TCABSE-J Research Article
ISSN: 2583- 2557, *Volume 2, Issue 2, pp18-22. 2026.*

TCABSE-J
Interdisciplinary Sciences Journal

Colorimetric monitoring of protein function during the protein purification under denaturing conditions that use chaotic agents

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Keywords: Protein purification, denatured conditions, chaotic agents, protein folding, protein function and chromophore.

Reporter genes play a pivotal role in molecular biology because they allow for real-time monitoring of protein expression and cellular response. Among these, Horseradish Peroxidase (HRP) is widely used because of its high sensitivity, stability, and ease of detection. Urea is a well-known protein denaturant that was hypothesized to impact HRP activity by disrupting its structure. This study aimed to evaluate the enzymatic activity of HRP under denaturing conditions, specifically focusing on the effects of urea at various concentrations. The results show that when urea concentrations increased, enzymatic activity dropped progressively, which is confirmed by the visual colour change of samples and also by analysing the samples by employing the digital technique ImageJ. These findings indicate that urea produces structural changes in the enzyme active site, compromising its catalytic effectiveness. Further, conformational changes can be monitored by using the various tools, like circular dichroism (CD), UV-vis spectroscopy, and fluorescence spectroscopy.

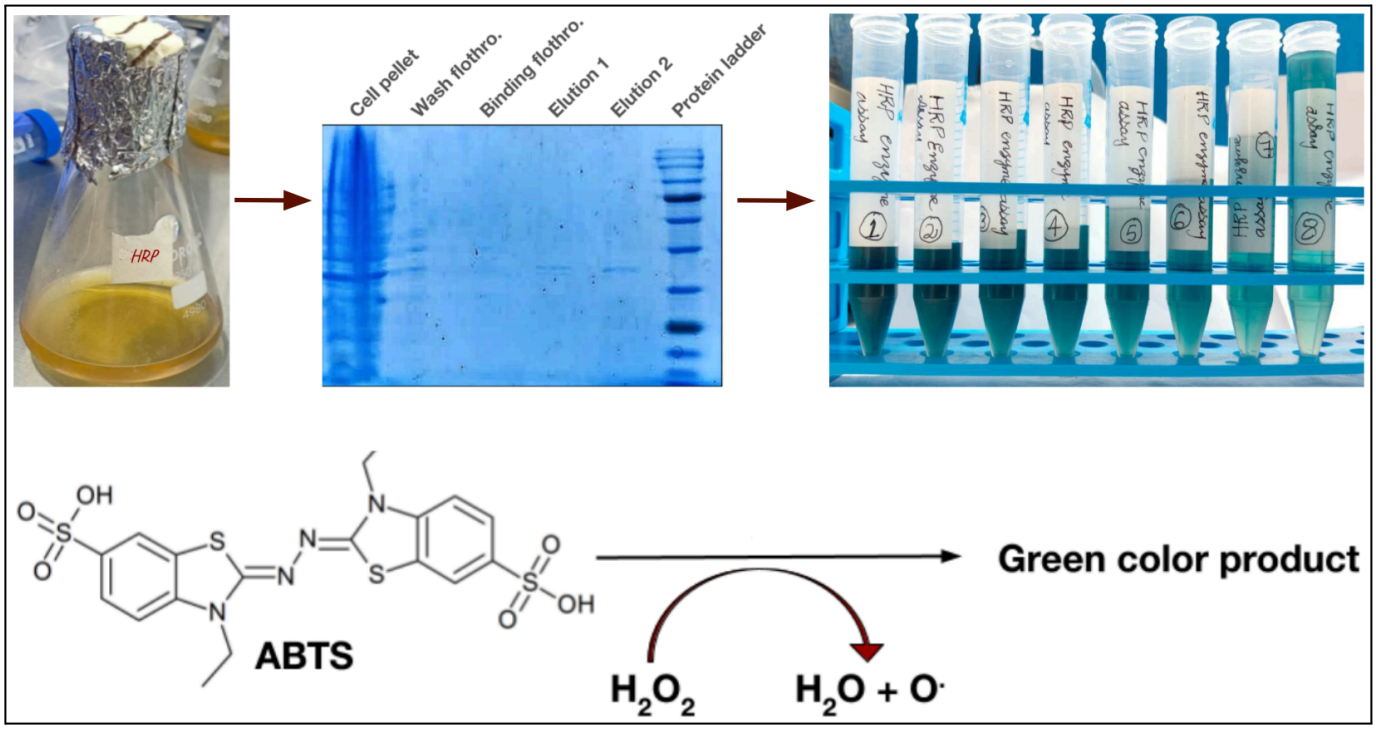


Figure 1. Overall process of expression, purification and denaturation studies of HRP.

Citation: Ruda, I., Sundaram, B., Sodasani, M. and Yedidi, R.S. (2026). Colorimetric monitoring of protein function during the protein purification under denaturing conditions that use chaotic agents. *TCABSE-J*, Vol. 2, Issue 2:18-22. Epub: October 20th, 2026.

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Reporter genes encode easily detectable proteins, allowing for visualization of gene expression, protein localization [1-6]. Among the most extensively used reporter genes are Green Fluorescent Protein (GFP) and its variants, which when activated by cell-specific promoters, enable precise labelling of various cell populations [7-9]. Other common reporter genes include LacZ gene that serves as a reliable histological signal of gene expression [10]. Reporter gene systems play an important role in neurobiological research, particularly in the visualisation of gene and protein expression within the neural tissue [11]. Moreover, reporter gene assays are becoming increasingly significant in natural product research, particularly for detecting the biological activity of herbal extracts, fractions, and purified compounds [12, 13]. Reporter genes are biological markers used either visually or quantitatively to indicate the activity of a gene, protein or cellular pathway *in vivo* [14].

