

## Abstract

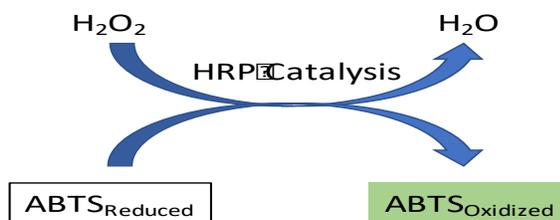
Horseradish peroxidase (HRP) is an enzyme extracted from horseradish roots that is classified as an oxidoreductase. HRP has proved to be a robust enzyme that maintains activity over an extended time, making it quite suitable for study in an undergraduate teaching lab. The overall focus of this project is to isolate HRP using multiple protein purification techniques, such as gel filtration, ion exchange and hydrophobic interaction chromatography, as a biochemistry lab experiment. Prior work found that some purification could be achieved using a hydrophobic interaction column (HIC) after an ammonium sulfate precipitation step. The project was then taken a step further and a 30 kDa cut off concentrator was used to concentrate our protein. The concentrated sample was then run onto a gel-filtration column. The fractions of active HRP fractions after being washed out of the gel-filtration column was tested for activity. Purity of the sample through this stage was evaluated using SDS-PAGE.

### Purification of Horseradish Peroxidase for Biochemistry Lab

The broad purpose of this project is to develop a reproducible ordered sequence of protein purification steps in order to isolate the peroxidase enzyme from a horseradish root. Horseradish peroxidase (HRP) has important clinical and industrial applications including: waste water treatment, reagent for organic synthesis, immunoassays, and diagnostic kits. Prior work shows the protein can be isolated from horseradish root and remain active over time. This project continued work towards developing a sequence of protein purification steps for use as an undergraduate biochemistry lab experiment. The main goal is to introduce students to various types of chromatography and other purification techniques by isolating active peroxidase enzyme from fresh horseradish root.

### Enzyme Activity

Peroxidase is an oxidoreductase that contains a heme-group. This heme-group binds to hydrogen peroxide which activates the oxidation-reduction reaction. ABTS (2,2'-azino-bis-3-ethylbenzthiazoline-6-sulphonic acid) produces a visible color change to green upon oxidation shown below (created by the research advisor, Dr. Miller.)



Using Beer's Law and the molar absorptivity of ABTS ( $3.6 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$ ), the change in absorbance at 420 nm can be used to calculate enzyme activity. Activity identifies fractions that contains HRP.

### **Extraction of Crude Sample**

The first step was extracting the crude horseradish extract. Horseradish root from a local market was peeled, diced, and blended with a phosphate buffer, pH 7.5. The blended sample was strained using a cheesecloth, then placed into a sonicator on ice to breakdown the cell walls and release HRP. Approximately 20 minutes after being sonicated, the sample was centrifuged to remove cell debris (Nguyen and Miller, 2016). The supernatant was collected.

### **Ammonium Sulfate Precipitation**

Ammonium sulfate was then be added to the supernatant. Proteins was salted out of solution at different ammonium sulfate concentrations, allowing separation of proteins in precipitate versus supernatant fractions via centrifugation. Prior work has shown that salting out with 30% ammonium sulfate should remove contaminating proteins and leave active HRP in supernatant fraction (Tulio and Miller, 2011).

### **Hydrophobic Interaction Column**

The active supernatant fraction will then be placed into a hydrophobic interaction column (HIC). Varying polarity can result in differential absorption of proteins to Phenyl Sepharose CL-4B HIC equilibrated with 50mM phosphate buffer, pH 7.5 with 1.0 M ammonium sulfate (Lavery et al., 2010). The HRP will bind to the column, and all the unbounded proteins can be washed away. The wanted, bound HRP protein can be eluted off the column by reducing the salt concentration in the buffer. High salt concentration from the ammonium sulfate step on the HRP caused it to bind effectively to the HIC. The HRP was eluted successively with buffer containing decreasing sulfate concentrations. Small fractions were collected one milliliter a time. Fractions with active HRP were identified and combined.

