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Research Article

Characterization of Peroxidase from Pepper (*Capsicum annuum* L. cv. Sera Demre 8) Seeds

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ABSTRACT

Peroxidases are effectively utilised in various industrial and biochemical applications for different purposes. Therefore, the purification and characterisation of this enzyme from new sources is of interest. Pepper seeds, which are an inexpensive, environmentally friendly, and readily accessible plant material, constitute a suitable source for peroxidase. In this study, peroxidase was isolated from pepper (*Capsicum annuum* L. cv. Sera Demre 8) seeds and characterised. The optimum pH and temperature of the enzyme have been calculated as 7.0 and 30 °C, respectively. The K_m and V_{max} values were calculated as 1.29 mM and 0.249 EU/mL·min for guaiacol, and 0.88 mM and 0.287 EU/mL·min for H_2O_2, respectively. The pH stability of the enzyme was assessed by incubating it at +4 °C for up to 72 hours at pH 6.0, 7.0, 8.0 and 9.0, and activity measurements were taken every 24 hours. After 72 hours, the enzyme retained 81.59%, 71.77%, 73.31%, and 74.00% of its initial activity at pH 6.0, 7.0, 8.0, and 9.0, respectively. These results indicate that the enzyme exhibits stability across the tested pH range, suggesting its potential suitability for applications under varying pH conditions. When the thermal stability of the enzyme was examined, it was observed that enzyme activity was maintained at various rates above 80% after 30 and 60 minutes of incubation at 20 °C, 30 °C and 40 °C. Enzyme activity was gradually lost as the temperature increased. The results obtained indicate that pepper seeds have the potential to be used as a new peroxidase source in various industrial applications.

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Biber (*Capsicum annuum* L. cv. Sera Demre 8) Tohumlarından Peroksidazın Karakterizasyonu

ÖZET

Peroksidazlar, çeşitli endüstriyel ve biyokimyasal uygulamalarda farklı amaçlarla etkin bir şekilde kullanılmaktadır. Bu nedenle, bu enzimin yeni kaynaklardan saflaştırılması ve karakterizasyonu ilgi çekicidir. Ucuz, çevre dostu ve kolayca erişilebilir bir bitki materyali olan biber tohumları, peroksidaz için uygun bir kaynak oluşturmaktadır.Bu çalışmada peroksidaz biber (*Capsicum annuum* L. cv. Sera Demre 8) tohumlarından izole edilmiş ve karakterizasyon işlemeleri gerçekleştirilmiştir. Enzimin optimum pH ve sıcaklığı sırasıyla 7,0 ve 30 °C olarak hesaplanmıştır. Guaiacol için Km ve Vmax değerleri sırasıyla 1,29 mM ve 0,249 EU/mL·dk, H_2O_2 için ise 0,88 mM ve 0,287 EU/mL·dk olarak hesaplanmıştır. Enzimin pH kararlılığı, 72 saate kadar pH 6,0, 7,0, 8,0 ve 9,0'da +4 °C'de inkübe edilerek değerlendirildi ve aktivite ölçümleri her 24 saatte bir alındı. 72 saat sonra, enzim pH 6,0, 7,0, 8,0 ve 9,0 değerlerinde başlangıç aktivitesinin sırasıyla %81,59, %71,77, %73,31 ve %74,00'ını korumuştur. Bu sonuçlar, enzimin test edilen pH aralığında kararlılık gösterdiğini ve değişken pH koşulları altındaki uygulamalar için potansiyel uygunluğunu göstermektedir.Enzimin ısıl kararlılığı incelendiğinde, 20 °C, 30 °C ve 40 °C'de 30 ve 60 dakikalık inkübasyondan sonra enzim aktivitesinin çeşitli oranlarda %80'in üzerinde korunduğu gözlenmiştir. Sıcaklık yükseldikçe enzim aktivitesini kademeli olarak kaybetmiştir. Elde edilen sonuçlar, biber tohumunun farklı endüstriyel uygulamalarda yeni peroksidaz kaynağı olarak kullanılma potansiyeline sahip olduğunu göstermektedir.

