity of wild-type BAM2 is shown in green and activity of B2-NDel1 is shown in red. The activities were plotted relative to the highest activity of each enzyme.

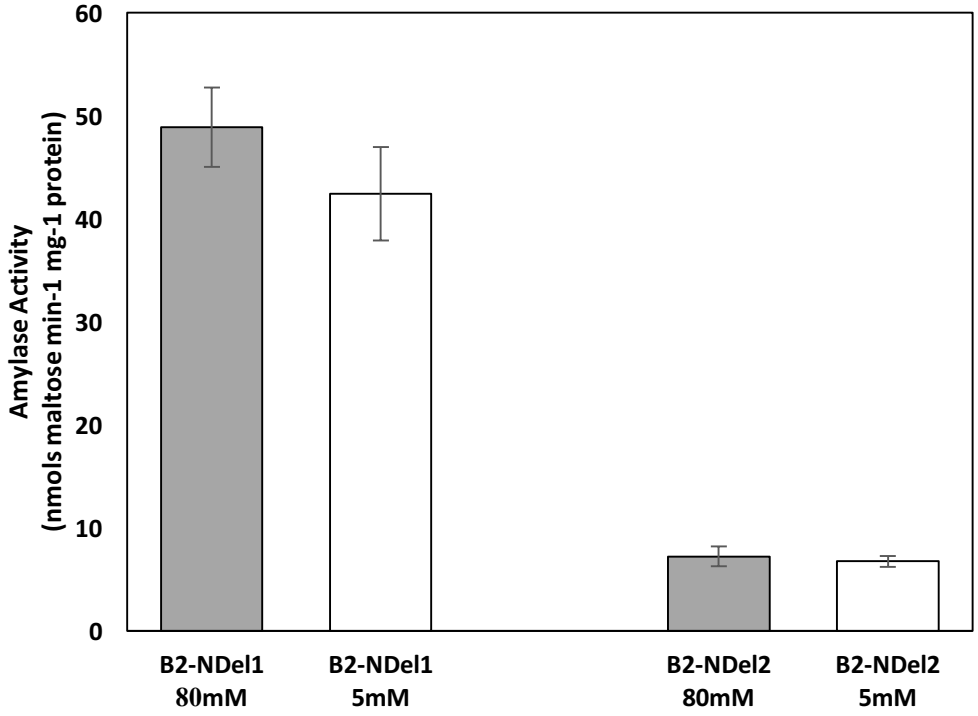

Figure 9. Activity of B2-NDel1, B2-NDel2 with high and low concentrations of KCl. 80 mM KCl was used to show activity at high KCl, and 5 mM KCl was used to show activity at low KCl. Error bars represent the standard deviation above and below the average (n=3).

