Characterizing Novel Peroxygenase Activity Found in Jalapeño and Habanero Seeds

LIBERTY UNIVERSITY

Anna Collins, Sally Hess, and Dr. Gregory Raner

Background and Objective

Peroxygenases are catalytic enzymes found in many plants to be active in oxidizing toxins and reactive oxygen species. Extensive research has been done to isolate and characterize these proteins for use in various industrial applications. Most often peroxidases and peroxygenases are used by textile plants to oxidize dyes in their wastewater. Research has been done to find peroxidases and peroxygenases with unique activity or with greater stability under stressful conditions like acidity or heat for use in these industrial applications.

In a previous project, a library of peroxidases was created that provided a simple characterization of 39 plant peroxidases. In completing this library, it was discovered that while jalapeño seeds do not have relatively high peroxidase activity, there is a unique activity in pepper seeds. Specifically, jalapeño and habanero pepper seeds, when reacted with fluorophenol and hydrogen peroxide, demonstrate a new product peak through a high-performance liquid chromatography method (Figure 1). Additionally, when quenched with glutathione, the new product peak disappeared on the HPLC chromatogram. The purpose of this study was to characterize the novel peroxygenase by isolating the primary reaction product through a secondary reaction with glutathione. Additionally, the optimal reaction time was determined for each peroxygenase, and long-term stability of the jalapeño enzyme was analyzed under varying hydrophobic storage conditions.

Introduction

Peroxidases and peroxygenases are ubiquitous to plant cells because they are responsible for oxidizing reactive oxygen species (ROS) that are made during cellular respiration (1). ROS and external toxins can be deadly to cells because these molecules can cause mutations in DNA and damage cellular machinery. Peroxidase from horseradish was likely the first to be discovered and given its ability to act as a biomarker and detoxifier it has been extensively used in many applications since then (2). Peroxygenases are very similar to peroxidases in that they oxidize ROS, but they are more selectively used in industrial applications, and their activity is not completely characterized.

The fluorophenol assay (Figure 2) indicates the capability of the peroxygenase to de-fluorinate a phenolic ring (3). In this assay, 4-fluorophenol is combined with hydrogen peroxide and treated with crude pepper seed extract seen to contain peroxygenase. The products of this reaction can be visualized through high performance liquid chromatography (HPLC)(3). The absorbance wavelength of the product is used to measure the amount of product in the sample. Increased absorbance indicates higher concentrations of the product. This reaction was originally used to check jalapeño seeds for peroxidase activity, but instead, it was found that the seeds had a different activity not seen in other plants tested, such as pumpkins (Figure 1). Habanero peppers were also tested to see if the peroxygenase is common to peppers. This activity was then characterized more in-depth by examining the increase in activity over reaction time, stability of activity in methanol and in other more hydrophobic storage solutions, and finally, under quenching conditions with glutathione.

Methods

- The seeds of fresh jalapeños and habaneros were crushed using a mortar and pestle. A one to two ratio of mass of seeds versus mL of extraction buffer was used. The extraction buffer was a 20 mM phosphate buffer with a 7.0 pH. The samples were stored at 4-8° C.
- To measure stability, three additional jalapeño samples were made by adding 10% glycerol to the first sample, 1% Tween 20 to the second, and 2% Tween 20 to the third. All samples were stored at 4-8° C.
- A fluorophenol assay was performed by making a stock solution of 5 mM 4-fluorophenol, 5 mM H_2O_2, and 50 mM phosphate buffer (pH 6.5). For jalapeño peroxygenase, reactions with each sample were conducted in duplicate with 490 µL of the reaction buffer, 10 µL of the extract, and 200 µL of methanol to demonstrate the stability of the enzyme. For habanero peroxygenase, reactions were conducted in duplicate with 500 µL of reaction buffer, 40 µL of extract, and 200 µL of methanol. The reactions for both pepper seeds were run at 30° C for variable reaction times, but usually 1 hour.
- Several different HPLC methods were used to highlight the specific facet of the protein by changing the polarity of the running buffer and reaction times.