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INTRODUCTION

Peroxidases (POD; E.C 1.11.1.7) are enzymes belonging to the oxidoreductase class, containing ferriprotoporphyrin IX as a prosthetic group. They can be found in bacteria, fungi, algae, plants, seeds, and animals. Peroxidases catalyze the oxidation of phenolic and non-phenolic compounds (catechol, guaiacol, indoles, pyrogallol, sulfonates, and aromatic amines) by consuming H_2O_2 as an electron acceptor (Huystee, 1987; Passardi et al., 2005). Plant peroxidases are favoured in clinical, industrial and environmental fields due to their versatile substrate utilisation properties. Peroxidases have been obtained from many different sources (Şişecioğlu et al., 2010: Zia et al., 2011: Altın et al., 2017: Landi et al., 2019). Although peroxidases are present in many plants, only horseradish root peroxidase is used commercially (Yuzugullu Karakus et al., 2018). There is growing interest in the purification and characterisation of this enzyme, which is actively used in industry, from new, low-cost, biocompatible and antioxidant sources (Altinkaynak et al., 2020).

Turkey ranks among the world's leading pepper producers. According to annual production figures for 2020, the world's six largest producers of hot peppers and peppers are China, Mexico, Indonesia, Turkey, Spain and the United States. Annual pepper production has been steadily increasing in recent years. Pepper (*Capsicum annuum* L.) seeds are mostly considered a byproduct. Consequently, large quantities of pepper processing waste are generated (Cvetković et al., 2022). Pepper seeds are good sources of some bioactive compounds, such as oil, protein, terpenes, carotenoids, and polyphenols (Zhang et al., 2019). The valuable bioactive components found in pepper seeds enable the use of this material as an alternative, inexpensive and innovative resource in various fields. Some of these can be shown as biodiesel production (Lee et al., 2017), obtaining protein isolates (Li et al., 2018), capia pepper seed flour in breakfast sauce production (Yilmaz, 2020). Although pepper seed is used as an alternative in many different areas, it has rarely been used as an enzyme source (Güler, 2015). This study aimed to characterise the peroxidase enzyme, which has industrial application potential, from pepper (*Capsicum annuum* L. cv. Sera Demre 8) seeds, a low-cost, environmentally friendly biological source with antioxidant properties.

MATERIAL AND METHODS

Preparation of crude enzyme extract

Crude enzyme extracts were obtained using a modified version of the method described by Geng et al. (2001). First, the pepper seeds were ground using an appropriate grinder. Two grams of seeds were weighed, and freeze-thawed, the samples were crushed using a mortar. Subsequently, 30 mL of 50 mM phosphate buffer (pH 7.0) was added, and the mixture was thoroughly homogenized. The homogenate was then filtered through four layers of cheesecloth and centrifuged at 10,000 rpm at 4 °C for 1 hour. The resulting supernatant was collected and used as the enzyme extract (Geng et al., 2001).

Determination of peroxidase activity

Peroxidase activity was determined using a UV-VIS spectrophotometer according to the method described by Şişecioğlu et al. (2010). One millilitre each of guaiacol (45 mM) and H_2O_2 (22.5 mM) was added to a 3 mL cuvette, along with 0.980 millilitres of acetate buffer (50 mM, pH 5.0) and 0.02 millilitres of enzyme extract. The absorbance at 470 nm was then measured for three minutes (Şişecioğlu et al., 2010). Enzyme activity was expressed in units, where one unit corresponds to the amount of enzyme required to catalyze the oxidation of 1 µmol of guaiacol per minute at 25 °C ($\varepsilon = 5000\ M^{-1}\ cm^{-1}$) (Kalin et al., 2014).

Protein assay

Protein quantities in the crude enzyme extract were determined using the Bradford method (Bradford,1976).