Horse Radish Peroxidase (HRP), extracted mainly from the roots of the horseradish plant (*Armoracia rusticana*), is known for its ability to speed up oxidation reactions using hydrogen peroxide (H_2O_2), as an electron acceptor [15]. From detecting diseases to cleaning up toxic waste, this enzyme plays an important role in research [16]. HRP contains a heme-ring with two major domains, the proximal and the distal, flanking the heme group [17, 18]. HRP remains a vital tool in modern science, combining a well-defined catalytic mechanism with structural stability and diverse acceptability [19]. ABTS (2,2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid)) is a chemical compound used as a substrate in assays to measure antioxidant capacity and enzyme activity of HRP. When oxidized, ABTS forms a green-colored radical cation, which can be measured spectrophotometrically to assess antioxidant or enzymatic reactions (Figure 1). The ABTS assay is highly valued for its simplicity, speed, and adaptability to both lipophilic and hydrophilic samples. Antioxidants neutralize the ABTS cation, causing a decrease in the absorbance at 734 nm, and can capture both hydrogen atom transfer (HAT) and single electron transfer (SET) mechanisms [20–24].

Hydrogen peroxide (H_2O_2) is a versatile and eco-friendly molecule with strong oxidizing ability typically produces only water as a byproduct, makes it a preferred "green" alternative to traditional oxidants like tert-butyl hyper oxides, nitrous oxide, and permanganate, which often generate harmful waste [25]. Electrochemical and photochemical processes are emerging as viable alternatives to the conventional anthraquinone-based approach toward greener production of H_2O_2 [26]. H_2O_2 is used as a chlorine-free bleaching agent in many industries and also it has numerous other applications [27, 28]. Urea is a chaotropic agent that is commonly used in protein purification because of its strong denaturing capabilities, which are necessary for solubilization of aggregated or misfolded proteins [29]. Depending on the desired level of unfolding, urea concentrations can range from moderate (1-4 M) to strong (5-8 M), allowing versatility for various experiments [30]. Its reversible effect on protein folding makes it an effective tool for investigating protein structure and dynamics because of its low cost. Urea remains an important reagent in protein research and biotechnology [31].

