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# The Influence of Polyethylene Glycol Precipitation Methods on Yield and Purity of White Radish Peroxidase

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## Abstract

Proteins are widely used in various industries as highly valued biotechnology products. One example is horseradish peroxidase isolated from horseradish (*Armoracia rusticana*) that used as enzyme label in immunochemistry. However, the cultivation of horseradish is limited to subtropical countries, making the dependency on horseradish peroxidase unsustainable for tropical countries. Numerous studies have explored alternative peroxidases, and white radish peroxidase isolated from *Raphanus sativus* L. has emerged as a promising candidate. In this study, white radish peroxidase is isolated using the polyethylene glycol (PEG) precipitation method which is widely used as a simple and cost-effective method. This study aims to evaluate the effectiveness of the one-step and two-step PEG precipitation method. The one-step PEG precipitation method used in this study was done by mixing the white radish juice with PEG 6000 30% (w/v), while the two-step method was done by mixing it with PEG 400 20% (w/v) and PEG 6000 30% (w/v) consecutively. This study compares the yield and recovery levels of total protein and white radish peroxidase, as well as the enzymatic specific activity of white radish peroxidase isolated both by the one-step PEG precipitation and the two-step PEG precipitation. The results indicate that both extraction methods yield the same level of white radish peroxidase. However, they differ in terms of purity. The two-step extraction method results in white radish peroxidase with higher purity, as evidenced by its specific activity towards the chromogen ABTS in the presence of $H_2O_2$.

***Keywords:*** *downstream processing, horseradish peroxidase, PEG, protein extraction, sequential extraction*

## 1. Introduction

Peroxidase is a significant biotechnology product with numerous diverse applications. The enzyme facilitates oxidative reactions or oxygen transfer between peroxides that serve as electron acceptors and the substrates that act as electron donors [1,2]. Horseradish peroxidase (HRP) is a widely used peroxidase in various sectors. It serves as an enzyme label in immunochemistry applications, such as in ELISA; as a catalyst for phenol removal in the bioremediation of wastewater; as bio bleaching and bio pulping in the paper industry, as well as decolorization of textile dyes [3–5]. Horseradish peroxidase is valued for its broad substrate specificity which allows it to oxidize an extensive range of chromogenic and chemiluminescent $H_2$ donors. It is also known for its ability to tolerate a wide range of pH and temperature, with optimum activity observed at pH 5.0-7.0 and 20-35°C [6,7]. However, the use of horseradish peroxidase may not always be a financially viable and sustainable option in certain parts of the world, such as Indonesia, because horseradish only grows in subtropical regions.

In recent years, researchers have been searching for a viable substitute for horseradish peroxidase. The alternative options include soybean peroxidase [8], red radish peroxidase [9], and white radish peroxidase [10,11]. White radish peroxidase is extracted from white radish (*Raphanus sativus* L.), which belongs to the Brassicaceae family--the same family as horseradish (*Armoracia rusticana*). It has been cultivated since 3000 BC and is now grown globally, including in tropical countries [12]. Research on peroxidase isolated from *Raphanus sativus* L. has been conducted since 1994, such as research on six isoperoxidases from Korean radish root [13], the isolation method & characterization of


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white radish peroxidase [10], immobilization of white radish peroxidase [14], and so on. Additionally, Barbosa et al. [14] found that white radish peroxidase shares about 70% of its amino acid chains with horseradish peroxidase. Given the global availability of *Raphanus sativus* L. and the structural similarity between white radish peroxidase and horseradish peroxidase, it could be used as a sustainable alternative to horseradish peroxidase.

There are several varieties of *Raphanus sativus* L. with different shapes and colors. In Indonesia, *Raphanus sativus* L. var. Hortensis Backer, also known as white radish, is cultivated as a horticultural commodity. White radish cultivation is distributed across various regions in Indonesia [15]. In 2020, the production of white radish reached 24,902 tons with an average yield of 15.96 tons/Ha. According to the Directorate of Food Corps [16], the average price for producers of white radish in 2022 was 492,975 IDR/quintal or approximately 5,000 IDR/kg. The use of white radish in Indonesia is currently limited to food consumption. Therefore, utilizing white radish for other purposes, such as in biotechnology, could increase its value.

