

Site-Directed Mutation of Switchgrass Ascorbate Peroxidase into Monomeric Form: Mutagenesis and Characterization

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Abstract

Ascorbate peroxidase, also known as APX, is an enzyme found in switchgrass. Although APX has been characterized from other plants, the switchgrass version (sgAPX) remains almost completely uncharacterized. Our goal here has been to characterize and alter the oligomeric state of sgAPX. Other APXs have been shown to function as a dimer. Here we demonstrate that sgAPX also functions as a dimer in its wild type. Also, using the structure for the pea APX, we have predicted mutation sites that appear to disrupt the dimer interface by placing like charges together. We have found that a mutation of the glutamate 112 to lysine causes a shift to the monomeric form of sgAPX.

Introduction

Switchgrass, *Panicum virgatum*, is a warm-season grass that is native to most of the United States. It is most common in the Midwest. Switchgrass grows 3-5 feet tall and seems to grow easily in even harsh conditions (USDA 2001). Switchgrass has recently been mentioned as a potential biofuel crop. This is because it grows fast and can grow in a variety of soil types. Though our research does not directly relate to using switchgrass as a biofuel source, it is still essential for scientists to understand more about the structure and the proteins of switchgrass.

APX is a protein enzyme found in the chloroplasts and cytosol of many plants. It is an enzyme that is vital for the plant. The function of APX is to detoxify hydrogen peroxide in the plants. Hydrogen peroxide is a toxin for the plant so it is important to get rid of it. Hydrogen peroxide is formed as the byproduct of many different processes including photosynthesis (Dalton 1991).

Proteins can be made up of only one subunit (monomer) or multiple subunits (oligomer). Studying the oligomeric association of proteins can help us understand how protein subunits interact. The APX wild type (WT) has two subunits that are the same (homodimer or simply dimer) with each having a catalytic center (Dalton, Hanus et al. 1987). It is not understood how or if this dimerization has any impact on the activity. In order to begin to understand this, we must be able to reliably generate the monomeric form APX. Although this has not been previously done for this protein, crystal structures of the protein give us a pretty good idea of how it might be accomplished (Patterson and Poulos 1995) First, we see from the structure of pea APX that there is a interface between monomers that can form where a number of ion pairs take place. We can take advantage of this information together with the sequence similarity of pea APX with sgAPX to predict a change that will likely disrupt this interface. Using a previously developed structural model of sgAPX based on pea APX (McCune 2006), we have selected an ion pair to mutate that is predicted to be in the center of the

interface region (Figure 1). If we can change the Glu to a Lys, replacing a negative with a positive charge, we will introduce two Arg(+)/Lys(+) pairs at the interface. We predict this will disrupt the interface and favor the monomeric form. The work described here is an effort to perform the mutation and then test the result.

Materials and Methods

Mutagenesis

From previous work we have obtained the gene for sgAPX cloned into pET28a, a 6x-His tag expression vector (Elson 2005). Using this plasmid we performed site-directed mutagenesis using mutant primers designed via the GeneTailor kit method (Invitrogen) and substitute the Glu112 codon (GAG) with a Lys codon (AAA). These primers were used together with the plasmid, pET28a.sgAPX, to perform the polymerase chain reaction (PCR). We used Platinum[®] Taq DNA Polymerase High Fidelity (Invitrogen) as is recommended for plasmids larger than 5kb and up to 8 kb. See Appendix A for full reaction recipe and thermocycle protocol.

Gel Electrophoresis

In order to verify that we have produced a full length mutated vector, we ran a sample of our mutagenesis reaction on an agarose gel.

Electrophoresis uses an electric current passed through a buffer/gel to cause a DNA sample to move towards the positive pole. After running current through the buffer for about 45 minutes the bands spread out depending on the size. By comparing our plasmid to the known markers, we can easily determine the approximate size. If our mutated DNA is the same size as the gene was before the mutation, then this is an indication that the mutagenesis has worked. When we observed plasmids that were the correct length, we transformed them into bacteria and used them to produce mutant plasmid for sequencing.