Optimum pH

To determine the optimum pH, enzyme activity was measured using buffer systems within the pH range of 4.0–9.0 (50 mM acetate buffer, pH 4.0–5.0; 50 mM phosphate buffer, pH 6.0–7.0; and 50 mM Tris-HCl buffer, pH 8.0–9.0), and pH versus relative activity (%) plots were generated (Geng et al., 2001).

Optimum temperature

The optimum temperature of the pepper (*Capsicum annuum* L. cv. Sera Demre 8) seeds POD was determined through activity measurements conducted at 10 °C intervals within the temperature range of 10–80 °C in the presence of guaiacol substrate. The optimum temperature graph was plotted using the obtained data (Somtürk et al., 2014).

K_m and V_{max} *values*

K_m and V_{max} values for guaiacol and H_2O_2 substrates of pepper (*Capsicum annuum* L. cv. Sera Demre 8) seeds POD were calculated using Lineweaver–Burk plots (Lineweaver and Burk, 1934).

The pH stability

The pH stability of pepper (*Capsicum annuum* L. cv. Sera Demre 8) seeds POD was examined by mixing the enzyme solution 1:1 with 50 mM phosphate buffer (pH 6.0–7.0) and 50 mM Tris-HCl buffer (pH 8.0–9.0). The mixtures were incubated at 4 °C, and enzyme activity was measured under optimum conditions every 24 hours. Based on the results, a graph of % remaining activity versus time was plotted (Şişecioğlu et al., 2010).

The thermal stability

The thermal stability of pepper (*Capsicum annuum* L. cv. Sera Demre 8) seeds POD was evaluated by incubating the enzyme at temperatures ranging from 20 to 70 °C at 10 °C intervals for 30 and 60 minutes. After incubation, the samples were cooled to room temperature, and enzyme activity was measured under optimum conditions. The enzyme activity without incubation was taken as 100%, and the remaining activity (%) was plotted against time (Elsayed et al., 2018).

RESULTS AND DISCUSSION

In this study, peroxidase enzyme was isolated from pepper (*Capsicum annuum* L. cv. Sera Demre 8) seeds. The protein concentration of the crude enzyme extract was determined as 5.25 mg/mL (Figure 1). Enzyme activity was calculated as 0.73 EU/mL·min and the specific activity as 0.14 EU/mg protein, based on spectrophotometric measurements using guaiacol as the substrate. In the literature, peroxidase isolated from apple seeds was reported to have a protein concentration of 3.54 mg/mL, an enzyme activity of 3.91 EU/mL, and a specific activity of 1.10 EU/mg protein, while in the same study, peroxidase isolated from orange seeds showed a protein concentration of 7.08 mg/mL, an enzyme activity of 4.20 EU/mL, and a specific activity of 0.59 EU/mg protein (Zia et al., 2011).

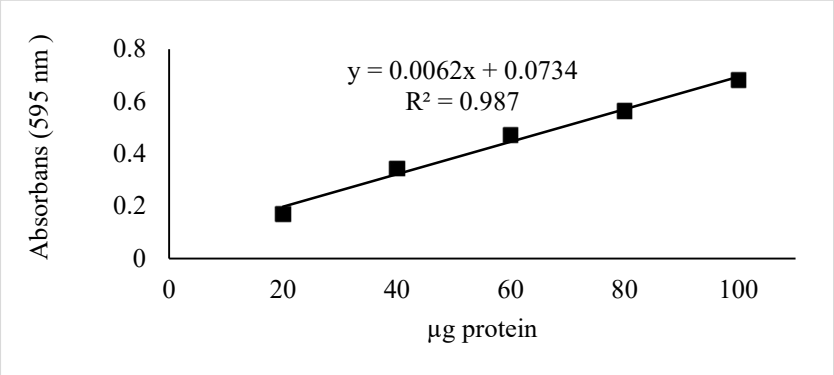

Figure 1. *Protein standard curve*

Optimum pH

The peroxidase enzyme extracted from pepper (*Capsicum annuum* L. cv. Sera Demre 8) seeds exhibited maximum activity at pH 7.0, as determined using guaiacol as the substrate (Figure 2). The optimum pH values