Other researchers have developed several methods for extracting and purifying radish peroxidase that can be used as a reference, as can be seen on Table 1. Some of those techniques include the typical downstream processing which involves a multi-step cascade, beginning with protein extraction from the biological matrix, followed by clarification or extract separation from impurities, precipitation, and protein purification . Each extraction method has its advantages and disadvantages.

Ammonium sulfate precipitation is widely considered the gold standard method for protein extraction. However, it has some back draws, including low extraction rates, low yields, cumbersome operations, and purification difficulties [17–19]. On the other hand, PEG can be performed at ambient temperature, does not denature protein, and exhibits fast precipitation kinetics [20,21]. Many past studies utilized both features of ammonium sulfate and PEG in an extraction technique called aqueous two-phase system (ATPS) to extract peroxidase [22–24], and yet several back draws remain, such as high cost or optimization complexity [25]. While studies dedicated on improving extraction methods continues, looking back at conventional extraction methods might give positive points as its usually employ simpler steps and great result. For example, in 1968, PEG had been used to extracted and purified an enzyme through fractional precipitation to its crystalline state [26]. Therefore, polyethylene glycol (PEG) precipitation alone can be used as an alternative extraction method.

On past study, Apriliani [11] employed two-step PEG precipitation with overnight incubation time for each step of PEG 400 and PEG 6000 addition to white radish extract. This procedure is time-consuming, taking at least three days to obtain an impure white radish peroxidase extract. Therefore, this study presents a modified isolation procedure. The study compares the white radish peroxidase (WRP) extracted using two-step PEG precipitation using PEG 400 and PEG 6000 to the one-step extraction using only PEG 6000. The incubation time was reduced from overnight to 30-35 minutes for both methods. The reduction of incubation time was decided by considering its fast kinetic and inspired by the fractional precipitation done by Jansen et al. [26] that stirring the sample with PEG for 30 minutes only. Moreover, PEG incubation time does not significantly affect the yield [20]. This study aims to analyse the potential of modifying the established procedure for isolating white radish peroxidase to a simpler

## 2. Methodology

### *2.1 Experimental Design*

The experimental design of this study is to compare two modified precipitation procedures using PEG as the precipitant. The experiment used white radish extract obtained from a single white radish which was bought from a local grocery. Each treatment is replicated six times. The incubation time was limited to 30-35 minutes. The concentrations of total protein and enzymatic activity of the white radish peroxidase (WRP) extract were analyzed with three to five replicates for each sample. As this study focuses on comparing one-step and two-step PEG precipitation effect on WRP yield, there is

**Table 1.** Radish Peroxidase Isolation Methods

| RADISH PEROXIDASE SOURCE | EXTRACTION METHOD | PURIFICATION METHOD | REFERENCE |
|---|---|---|---|
| Cell suspension culture from white radish seed | Ammonium sulfate precipitation | Dialysis and DEAE-cellulose column chromatography | [10] |
| Red radish extract | Ultrafiltration using Amicon Cell with a membrane cut-off of PM10 | Dialysis and DEAE-cellulose column chromatography | [9] |
| White radish extract | Two-step PEG precipitation using combination of PEG 400 20% & PEG 6000 30% | None | [11] |


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no comparison between PEG precipitation method and other extraction methods.

### 2.2 White Radish Crude Extract Preparations

After washing the white radish (*Raphanus sativus* L.) with tap water, it was cut into small cubes. Next, phosphate buffer saline (PBS) was added, and the mixture was blended using a kitchen blender to obtain a smooth and homogenized white radish juice. The resulting mixture was then filtered through a cheesecloth, transferred into several Falcon tubes, and centrifuged at 10,000 rpm for 20 min at 4 °C, resulting in the separation of the white radish extract from cell debris. The supernatant was collected and used directly for the extraction of WRP . The resulting supernatant is referred to as white radish extract.

### 2.3 Preparation of samples & white radish peroxidase extraction

The white radish extract was divided into 12 Falcon tubes. Six of the tubes were used for one-step extraction samples, and the other six were used for two-step extraction samples. A small amount of white radish extract was taken from each tube for further analysis.