Protein Expression and Purification

We expressed WT and mutant proteins in *E. coli*, BL21 (DE3). Cells were grown in 200 mL LB cultures (500 mL flasks) for 48 hrs at 31°C with shaking at 150 rpm without induction. Cultures appeared dark red in color at end of growth. Cells were lysed with a combination of 45 min room temperature lysozyme (Sigma) (1 mg/mL final concentration) treatment, Fastbreak solution (Fisher) and sonication with a tip sonicator at full power for 15 sec blasts while on ice. Lysed cells were centrifuged at 25,000 x G for 20 minutes and cleared lysate (reddish in color) was collected for purification via Ni-NTA chromatography on a GE Healthcare AKTA Prime FPLC (fast protein liquid chromatography) system.

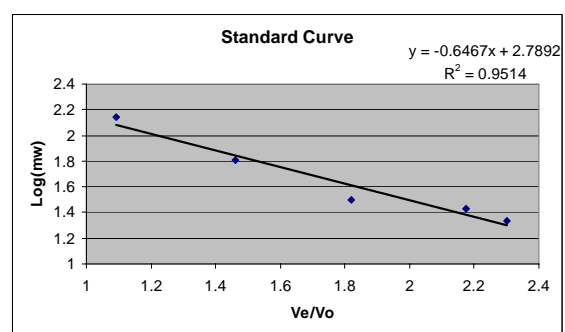


Figure 2: Molecular weight standard curve.

Table 1: List of MW standards and their sizes	
MW Standard	Size (kDa)
Blue Dextran	2,000
β-Amylase	200
Alcohol Dehydrogenase	150
Bovine Serum Albumin	66
Carbonic Anhydrase	29
Cytochrome c	12.4

Size Exclusion HPLC

We performed size exclusion high performance liquid chromatography (SE-HPLC) to determine the size of the APX using a column that separates proteins by their size. It does this by using tiny beads with microscopic holes. These holes allow molecules to be separated by size based on different amounts of interaction with the beads also called the resin. Smaller molecules can pass further into the beads and are retained longer. Larger molecules move more quickly through the resin and come off of the column sooner.

We used a HPLC system with a Waters 1525 Binary HPLC pump and a Waters 248 Dual λ Absorbance Detector. We monitored at wavelength of 280 nm with an AUFS of 0.05. Our data was collected using a Labview program that was set to take five readings per second with a voltage was set at a range of -500mV to 500mV. Our column was a 300 mm x 4.6 mm analytical SE-HPLC column (Alltech Prosphere SEC 250HR 4 μ 250A). We used a 50 mM Tris-HCl, 100 mM KCl buffer for our isocratic runs. The flow rate was 0.35 mL/min and giving a pressure around 1300 psi.

We began by running size standards (Gel Filtration Molecular Weight Markers, Sigma – Table 1) to create a standard curve (Figure 2). We created our standard curve by first determining the void volume (V_0). The void volume is the actual volume of the column and is found by running a compound too large to be slowed down by the column. Blue Dextran (MW ~ 2 million) came out of our column at about 5.18 min. We could then take our known flow rate of 0.35 mL/min and multiply them together. This told us our void volume was 1.798 mL. We then ran rest of our standards and found the elution volume (V_e) for each. We made our curve by plotting the log of the MW vs. V_e/V_0 and used the equation for the line to determine the MW for our unknowns, wild type and mutant APX. We performed multiple runs of each standard and observed very small deviations in V_e/V_0 of all less than 0.5%. Unknowns were all run under same conditions as our standards. For the unknowns we did collect some of the peaks that elute in order to concentrate and rerun them. Collected fractions were concentrated using Pierce iCon concentrator tubes (Fisher) with a 9 kDa cutoff.

Results and Discussion

Mutagenesis

We were able to demonstrate that our mutagenesis experiments worked based on DNA sequencing results. Shown in Figure 3, each base type is represented by a different colored chromatogram and all four are superimposed to deduce the sequence which is shown as A, G, C or T's above the peaks. These sequencing chromatograms show clearly that the WT sequence containing the **GAG** codon has been changed in the mutant sequence to **AAA** (Figure 3) by the absence of the Black (G) peaks in the combined chromatogram.