Typically, most of the recombinant proteins are expressed in bacterial systems for the ease of simplicity. Purification of such recombinant proteins may be done under denaturing conditions using chaotropic agents such as urea. In this study we evaluated the effect of urea concentration on HRP enzyme activity, using ABTS and H_2O_2 in order to monitor the protein function during its purification under denaturing conditions using urea as the chaotropic agent.

Materials & Methods:

Bacterial expression of HRP gene: The *E. coli* (BL21-DE3) bacterial cells were transformed with a plasmid containing HRP gene and ampicillin selection marker using the standard heat-shock method. Transformants were plated on LB agar plates containing 50 µg/ml ampicillin. The plate was incubated overnight at 37 ℃. A loop full of the colonies obtained from the overnight incubation were used to inoculate 10 ml of autoclaved LB broth containing 50 µg/ml ampicillin. The inoculated broth was then incubated overnight at 37 ℃. The next day, IPTG was added to the culture to a final concentration of 0.1 mM in order to induce the HRP gene expression. Cells were harvested by centrifugation after 3 hours of IPTG-induction.

Affinity chromatographic purification of HRP: HRP purification was performed using Concavalin A agarose beads. Five hundred µl of these beads were taken into a gravity-based column and were equilibrated with 5 ml of sodium acetate buffer followed by the addition of crude HRP extract (900 µl) prepared by lysing the *E. coli* (BL21-DE3) pellet. The column was washed with 4 ml of sodium acetate buffer followed by the elution of HRP into 1 ml of the elution buffer.

SDS-PAGE analysis of HRP: A 12% sodium dodecyl sulfate polyacrylamide separating gel was prepared using 30% Acrylamide-bis acrylamide stock solution in Tris-SDS Buffer (pH 8.8) supplemented with 10% ammonium persulfate and TEMED. A 5% Stacking gel was used and the gel was loaded with protein samples from various fractions collected during the chromatographic purification of HRP. Five µL of prestained protein ladder and 10 µL of the HRP samples were loaded onto SDS-PAGE. SDS-PAGE was run using the Tris-Glycine-SDS buffer at 120 volts, 90 mA for 1 hour. The gel was then stained in Coomassie blue and was destained before taking the picture.

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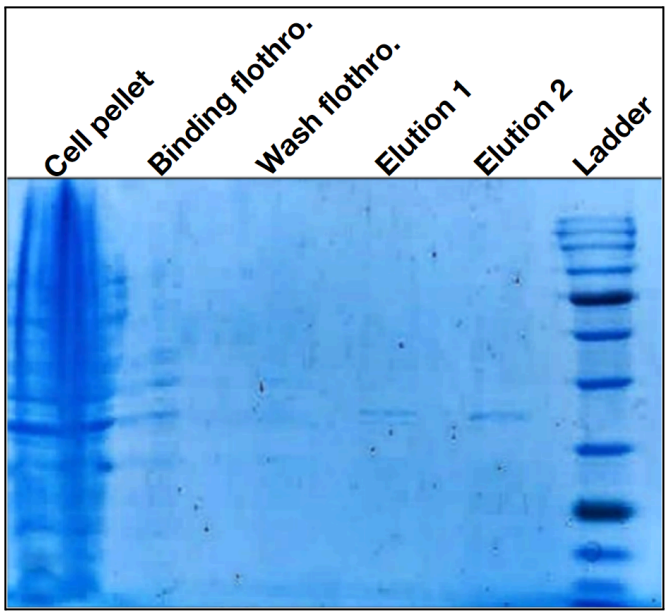


Figure 2. Qualitative analysis of HRP purification using SDS-PAGE. Five fractions were collected during the affinity chromatographic purification of the recombinant HRP. A clean and clear single band of HRP is seen in both elutions.

Colorimetric assay of HRP function: A substrate mixture was prepared by the addition of 25 ml of ABTS and 5 µL of hydrogen peroxide. Three mL of substrate mixture was added into each of the eight test tubes, which were used for visualizing the color change. Three µL of HRP enzyme was added to all tubes, followed by the addition of urea in each of the test tubes with different concentrations. Color changes were monitored before and after 1 hour incubation at 37 ℃.