For the one-step extraction, approximately 30% (w/v) PEG 6000 was added to the final volume of each of the 6 tubes. The mixtures were then homogenized using a Falcon rotary machine for 30-35 minutes and centrifuged using the same procedure for white radish extract preparation. The pellet obtained from the centrifugation was then separated from the supernatant and gently rinsed with PBS to remove the remaining precipitant.

The six remaining tubes of white radish extract were used for a two-step extraction treatment. Approximately 20% (v/v) of PEG 400 was added to the final volume of white radish extract. The mixtures were homogenized and centrifuged using the same procedure as the one-step extraction. The resulting supernatants were transferred to new Falcon tubes, and a small volume of each tube was collected for further analysis. After that, approximately 30% (w/v) PEG 6000 was added to the final volume of each supernatant. The mixtures were homogenized and centrifuged again using the same procedure as before. The resulting pellets were then separated from the supernatant.

The pellets obtained from one-step and two-step extractions were resuspended with PBS consecutively for each treatment. A small amount of the pellet resuspensions from each treatment tube were collected for analysis.

### 2.4 Protein Quantification

The total protein of each treatment was measured using NanoDrop 280 nm. PBS was used as a blank. A standard curve was generated using BSA protein in the concentration range of 0.1678 - 1.678 mg/mL

### 2.5 White radish peroxidase enzymatic activity measurements

The WRP extract samples were diluted with PBS in the range of 50-100 times. Then, 10 µL of each diluted sample was added to a 96-well plate followed by the addition of 90 µL of ABTS solution containing 0.01% (v/v) $H_2O_2$. The ABTS solution used in this study is a 0.3645 mM (0.2 mg/mL) ABTS in sodium acetate buffer pH 4.5. The absorbances of the mixtures were measured every 5 minutes for 25 minutes using an ELISA plate reader spectrophotometer at a wavelength of 415 nm. The dilution factor was selected based on the linearity of the absorbance over time graph. The slope of the resulting graph was used to determine the unit peroxidase of the WRP extract.

One unit of white radish peroxidase is defined as the amount that increases the absorbance by 0.001 per minute of incubation at 415 nm. The concentration of white radish peroxidase concentration was calculated as follows:

$$Unit\ Peroxidase\ = \frac{Slope}{0.1}$$

$$WRP\ Concentration\ = \frac{Unit\ peroxidase}{Analite\ volume} \cdot\ Dilute\ factor$$

### 2.6 SDS-Page

The samples are then analyzed with SDS-PAGE using the Laemmli procedure [27]. The samples were loaded in 5% stacking gel and 10% resolving gel. The electrophoresis was run until the dye almost reached the edge of the gel.

### 2.7 WRP Lyophilization

The extracted WRP samples were lyophilized into a solid form. The resuspended samples were mixes with sucrose as lyoprotectant and PVP as wetting agent to minimize negative effects of lyophilization process. The lyophilization process was carried out for a day.

### 2.8 Specific activity comparison of WRP and HRP

An amount of lyophilized WRP was resolved in deionized water and the concentration was measured with NanoDrop using the same procedure as mentioned above. An amount of the commercially available HRP powder was resolved in deionized water in the same concentration as the resolved lyophilized WRP. The specific activities were measured using the procedure mentioned above (three replication data).

### 2.9 Statistical analysis

The protein quantification and enzymatic activity analysis data were statistically calculated. Normality, t-tests and two-way ANOVA were used to determine the data distribution and significance of each treatment. The parameters used for statistical analysis were protein recovery degree, white radish

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peroxidase recovery degree, and white radish peroxidase-specific activity.

## 3. Results and Discussion

### *3.1 Total protein recovery level*

Total protein concentrations of white radish extracts and WRP extract were measured using Nanodrop. The protein recovery level of the final products compared to white radish extract is 10.33% and 5.17%, respectively (Figure 1). The two-step extraction shows a lower protein recovery level compared to the one-step extraction which means that fewer protein contaminants co-precipitated with the protein of interest.