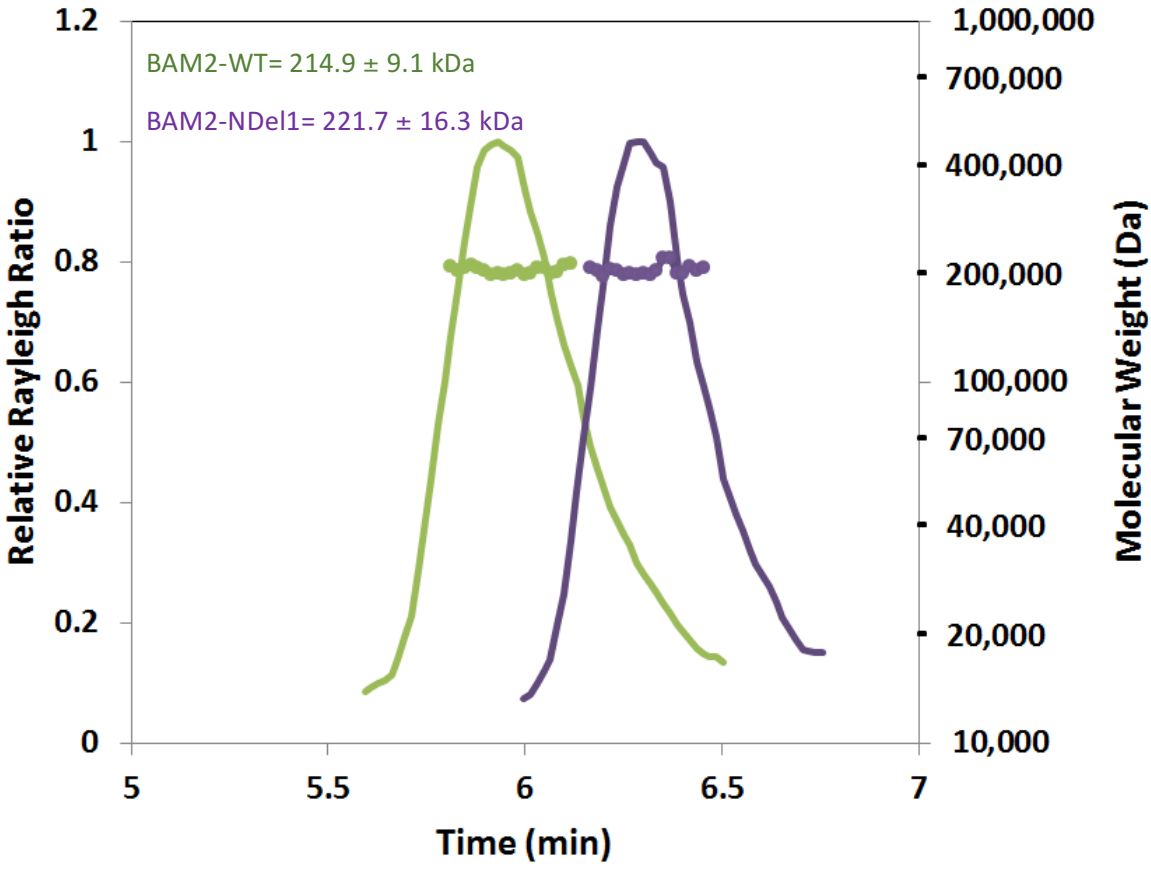

Figure 10. SEC-MALS data of BAM2-WT and BAM2-NDel1. The primary Y axis represents the relative Reyleigh ratio of light scattering, the secondary Y-axis represents the molecular weight of the protein in Daltons, and the X-axis represents the time the sample eluted from the column. BAM2-WT is in green, and BAM2-NDel1 is in purple. The averaged molecular weights of each protein is in the corresponding color in the top left of the figure.

Discussion

β-amylase2 in Arabidopsis is an evolutionarily conserved BAM that was widely believed to be inactive (Fulton et al., 2008; Li et al., 2009). Recently, BAM2 was shown to be catalytically active in the presence of high ionic strength, and is likely activated by KCl *in vivo* (Monroe et al., 2017). This insight into the requirements for activity allowed us to discover that BAM2 also displays sigmoidal kinetics due to a secondary binding site, and that BAM2 is tetrameric in its native state. While the secondary binding site and tetramerization had been previously described in Monroe et al. (2017) and Pope (2017), respectively, the salt dependence had not been further analyzed.

As previously reported in the Senior Honors Thesis of Lauren Pope, using a tetrameric model of BAM2, we predicted two specific interfaces that are responsible for tetramerization, interface A and B. Interface A binds two monomers together to create a functional dimer, and interface B binds two dimers together to create a functional tetramer. To test these interfaces, Pope (2017) mutated perfectly conserved residues in BAM2 that were not conserved in BAM5. As expected, the residues identified as a possible interface in the homology model appeared to be supported in the data obtained from the mutagenized proteins. After these data were obtained, the tetramer model was adjusted to better match the data collected. Because of this, the tetramer model was supported and was used to analyze the structure of BAM2.