ImageJ analysis of the pictures: Each image from the eight tubes were analyzed using the ImageJ software. The pixel counts in each tube were quantified and compared to evaluate any differences in the color intensity with varying urea concentrations. A control tube without urea was taken as a reference for the full enzyme activity of HRP. The final results were tabulated and were pictorially depicted as a bar graph.

Results:

Qualitative analysis revealed good quality of rec.HRP: The qualitative analysis of fractions that were collected during the affinity chromatographic purification of the recombinant HRP using SDS-PAGE revealed highly pure protein in both the elution fractions. No protein bands were seen either in the binding flow through or the wash flow through fractions suggesting that the total loaded amount of recombinant HRP was bound to the beads within the column with high affinity. A corresponding thick band of recombinant HRP is also seen among the other bands in the cell lysate (leftmost lane in Figure 2) suggesting the overexpression of the protein induced by the IPTG. Both elution fractions exhibit almost 95% purity on a qualitative basis.

Incubation of rec. HRP with ABTS improved enzyme activity: The purified recombinant HRP was mixed with the ABTS and H_2O_2 followed by incubation in order to allow the enzyme-substrate interaction. As shown in Figure 3A the green color was seen intensely in all the tubes irrespective of the urea concentration before incubation. However, the green color faded with time after incubation where the recombinant HRP was denatured by the increasing concentrations of urea (Figure 3B). HRP catalyzes the oxidation of ABTS by hydrogen peroxide, producing a green colour, which indicates enzymatic activity. Tubes with higher urea concentrations showed less colour change, indicating the inhibition or denaturation of the enzyme. Tubes with lower urea concentrations showed stronger colour change, indicating the higher enzyme activity. This experiment effectively visualizes how increasing concentration of a denaturant (urea) impacts the enzymatic activity of recombinant HRP.

An 8.7-fold decrease in enzyme activity occurred at high urea concentrations: The overall analysis of the recombinant HRP enzyme activity revealed an 8.7-fold decrease in the enzyme activity with increasing amounts of urea from 1 M to 8 M. ImageJ allowed us to accurately measure colour intensity changes, providing a more objective representation of enzyme activity. As shown in Figure 3, the X- and Y-axes of a bar graph refer to the concentration of urea and pixel count, respectively. The analysis confirms our initial manual inspection-based color fading findings by quantifying the rate of colour degradation across different urea concentrations.

Discussion:

In this study the initial observations revealed that a gradual but noticeable loss of colour occurred as urea concentration was increased, suggesting that the HRP activity was compromised. This loss in colour intensity can be attributed to the unfolding of the enzyme's tertiary structure, leading to the inactivation of the active site and subsequent decrease in the oxidative capabilities. While these visual cues provide a strong indication of enzyme denaturation, they remain qualitative in nature. This effect is consistent with the known action of urea as a chaotropic agent, which destabilizes hydrogen bonds and hydrophobic interactions critical for maintaining protein folding.

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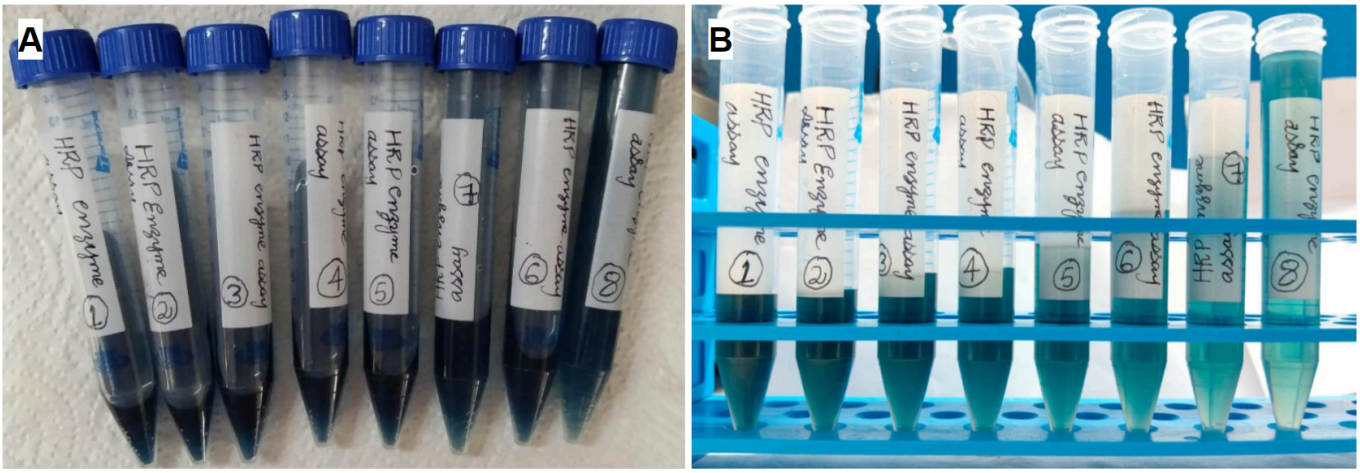