One of the theories behind protein precipitation is the free volume exclusion effect [28]. It is influenced by various factors such as pH, temperature, molecular weight, and concentration of PEG used. Ignoring sample environments such as pH and temperature, protein precipitation using PEG mainly depends on PEG molecular weight and concentration as it directly affects the protein partition coefficient (Kp) needed for separating the protein of interest from other substances in solution. The protein partition coefficient (Kp) in PEG precipitation is defined as the ratio of protein concentration in the supernatant to that in the precipitate. A low Kp value indicates that the protein is present in the precipitate phase rather than in the supernatant phase. PEG with a molecular weight below 1540 is efficient in precipitating proteins with a low Kp. On the other hand, PEG with a molecular weight above 1540 can precipitate proteins with higher Kp values, as it reduces more water molecules through hydrophobic interactions between the PEG chain and the hydrophobic region of proteins [24]. Therefore, PEG with lower molecular weight can remove protein contaminants with a lower Kp value while retaining proteins with a higher Kp value in the supernatant.

The addition of PEG 400 20% (v/v) before to PEG 6000 30% (w/v) eliminates protein contaminants with a Kp value lower than white radish peroxidase by precipitating it. The remaining impurities were then eliminated by the addition of PEG 6000 by precipitation of the remaining higher Kp proteins, which in this case are white radish peroxidase and a few other protein impurities with similar K values.

### *3.2 White radish peroxidase recovery level*

The white radish peroxidase was quantified in peroxidase units against the ABTS chromogen. The recovery level of white radish peroxidase from one-step extraction is 62.84%, which is slightly higher compared to two-step extraction at 56.65%. However, the difference is not statistically significant (Figure 2). Therefore, the extraction of white radish peroxidase by one-step extraction (PEG 6000 only) and two-step extraction yields the same amount of white radish peroxidase as the product.

This data shows that the addition of PEG 400 20% (v/v) to white radish extract does not necessarily precipitate or interfere with white radish peroxidase. The addition of PEG with a molecular weight higher than 1540 leads to peroxidase precipitation. According to the study conducted by Apriliani [11], white radish peroxidase started to precipitate after the addition of PEG 4000, and optimal precipitation was achieved

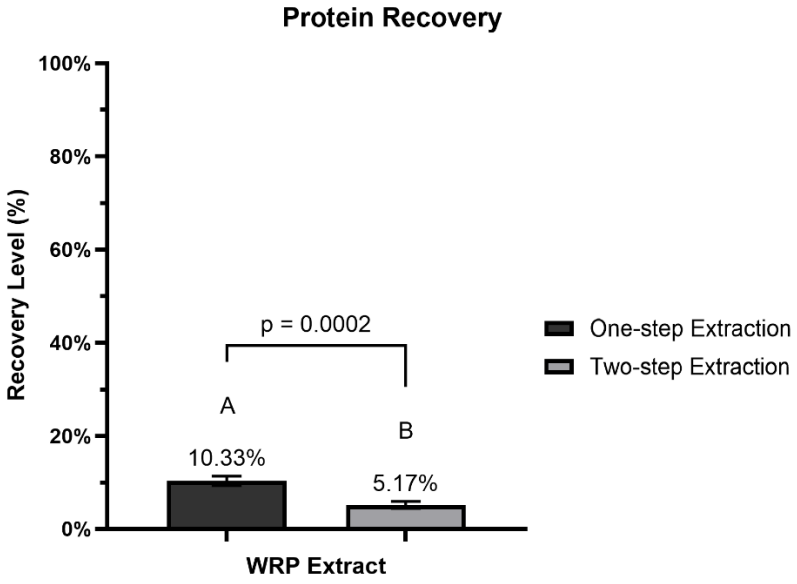


**Figure 1.** Protein recovery of one-step and two-step extraction compared to each method and its respective white radish extract. Statistical analysis using two-tailed t-test resulted p-value of 0.0002 between two experiments.