for peroxidase (POD) have been reported as 7.0 for papaya (*Carica papaya*)(Pandey et al., 2012), 5.5 for wheat (*Triticum aestivum* ssp. vulgare)(Altın et al., 2017) and 6.0 for black radish (*Raphanus sativus* L.)(Şişecioğlu et al., 2010). Variations in optimum pH values for peroxidases can be attributed to the enzyme source, the type of buffer system used, and the substrate employed. These factors influence the ionization state of amino acid residues in the active site and substrate binding, leading to source-specific pH optima (Doğan et al., 2007).

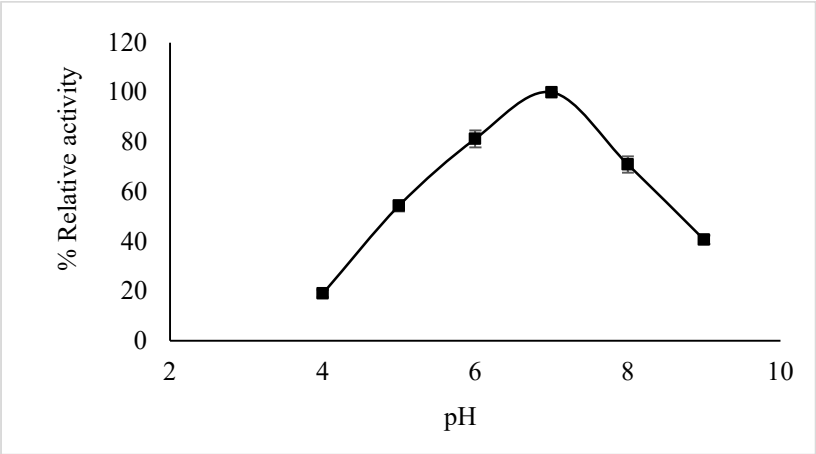

Figure 2. *Optimum pH*

Optimum temperature

The activity of POD (peroxidase) was investigated within the temperature range of 10–80 °C, and the optimum temperature was determined as 30 °C (Figure 3). Beyond this temperature, peroxidase activity in pepper seeds gradually decreased. Previous studies have reported that the optimum temperature values of POD enzymes obtained from different plant sources vary. For instance, the optimum temperature was found to be 30 °C for Turkish black radish (*Raphanus sativus* L.) (Şişecioğlu et al., 2010), 30 °C for red cabbage (*Brassica oleracea* var. *capitata* f. *rubra*) (Somtürk et al., 2014), 30 °C for Turkish black radish and turnip roots (Kalin et al., 2014), and 50 °C for cress (*Lepidium sativum* subsp. *sativum*) (Altay et al., 2018).