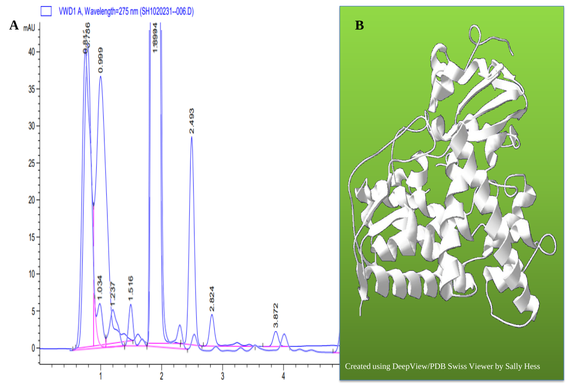

Figure 1. Novel peroxygenase activity seen in jalapeño seeds. (A) Peroxidase and peroxygenase activity in pumpkin skin and jalapeño seeds with the peak at 1.516 minutes representing peroxidase activity and the peak at 2.493 minutes representing the new peroxygenase activity. (B) The crystallographic structure of a common peroxygenase, cytochrome p450 peroxygenase. The structure of the jalapeño seed peroxygenase likely would vary from the image shown.

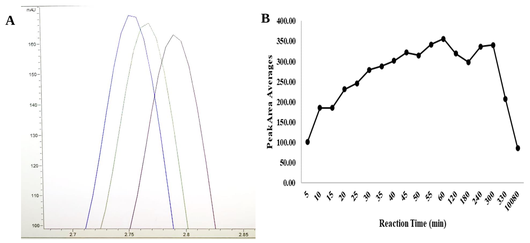

Figure 3. The optimal reaction time for habanero and jalapeño seed peroxygenase was determined. (A) Based on reaction peaks for habanero peroxygenase at 15 minutes (purple), 90 minutes (green), and 180 minutes (blue) measured at 254 nm the optimal reaction time for habanero seed peroxygenase was found to be at 180 minutes as activity after 180 minutes did not significantly increase. (B) The optimal reaction time for jalapeño peroxygenase was determined to be 60 minutes The primary reaction product appears to be unstable after 300 minutes since the product peak area decreases exponentially after that time.

Obtained from 41olive.com and crystalvalleyfoods.com

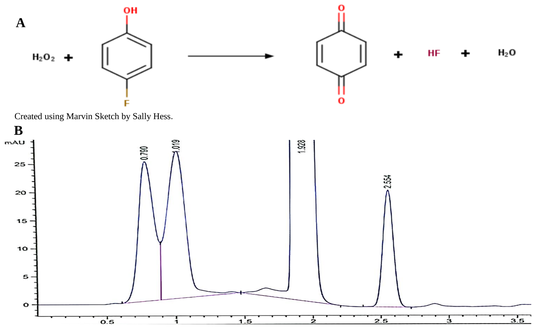

Figure 2. Simplified defluorination reaction mechanism. (A) From this simplified reaction mechanism, the substrate 4-fluorophenol and the likely product, quinone, can be seen. (B) This HPLC chromatogram of habanero peroxygenase shows the substrate peak at 1.928 minutes, and the main product peak of the peroxygenase reaction at 2.554 minutes while the main product peak of the peroxidase reaction is normally found at about 1.5 minutes. Minutes are along the x axis.

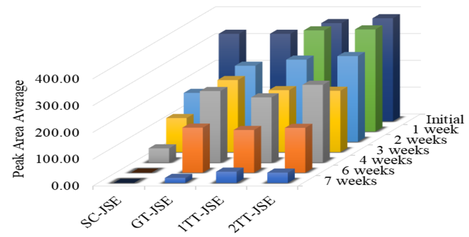

Figure 4. The long-term storage stability of jalapeño peroxygenase in hydrophobic solutions was increased. The stability of jalapeño peroxygenase was determined to be the highest when treated with 2% Tween 20. Relatively high stability was maintained in the three samples treated with glycerol (GT-JSE), 1% Tween 20 (1TT-JSE), and 2% Tween 20 (2TT-JSE) through week 4, with a gradual decrease through week 7. Untreated jalapeño peroxygenase (SC-JSE) rapidly lost stability after week 1, and completely lost activity between week 4 and week 6.

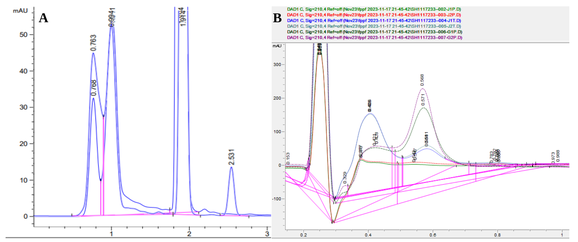

Figure 5. The destruction of the primary product by glutathione to form a more stable secondary product as seen for jalapeño peroxygenase. (A) Overlayed chromatographs of jalapeño seed extract reacted without glutathione (peak at 2.531 minutes) and reacted with glutathione (no peak at 2.5 minutes), demonstrate the secondary reaction between the primary product and glutathione. (B) Overlayed chromatographs of two controls with one sample help to visualize the hypothesized secondary reaction product. The green and red lines represent crude jalapeño extract run without glutathione added (J#P). The dark blue and teal green lines represent crude jalapeño extract run with glutathione added at the end (J#T). The dark green and burgundy lines represent reaction buffer with glutathione, but without crude jalapeño extract (G#P). The peak seen at 0.406 minutes appears to be the secondary product of the reaction between the primary product of the peroxygenase and glutathione.