Figure 3. Colorimetric analysis of recombinant HRP activity with varying concentrations of urea. Panels A and B represent before and after incubation, respectively. The tubes are numbered from 1 to 8 representing the urea concentration 1 M to 8 M.

In order to support the quantitative evidence for our findings, imageJ software was used to quantify the colour intensity in a more accurate and uniform manner. This digital technique allowed us to generate reliable data that clearly illustrates the negative association between the urea content and the HRP activity. This work provides meaningful data about the denaturation behaviour of the HRP under the urea-induced stress and demonstrates the need to combine traditional and modern digital methods.

Conclusion and Future directions:

In conclusion, our data shows a clear and consistent decline in HRP activity as urea concentrations increased. Visually, this was observed as a steady decrease in the intensity of the colour development in the reaction mixture, a direct result of poor substrate oxidation. The imageJ analysis allowed for the precise and reproducible results of enzymatic activity. A clear correlation between the enzyme activity and the urea concentrations was established, confirming the visual patterns and emphasising the reliability of combining traditional biochemical assays with modern digital tools for data analysis. This work not only confirms the known effects of the denaturants like urea on protein functions but also contributes to a broader understanding of the enzyme stability under the denaturing conditions. To explore the scope of this work further orthogonal approaches using advanced spectroscopic techniques like circular dichroism (CD), UV-vis absorption spectroscopy and fluorescence spectroscopy are needed to directly monitor the conformational changes, offering the identification of secondary structural changes at the molecular level. Comparative studies with other denaturing agents would help to establish the broader profile of HRP stability under different types of denaturing conditions.

Acknowledgements: We thank The Yedidi Institute of Discovery and Education, Toronto for scientific collaborations.

Conflict of interest: The authors declare no conflict of interest in this study.

Author contributions: I.R. and B.S. performed all the wet lab experiments. M.S. established the protein purification protocol for HRP. B.R. co-supervised I.R. R.S.Y. is the principal investigator who designed the project, trained I.R. and B.S., secured required material for the project, provided the laboratory space and facilities needed. R.S.Y. wrote, edited and finalized the manuscript. All authors approve the final manuscript.

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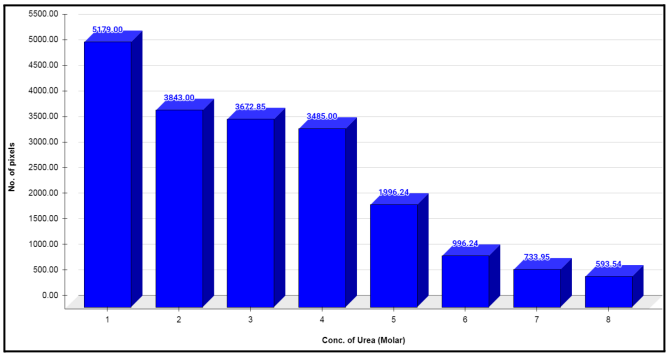


Figure 4. Quantitation of rec. HRP activity using ImageJ. The pixel count of the green showed an 8.7-fold decrease as the urea concentration rose from 1 M to 8 M due to enzyme denaturation.

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