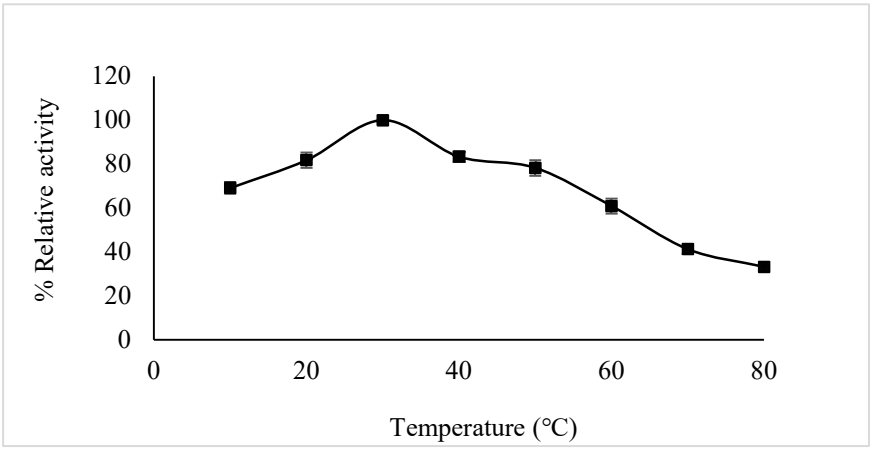

Figure 3. *Optimum Temperature*

K_m and V_{max} values

The determination of K_m and V_{max} values for enzymes derived from novel sources is of considerable importance, particularly in view of their potential industrial applications. Accordingly, the kinetic parameters of pepper seed POD toward guaiacol and H_2O_2 substrates were evaluated using Lineweaver–Burk plots (Figure 4). The K_m and V_{max} values were calculated as 1.29 mM and 0.249 EU/mL.min for guaiacol, and 0.88 mM and 0.287 EU/mL.min for H_2O_2, respectively (Figure 4).

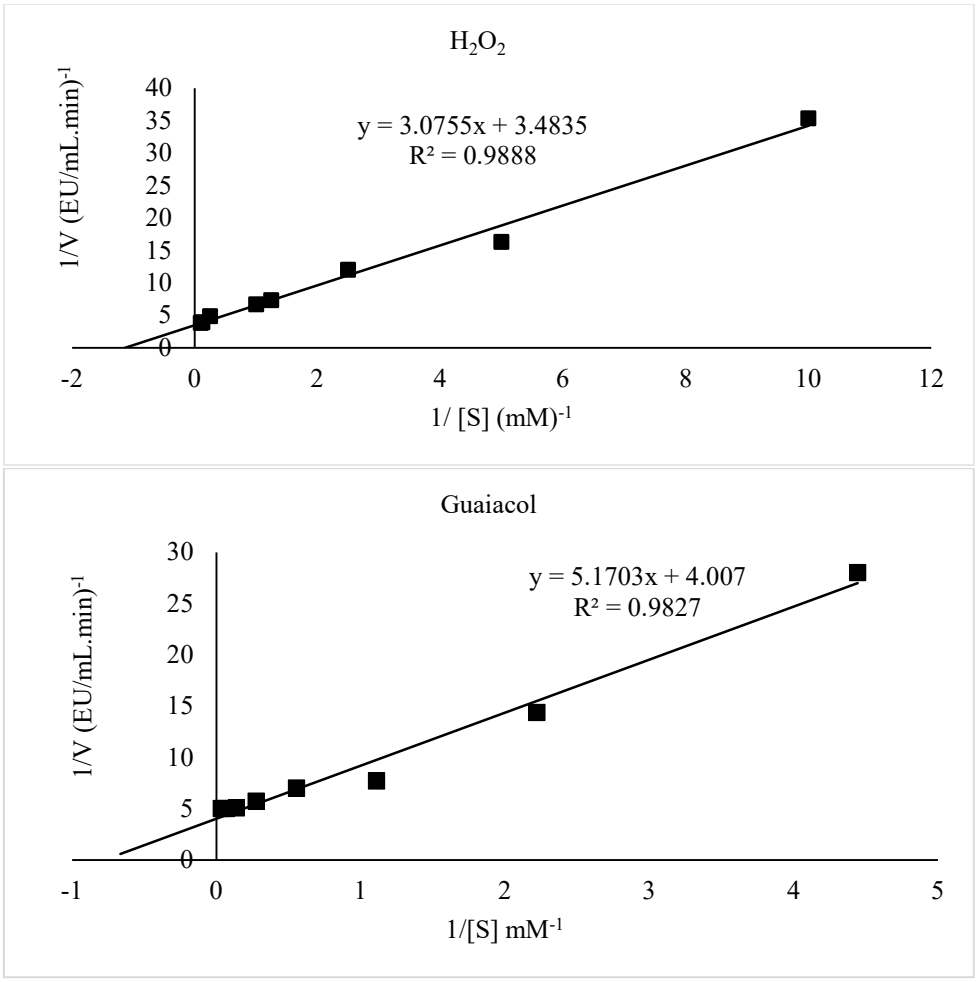

Figure 4. *Lineweaver-Burk curves showing K_m and V_{max} for the substrates of guaiacol and H_2O_2*