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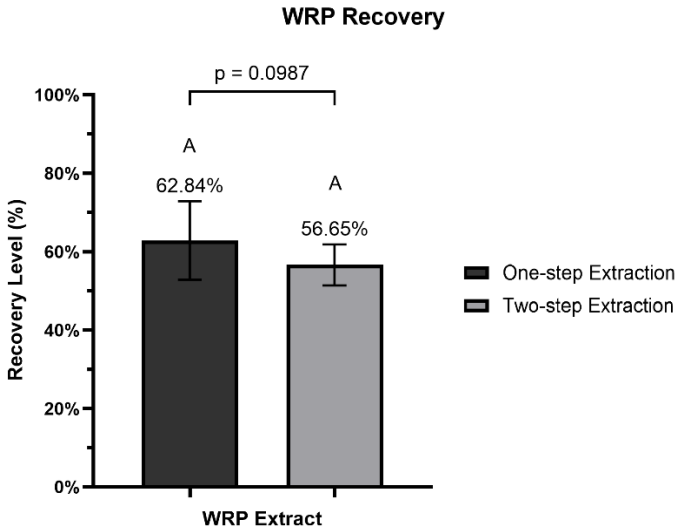


**Figure 2.** WRP recovery level in percentage compared to its initial white radish extract. Total white radish peroxidase was measured in unit peroxidase (UP), which indicates the amount of WRP capable of converting the ABTS chromogen and increasing the absorbance by 0.001 within one minute at a wavelength of 415 nm. Statistical analysis using two-tailed t-test resulted p-value of 0.0987 that indicates no differences in WRP yield between two experiments

with the addition of PEG 6000.

It is also known that PEG 6000 is commonly used to precipitate the protein of interest at different concentrations depending on its properties. Protein solubility decreases as the PEG concentration increases, resulting in protein precipitation [29,30]. As the equilibrium of protein solubility is affected, the Kp of white radish peroxidase is expected to decrease, leading to its precipitation. Although the Kp of white radish peroxidase was not measured in this study, according to Apriliani [11], 30% (w/v) PEG 6000 optimally precipitates white radish peroxidase. While 20% (w/v) PEG 6000 was able to precipitate about 41% white radish peroxidase, and PEG 6000 40% (w/v) precipitate around 66% white radish peroxidase with higher total protein as more protein contaminants co-precipitated.

### *3.3 White radish peroxidase specific activity & purity*

The specific activity of white radish peroxidase was determined by measuring peroxidase units per milligram of total protein. The average specific activity of white radish peroxidase for one-step extraction is 523.61 UP/mg protein, while for two-step extraction, it is 941.01 UP/mg protein (Figure 3). Although there is no difference in the total amount of white radish peroxidase extracted by one-step or two-step extraction, there is a significant difference in the specific activity of white radish peroxidase as shown in Figure 3. The two-step extraction yielded white radish peroxidase with a specific activity 1.8 times higher than white radish peroxidase extracted with PEG 6000 alone. As the specific activity indicates the enzyme activity per total mg of protein in solution, it has a direct linear relationship with the purity of the white radish peroxidase. The use of PEG 6000 30% (w/v) alone (one-step extraction) did not result in a higher purity product because it has higher total protein, higher protein recovery, and lower specific activity compared to the two-step extraction using PEG 400 and PEG 6000 sequentially.

Another way to measure the purity of white radish peroxidase is to compare the specific activity of white radish peroxidase before and after the extraction process. As shown in Figure 4, the one-step extraction produced white radish peroxidase with a specific activity 6.16 times higher than the crude extract, whereas the two-step extraction was approximately 11.35 times higher. These results are consistent with the study done by Hammerschmidt et al. [21] where they optimized sequential precipitation of multiple mAb with a different pH range as the impurities were eliminated based on the pI value. In their study, the sequential precipitation which includes a two-step precipitation, helps to remove impurities in the first step and precipitate the final product in the second step. It also shows that sequential precipitation with two-step precipitation produces a yield with higher purity.

In most cases, the selection of the best extraction method depends on the extraction efficiency, expressed as the yield recovery levels [31]. However, in this case, since both methods gave the same recovery degree of white radish peroxidase, the efficiency of the extraction method was measured by comparing the specific activity of white radish peroxidase per mg of recovered protein. Figure 4 shows that the two-step extraction is almost two times more efficient than the one-step extraction in terms of white radish peroxidase purity and specific activity.