SE-HPLC

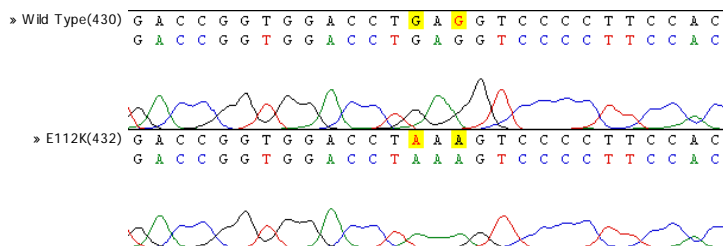


Figure 3: Comparison of WT and E112K mutant sequences. Bases that were changed are highlighted in yellow.

The results for our standard data are shown in Figure 2. Using the same conditions, we collected data on numerous runs of WT and mutant sgAPX. Shown in Figure 4 are examples of the HPLC data we obtained for these samples. It is clear from this data that the WT main peak appears to be larger than the mutant since it comes out earlier. The actual sizes for the main peaks, determined from the standard curve, are WT = 44 kDa and E112K = 28 kDa. Using the gene DNA sequence we have calculated the size of sgAPX with the His tag that comes from the plasmid to be 29.5 kDa (See Appendix B). This would indicate that the E112K APX appears to be a monomer in solution.

It is readily seen that the apparent MW of the WT APX is not two times the monomer (60 kDa). We believe that even though our dimer is not quite twice the size of our monomer that this is still indeed what we have. We believe that our size difference has more to do with the way that the dimer interacts with the column. The APX monomer has a spherical structure which is absolutely ideal for the type of column we are using. When we look at the sgAPX dimer (Figure 1) we see that it is made by placing two monomers side by side. This is not the same as simply doubling the size of a sphere (Figure 5). Two monomers interacting with each other will not have the same profile or in other words they will look smaller in certain directions than in others (Figure 5). Basically, this means that the shape of the dimer causes it not to move like a monomer of double the size. Also, sometimes proteins have interactions with the size exclusion resin that are not size related. This can also cause the size to appear different from the predicted.

Conclusion

The data that was collected supports our mutant design. We proposed that changing amino acid Glu (E) which has a negative charge to amino acid Lys (K) which has a positive charge would cause the monomer to be favored. It was shown that after this mutation the protein appears smaller based on SE-HPLC. It was not half as big but it was definitely smaller. A logical conclusion is that we have the monomer after we make this mutation.

Future research with the dimer and monomer of APX would need to be done to test the enzyme activity differences in the two. It could be tested to see if the dimer or monomer breaks down hydrogen

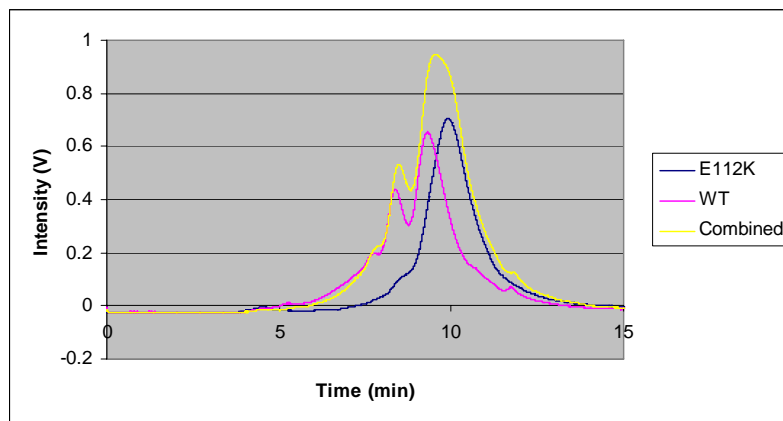


Figure 4: Chromatogram of wild type and E112K mutant. The combined chromatogram is of a mixed sample of mutant and WT.