Table 1. *Comparison of the kinetic parameters calculated for guaiacol and H_2O_2 substrates of POD enzyme obtained from pepper (Capsicum annuum* L. cv. Sera Demre 8*) seeds with those reported in previous studies*

Enzymes source	Substrate	K_m (mM)	V_{max} (EU/mL.min)	Reference
Pepper (*Capsicum annuum* L. cv. Sera Demre 8) seed	H_2O_2	0.88	0.287	In this study
	Guaiacol	1.29	0.249	
Lettuce (*Lactuca sativa* L.)	H_2O_2	-	-	Hu et al., 2012
	Guaiacol	4.74	10585	
Chard (*Beta vulgaris Subspecies cicla*) Leaves	H_2O_2			Bursal, 2013
	Guaiacol	15.8	3840	
Pomegranate arils (*Punica granatum* L. cv. Wonderful)	H_2O_2	8.00	-	Rayan and Morsy, 2020
	Guaiacol	8.33	-	
Jerusalem Artichoke (*Helianthus Tuberosus* L.)	H_2O_2	0.208	1.481	Kalin, 2023
	Guaiacol	94.33	12.74	

Table 1 compares the kinetic parameters of the peroxidase enzyme isolated from pepper (*Capsicum annuum* L. cv. Sera Demre 8) seeds against the substrates H_2O_2 and guaiacol with those reported for peroxidases in previous literature. In this study, the K_m value of POD obtained from pepper seeds was calculated as 0.88 mM for H_2O_2 and 1.29 mM for guaiacol. These K_m values indicate that the pepper seed derived POD exhibits high substrate affinity for both substrates. In addition, the V_{max} values were determined as 0.287 EU/mL.min for

H_2O_2 and 0.249 EU/mL.min for guaiacol. Overall, the table shows that the K_m and V_{max} values of peroxidase enzymes isolated from different plant sources can vary considerably for both substrates. This suggests that the source of the enzyme may have a significant impact on its kinetic properties.

pH and thermal stability

In industrial applications of enzymes, determining pH and thermal stability are important, as processes are often prolonged and enzymes are exposed to these conditions for extended periods (Robinson, 2015). The pH stability of the enzyme was evaluated by incubating it at pH 6.0, 7.0, 8.0, and 9.0 at +4 °C for up to 72 hours, with activity measurements taken every 24 hours. As illustrated in Figure 5, after 24 hours, the enzyme retained 98.06%, 85.20%, 93.18%, and 81.06% of its initial activity at pH 6.0, 7.0, 8.0, and 9.0, respectively. Following 72 hours of incubation, the residual activity was 81.59%, 71.77%, 73.31%, and 74.00% at the corresponding pH values. These findings demonstrate that the enzyme maintains good stability across the tested pH range, indicating its suitability for applications under varying pH conditions. When the heat stability of pepper seed POD was examined, it was observed that enzyme activity was maintained above 80% at various rates after 30 and 60 minutes of incubation at 20 °C, 30 °C, and 40 °C (Figure 6). The enzyme was found to retain 71.18% and 68.69% of its activity after 30 and 60 minutes of incubation at 50 °C, respectively (Figure 6). It was found that the enzyme lost more than 50% of its activity after 30 and 60 minutes of incubation at 60 °C and 70 °C (Figure 6). Literature reports indicate that activity decreases due to structural deterioration of the enzyme at temperatures above 65 °C (Rajan and Murugan, 2010).