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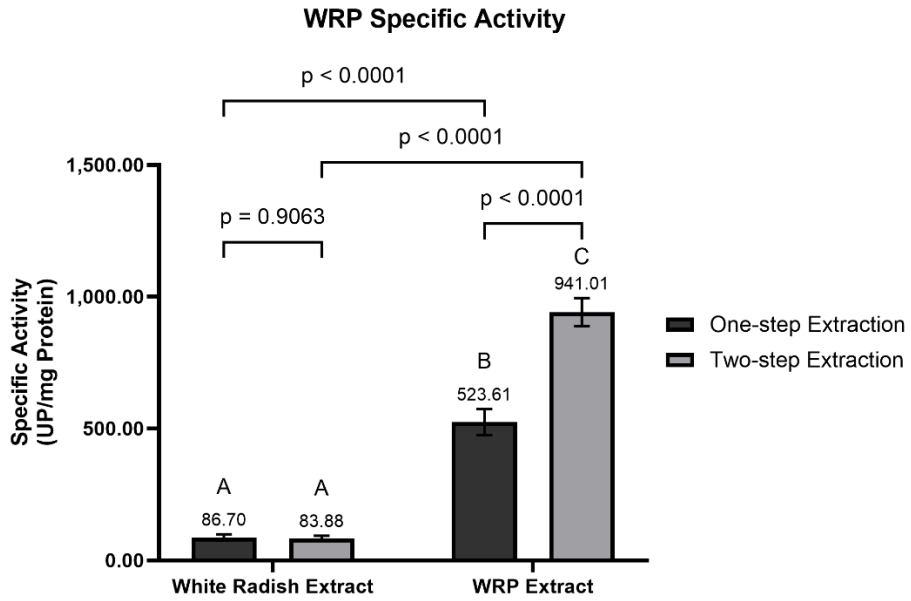


**Figure 3.** The specific activity of white radish peroxidase measured as unit peroxidase (UP) per mg of total protein. It was compared for each extraction method in both white radish extracts and WRP extract. Statistical analysis using two-way ANOVA resulted a p-value of 0.9063 for the initial specific activity of white radish extract (before enzyme extraction) that emphasized there is no difference between in starting material's specific activity value. While the comparison analysis of the specific activity of WRP extracts from one-step and two-step extraction resulted a p-value of <0.0001, indicates a significant difference between two conditions.

*3.4 Molecular confirmation of white radish peroxidase isolate*

The SDS-PAGE of WRP extract from both extraction methods shows the same amount of protein bands (Figure 5). Therefore, it indicates both samples contain the same proteins which support the white radish peroxidase recovery level regardless of the addition of PEG 400. The product solely depends on the addition of PEG 6000 which precipitates white radish peroxidase with several other protein contaminants of similar Kp values.

White radish peroxidase has several isoenzymes including the cationic and the anionic ones. Young Lee and Soo Kim [13] reported six isoenzymes with molecular weights of 31, 43, 43, 44, 45, and 50 kDa. Within all the bands that occurred on the gel, according to the migration and Rf values, the WRP extract might contain white radish peroxidase enzyme with a molecular weight indicated as 43 kDa. It can also be seen that there are many impurities in the sample. Based on band

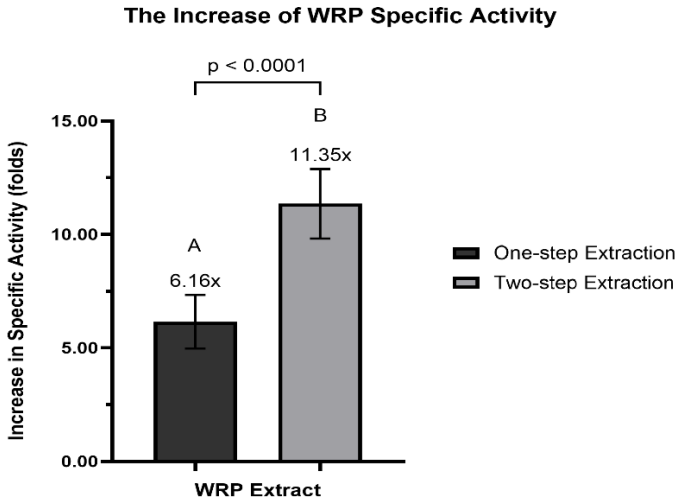


**Figure 4.** Comparison of the increase in specific activity of white radish peroxidase after extraction between one-step and two-step extraction with PEG precipitation. The increase in specific activity was measured by comparing the specific activity in WRP extract and white radish extract. Statistical analysis using two-tailed t-test results p-value of < 0.0001 that indicates significant differences between two experiments.