Results and Discussion

Early confirmation of the new activity showed that jalapeño seed extract and pumpkin skin extract demonstrated distinct products in the HPLC chromatograph. In Figure 1, the jalapeño seeds had a product appear at 2.493 minutes that was not seen in the pumpkin skin, indicating that the product was unique to the jalapeño peroxygenase. Evidence that the jalapeño peroxygenase was in fact a peroxygenase was established through the enzyme not being denatured in 20% methanol. Additionally, the reaction completed by the peroxygenase is likely different than the one completed by peroxidase, also resulting in a new product (Figure 2A). In Figure 2B, habanero seeds were found to have a product form at 2.554 minutes signifying that habanero seeds had similar activity to jalapeño seeds.

As the next step, jalapeño and habanero seeds were tested to determine the optimal reaction length using the same reaction buffer and HPLC programming. Habanero seed peroxygenase was found to have an optimal reaction time at 180 minutes while jalapeño seeds had an optimal reaction time at about 60 minutes (Figure 3). Both peroxygenases demonstrated continued optimal activity after this time. However, it was also seen that the stability of the reaction product decreased rapidly after 300 minutes had passed for the jalapeño peroxygenase since the product peak decreased.

In addition to the stability of the product decreasing, peroxygenases stored long-term in their crude form seemed to rapidly lose activity. The long-term stability testing showed that untreated enzyme quickly deteriorated into being inactive given the lack of a product peak (Figure 4). In contrast, all three jalapeño samples treated with a hydrophobic solution to mimic the hydrophobic environment of the seed all maintained relatively high levels of activity through the fourth week. The sample that maintained the highest activity throughout the entire experiment was the sample treated with 2% Tween 20.

Since the product of the primary reaction by peroxygenase seemed to be unstable, and the reaction could not be quenched by methanol, another quenching molecule, glutathione was used. In addition to quenching the reaction, glutathione appeared to react with the primary product, since after a normal reaction quenched by glutathione, the normal product peak disappeared (Figure 5A). This disappearance could indicate a more stable product was formed as a complex of the primary product and glutathione. Since isolating this secondary product could prove useful to identifying the specific activity of the peroxygenase, Figure 5B might indicate the product peak of the secondary reaction. This product must be amplified and investigated further for confirmation of the new product. Once the product is isolated, this could give more insight into the activity of the peroxygenase.

Future Work

1. Fully characterize the activity of the peroxygenase by amplifying the signal of the new product peak.
2. Completing LC-MS on the newest product peak to find its molecular structure and further understand the characteristics of the peroxygenase.
3. Make purified jalapeño and habanero seed peroxygenase and determine possible yields.
4. Establish ideal conditions (pH, temperature, ionic strength, time) for the peroxygenase reaction and lengthen the storage-life of purified enzyme.
5. Perform crystallographic analysis of the enzyme and conduct complete kinetic characterization of the enzyme for further industrial and scientific applications.
6. Test jalapeño and habanero seed peroxygenase with dopamine solutions to examine product formation specificity.

References

1. Achar, R. R., Venkatesh, B. K., Vivek, H. K., Priya, B. S., & Swamy, S. N. (2017) *Caralluma umbellata* peroxidase: Biochemical characterization and its detoxification potentials in comparison with horseradish peroxidase. *Applied Biochemistry and Biotechnology*, 181(2), 801-812.
2. Veitch, N.C. (2004) Horseradish peroxidase: a modern view of a classic enzyme. *Phytochemistry*, 65, 249-259.
3. Buttner, E., Ullrich, R., Strittmatter, E., Piontek, K., Büttner, E., Ullrich, R., Plattner, D. A., Hofrichter, M., & Liers, C. (2015). Oxidation and nitration of mononitrophenols by a DyP-type peroxidase. *Archives of Biochemistry and Biophysics*, 574, 86-92.