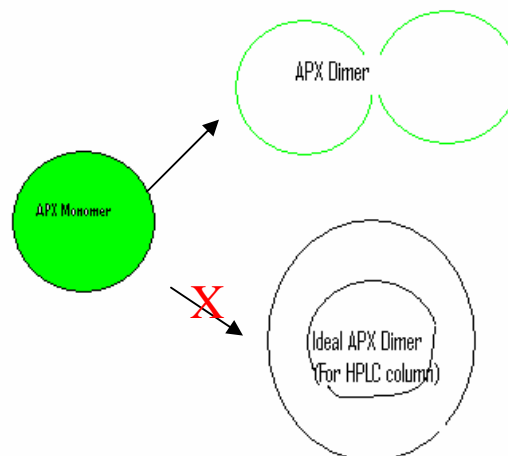


Figure 5: Model for difference smaller apparent size of sgAPX dimer.

peroxide faster or with less energy. Another thing that could be done is to see the effects of more mutations at the interface to see if a more clear cut monomer can be made that is half as big as the wild type dimer.

References

- Dalton, D. A. (1991). Ascorbate Peroxidase. Peroxidases in Chemistry and Biology. J. Everse, K. E. Everse and M. B. Grisham. Boca Raton, CRC Press. **2**: 139.
- Dalton, D. A., F. J. Hanus, et al. (1987). "Purification, Properties, and Distribution of Ascorbate Peroxidase in Legume Root Nodules." Plant Physiol. **83**: 789-794.
- Elson, J. A. a. K., F. A. (2005). "Expression of Ascorbate Peroxidase from Switchgrass." SSRP **1**: 1.
- McCune, J. M. a. K., F. A. (2006). "Site Directed Mutagenesis of Ascorbate Peroxidase in Switchgrass." Chemistry 499 Honors Project **1**: 1.
- Patterson, W. R. and T. L. Poulos (1995). "Crystal structure of recombinant pea cytosolic ascorbate peroxidase." Biochemistry **34**(13): 4331-41.
- USDA (2001). "Plant Fact Sheet: switchgrass - panicum virgatum." NRCS Plant Mater. Prog.: 1-2.

Appendix A: Mutagenesis reaction recipe and thermocycle protocol

50 uL Mutagenesis reaction

5.0 uL 10X Buffer
1.5 uL 10 mM dNTP
1.0 uL MgSO₄
1.5 uL Forward Primer
1.5 uL Reverse Primer
2.0 uL Plasmid DNA (30 ng)
0.2 uL Platinum[®] *Taq* Polymerase
37.3 uL Sterile nuclease-free ddH₂O

50.0 uL

Thermocycle Protocol

1. 94°C for 2 min. - Denature
2. 94°C for 1 min.
3. 56°C for 1 min. – Anneal primers
4. 70°C for 9 min - Elongation
5. Loop to Step 2 – 29 times
6. 70°C for 10 min - Final elongation

Appendix B – Calculated MW for Recombinant Switchgrass APX (rsgAPX)

We obtained a calculation of the MW using the DNA sequence for the APX switchgrass gene cloned into the His tagged vector from:

<http://www.scripps.edu/cgi-bin/cdputnam/protcalc3>

The following is the output from this web site:

rsgAPX with 6X His Tag

Accepted Sequence

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mgsshhhhhh  ssglvprgsh  makcypvtvsa  eyqdavekar  rklraliaek  scaplmlrla  60
whsagtdfvs  sktggpfgtm  knpaeqahga  nagldiavrm  lepvkeefpi  lsyadlyqla  120
gvvavevtgg  pevpfhpgre  dkpqpppegr  lpdatkgsdh  lrqvfgkqmg  lsdqdivals  180
gghtlgrchk  ersgfeqpw  rnplvfdnsy  fkellsgdke  gllqlpsdka  llsdpvfrpl  240
aekyaadeka  ffddykeahl  klseifgafa  270
  
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Amino Acid Count

Residue	Number Found
A Ala Alanine	27
R Arg Arginine	13
N Asn Asparagine	4
D Asp Aspartate	17
Q Gln Glutamine	8
E Glu Glutamate	19
G Gly Glycine	25
H His Histidine	14
I Ile Isoleucine	4
L Leu Leucine	28
K Lys Lysine	18
M Met Methionine	6
F Phe Phenylalanine	12

P	Pro	Proline	20
S	Ser	Serine	20
T	Thr	Threonine	8
Y	Tyr	Tyrosine	7
V	Val	Valine	15
W	Trp	Tryptophan	2
C	Cys	Cysteine	3
U	SeC	Selenocysteine	0

Isotopically Averaged Molecular Weight = 29490.2441