Using sequence alignments of BAM2 orthologs, we found a domain on the N-terminus of the protein that was enriched in acidic residues in comparison to the catalytic domain and the chloroplast transit peptide. Additionally, a short, perfectly conserved sequence made up of a glutamic acid, arginine, aspartic acid, and phenylalanine (ERDF) was found directly adjacent to the C-terminal end of the hypothesized acidic domain that was not conserved in other active BAMs (Monroe, unpublished). Because of their close proximity to the starch-binding site, both the acidic domain and the ERDF sequence were hypothesized to interact with starch binding to the SBS and thus activation of the enzyme. Two mutants were made—one lacking the acidic domain and containing the ERDF sequence (B2-NDel1), and one lacking both the acidic domain and the ERDF sequence (B2-NDel2). Using activity assays to analyze the activity of each of the B2-NDel mutants, we found that while wild-type BAM2 requires KCl to function, both of the B2-NDel mutants have similar activity with and without KCl, indicating that the acidic domain might play a role in the salt requirement of BAM2 (Figure 9). Additionally, preliminary data using MALS shows that removing the acidic domain did not disrupt tetramerization of B2-NDel1 (Figure 10).

In the homology model of BAM2, this acidic domain has minimal secondary structure, suggesting that it might have greater flexibility than other parts of the enzyme. It is possible that this flexible, negatively charged sequence could bind to a positive area of the enzyme not yet observed by our lab. If this is the case, the addition of salt could alter the position of the acidic domain, allowing for starch to bind to the SBS leading to the activation of the enzyme. In this

scenario, if this acidic domain is then removed, the enzyme would essentially be "locked" in the same active state that it would be if salt was continuously present. Interestingly, the salt curve of wild-type BAM2 is sigmoidal. If K^+ binds to multiple negative residues on the N-terminus, and there is a specific threshold of residues that must be bound to activate the enzyme, as the concentration of salt increases, the activity of BAM2 might increase sigmoidally.

While the tetramer model is supported by the mutations created in the tetramer interfaces, homology models are often not able to predict the structure of termini due to the variability of terminus length in homologs. Therefore, the acidic domain might actually form a more complex structure that we cannot observe at this time. We are currently working on obtaining a crystal structure of BAM2, which will likely help us to form more accurate hypotheses.

Focusing specifically on B2-NDel2, which has much lower activity than both wild-type BAM2 and B2-NDel1, we can also hypothesize that the ERDF sequence plays an important role in activation of the enzyme. Due to the close proximity of the ERDF sequence to the acidic domain, and to the starch binding groove, it is not unreasonable to think that a necessary interaction could be taking place between the ERDF sequence and the starch binding groove, therefore impacting starch binding and thus the activity of the enzyme when the ERDF sequence is removed. While it is certainly possible, it is unlikely that two unique characteristics of BAM2, the salt requirement and the sigmoidal kinetics, would have impactful structures directly next to each other and have no interaction.

To further investigate the role of the acidic domain and the ERDF peptide in BAM2, it would be beneficial to obtain a resolved crystal structure of both the wild-type enzyme and the two B2-NDel mutants. Additionally, by mutating the acidic residues on the acidic domain to uncharged residues and testing the activity of this enzyme with different concentrations of salt, we can further test the hypothesis that the negatively charged residues might be interacting with salt.

In summary, the tetramer model has been supported through data obtained from several mutagenized proteins, and is therefore beneficial when hypothesizing potential structural characteristics of BAM2. Using this model and BAM2 ortholog sequence alignments, we identified two areas of interest near the N-terminus—an acidic domain and a four amino acid sequence that is highly conserved in BAM2 orthologs. From data collected using proteins lacking the acidic domain, we hypothesize that an approximately 30 residue sequence interacts with the cation of stromal salts, specifically K^+. Additionally, using a mutated BAM2 protein lacking both the acidic domain and the ERDF sequence, we have reason to believe that the ERDF sequence plays a significant role in the activation of BAM2. These data help to differentiate BAM2 as one of the more unique BAMs in *Arabidopsis*, and therefore is worthy or further investigation.

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