### **Centrifugal Concentrator**

The combined active sample was then concentrated down to a smaller volume using a concentrator. A centrifugal concentrator uses a filter to allow small molecules and solvent to be removed, while proteins larger than the specified molecular weight cut off are retained. The sample was centrifuged in a 30 kDa filter, retaining the 44 kDa HRP. This reduces the volume of active HRP and increases the concentration of HRP. This process also gets rid of the ammonium sulfate, which allows it to be evaluated by an SDS-PAGE.

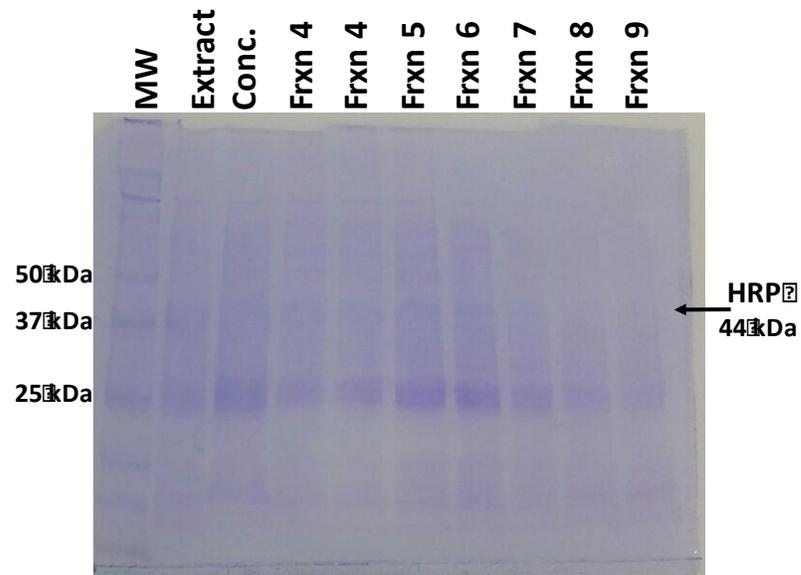
### **Gel-Filtration Column**

This reduced volume of concentrated sample was further purified using a gel-filtration column. Gel-filtration separates proteins based on size and the path each protein takes as it passes through the resin bed. Large proteins will pass quickly down the column without entering the bead interiors. Proteins in the fractionation range will partition into the beads and elute in later fractions. The concentrated, small-volume HRP was separated over a Sephadex G-100 gel filtration column. Substantial HRP activity was present in at least six fractions from the elution. This represents most of the total volume of the column as determined using DNP-glycine. A longer column or smaller protein sample is needed to improve separation using gel filtration.

HRP activity was detected in fractions 4-9 (each fraction represents 1 mL that came off the column).

### Gel Electrophoresis

The six samples were loaded using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), which separates proteins based on molecular weight. HRP is expected to be 44 kDa. The gel of this experiment is shown below.



HRP fractions from the gel filtration step were analyzed, along with the concentrated HRP sample loaded and the original extract from the horseradish root. Fractions 4 through 9 had indications of HRP activity. Bands consistent with the expected 44 kDa size for HRP were observed in each sample. The strongest HRP bands corresponded to fractions with the highest HRP activity. Fraction 5 had the darkest band, as well as the highest activity.

### Conclusion

Overall, we improved visibility of our bands in the SDS-PAGE. There are stronger bands indicating that using the concentrator to remove ammonium sulfate and increase HRP concentrations worked. We were also able to extract active HRP within these sequence purification steps. We were able to continue work from the hydrophobic interaction, as well as add improvements to it, and used a concentrator, gel-filtration, and an SDS-PAGE to successfully visualize darker bands onto the gel. Some future work is to take a look into the binding of the hydrophobic interaction column and determine if more binding is necessary and look into another purification technique like Ion Exchange chromatography, which is a technique that separates based on charge (Shannon et al., 1966).

## References

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