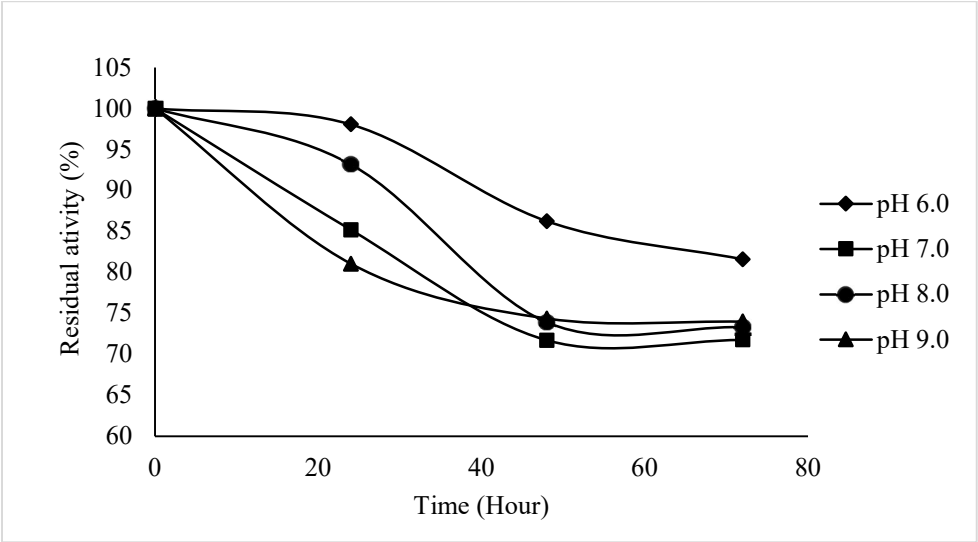

Figure 5. *pH stability*

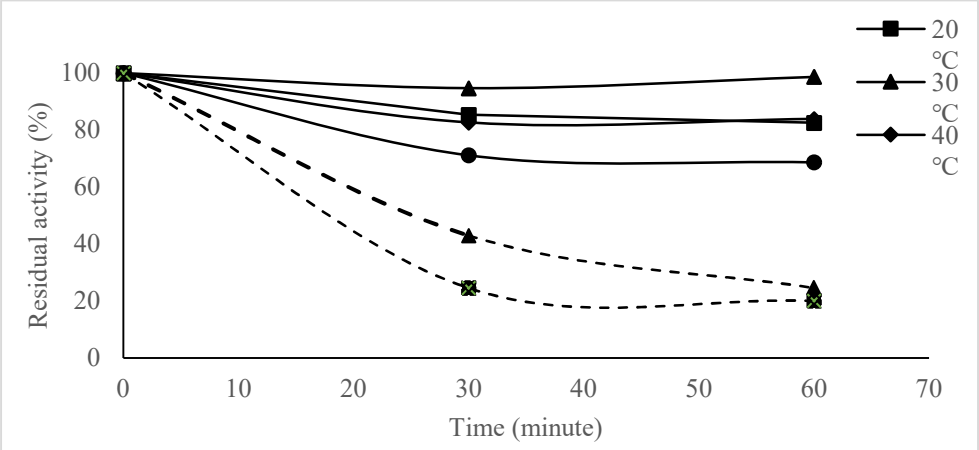

Figure 6. *Temperature stability*

CONCLUSION

Peroxidases are commercially valuable enzymes due to their widespread use in many areas of industry. There are limited commercially available sources for peroxidases. Therefore, it is important to obtain and characterize them from cost-effective and environmentally friendly novel plants. Pepper seeds are waste products in many food processing industries. This study, to the best of our knowledge in the literature, has yielded and characterized peroxidase from pepper (*Capsicum annuum* L. cv. Sera Demre 8) seed for the first time. The characterization results are consistent with the enzyme's properties reported in the literature. This is important because it paves the way for the use of pepper seeds as a peroxidase source.

CONFLICT OF INTEREST

The Author(s) declare(s) that there are no conflicts of interest.

AUTHOR CONTRIBUTIONS

The authors contributed equally to this work.

ADDITIONAL INFORMATION

This study was previously presented as an oral presentation (abstract) at the IV International Van Scientific Research Congress, March 21–23, 2025, Van, Türkiye.

ETHICAL APPROVAL

Ethical approval: Not applicable.

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DATA AVAILABILITY

The datasets generated during and/or analyzed during the current study are available from the corresponding author upon reasonable request.

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