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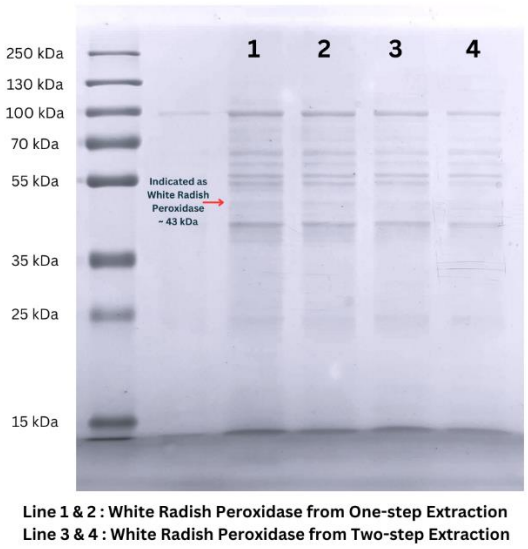


**Figure 5.** The SDS-PAGE protein band migrations of WRP from one-step and two-step extraction samples. Rows 1 & 2 represented WRP extract from one-step extraction samples, while rows 3 & 4 are from two-step extractions. Bands indicated as WRP were observed at 43 kDa.

intensity, the purity level of one-step extraction and two-step extraction respectively is 17% and 19%.

### *3.4 Comparison of WRP and HRP*

In order to evaluate the ability of WRP as an alternative to HRP, the specific activity of powder form (lyophilized) WRP and HRP was compared. The results showed that the specific activity of lyophilized WRP cannot match the commercial powder form HRP with values of 2,093.59 UP/mg protein and 1,385,714.28 UP/mg protein respectively (Figure 6). The reason for WRP low specific activity is because of its low purity level whilst the commercial HRP is in high purity level. Moreover, the extreme condition of lyophilization might also affect the WRP enzymatic activity resulting in much lower value though it is not further analyzed in this study.

## 4. Conclusion

Simplification of the white radish peroxidase extraction method using only PEG precipitation with two-step extraction using 20% (v/v) PEG 400 and 30% (w/v) PEG 6000 consecutively demonstrates its potential as an alternative downstream process for white radish peroxidase extraction. The difference between one-step and two-step extraction also shows a significant difference in the yield of white radish peroxidase purity and specific activity. Further research is needed to include other parameters such as optimal pH, temperature, homogenization time, etc.

## Conflict of Interest

The authors declare no competing interest in preparing this article. This research received no specific grant from any funding agency in the public, commercial, or non-profit sectors.

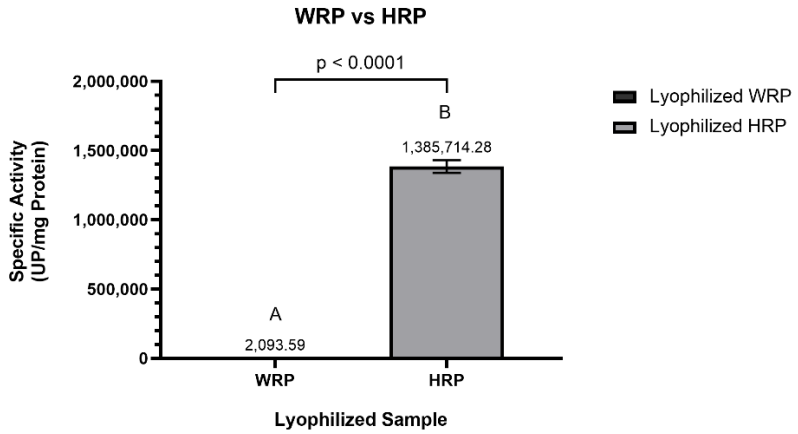


**Figure 6.** The comparison of specific activity of solid form WRP and commercial HRP. The extracted WRP was lyophilized first to turn it into solid form to match the available HRP form. Statistical analysis using two-tailed t-test results p-value of < 0.0001 indicated a significant difference between two comparisons.


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