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A peroxidase from bitter gourd (*Momordica charantia*) with enhanced stability against organic solvent and detergent: A comparison with horseradish peroxidase

Aiman Fatima [a], Qayyum Husain [a,*], Rizwan Hasan Khan [b]

[a] *Department of Biochemistry, Faculty of Life Science, Aligarh Muslim University, Aligarh 202002, UP, India*
[b] *Interdisciplinary Biotechnology Unit, Aligarh Muslim University, Aligarh 202002, UP, India*

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Abstract

A detailed comparative stability study of the purified bitter gourd peroxidase and commercially available pure horseradish peroxidase has been undertaken against various denaturants. Stability of the enzymes was monitored spectrophotometrically as well as by ellipticity changes in far-circular dichroism region. Bitter gourd peroxidase was more thermo-stable. The disruption of secondary structure and enzymatic activity at various temperatures was greater for horseradish peroxidase. Bitter gourd peroxidase retained remarkably greater fraction of catalytic activity as compared to horseradish peroxidase in the alkaline pH range. The difference in catalytic activity of bitter gourd peroxidase by varying the pH was related to the change in secondary structure as manifested by the change in CD value at 222 nm. It was further complemented by the far UV-CD spectra, which showed greater retention of secondary structure at pH 6.0 and 10.0. BGP had remarkable stability in the presence of urea, sodium dodecyl sulphate and dimethyl formamide. In view of its higher stability, bitter gourd peroxidase can serve as a better alternative to horseradish peroxidase in clinical and environmental analyses as well as in various biotechnological applications.

Keywords: Horseradish peroxidase; Bitter gourd peroxidase; Circular dichroism; Urea; Stabilization

1. Introduction

Peroxidases (E.C. 1.11.1.7) are ubiquitous heme-proteins, which utilize hydrogen peroxide to catalyze the oxidation of a wide variety of organic and inorganic substrates [1]. Plant peroxidases are receiving increasing attention due to their extensive bioactivation property and potential applications in clinical, biochemical, biotechnological and related areas [2]. Advances have recently been made in using them to synthesize, under mild and controlled conditions, chiral organic molecules, which are highly valuable compounds [3]. They have also been successfully employed in the development of new bioanalytical tests, improved biosensors and in polymer synthesis [4].

Peroxidases have been used for various analytical applications in diagnostic kits, such as quantification of uric acid, glucose, cholesterol and lactose. Due to its ability to convert colorless substrates into chromogenic products, these enzymes are most well suited for the preparation of enzyme-conjugated antibodies, which are used in enzyme-linked immunosorbent assay (ELISA) tests [5,6]. It has also been described that peroxidases can be used in the detoxification of various phenols and aromatic amines present in polluted water [7–9]. More recently some investigators have reported the decolorization and removal of textile dyes from polluted water and dyeing effluents by using soluble and immobilized peroxidases [10–16].

Horseradish peroxidase (HRP) is the most widely studied peroxidase [17]. It has enormous diagnostic, biosensing and biotechnological applications [18]. The availability and cost of commercially available HRP restricts its applications. Horseradish roots are not available in most parts of India whereas bitter gourd is easily available in all parts of the country throughout the year. Peroxidases from other plant sources have also been explored; however these investigations have been unsuccessful

Abbreviations: HRP, horseradish peroxidase; BGP, bitter gourd peroxidase; far UV-CD, far ultra violet-circular dichroism; DMF, dimethyl formamide; SDS, sodium dodecyl sulphate; ELISA, enzyme-linked immunosorbent assay

* Corresponding author. Tel.: +91 571 2700741; fax: +91 571 2706002.
E-mail address: qayyumhusain@rediffmail.com (Q. Husain).

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in terms of identifying peroxidases able to knock out HRP as the preferred plant peroxidase in biotechnology. The availability of highly stable and active peroxidases from sources other than horseradish would go a long way towards the development of a catalytic enzyme with broad commercial and environmental applications.

In the present study, a systematic effort has been made to compare the stability of a homogeneously purified novel peroxidase from bitter gourd and commercially available HRP. The studies were carried out at different temperatures and pH values employing a combination of circular dichroism in far UV-CD region as well as by monitoring the activity of the enzyme. The stability of bitter gourd peroxidase (BGP) against urea, SDS and DMF was also compared with HRP by measuring the activity of the enzymes.

2. Experimental

2.1. Materials

Horseradish peroxidase (205 U/mg) and bovine serum albumin were obtained from Sigma Chem. Co. (St. Louis, MO) USA. *o*-Dianisidine–HCl was the product of IGIB, New Delhi, India. Hydrogen peroxide was obtained from Merck, India. Concanavalin A (Con A)-Sepharose was obtained from Genei Chemicals, Bangalore, India. Ammonium sulphate, urea, dimethyl sulphoxide, dimethyl formamide and sodium dodecyl sulphate were purchased from SRL Chemicals, Mumbai, India. Bitter gourd was obtained from the local market. All the other chemicals and reagents used were of analytical grade and were used without any further purification.

2.2. Purification of BGP

Bitter gourd proteins were precipitated by ammonium sulphate fractionation [12]. Further, purification of BGP was carried out by employing gel filtration on Sephacryl S-100 column and affinity chromatography on Con A-Sepharose [19,20]. The salt fractionated and dialyzed BGP was filtered through Whatman filter paper. The Sephacryl S-100 column (49 cm × 1.7 cm) was equilibrated with 100 mM sodium acetate buffer, pH 5.6. The ammonium sulphate fractionated and dialyzed BGP was then loaded on the column. Fractions of 2.0 mL were collected using 100 mM sodium acetate buffer, pH 5.6. The flow rate of the column was 8 mL/h. Protein concentration and peroxidase activity were determined in all collected fractions. Con A-Sepharose column was equilibrated with 100 mM sodium acetate buffer, pH 5.6 containing 1 mM each of $CaCl_2$, $MgCl_2$, $MnCl_2$ and 0.15 M NaCl. The fractions obtained from the main peak of Sephacryl S-100 column exhibiting peroxidase activity were then pooled and passed through it. Bioaffinity adsorbed proteins were eluted by passing 100 mM sodium acetate buffer, pH 5.6 containing 0.5 M methyl α-D-mannopyranoside. The flow rate of Con A-Sepharose column was 15 mL/h. Both the columns were run at a temperature of 25 °C.

2.3. Effect of temperature

Activity of BGP and HRP (0.4 U/mL) was determined at various temperatures (30–80 °C) in 100 mM sodium acetate buffer, pH 5.6. The percent remaining enzyme activity was calculated by taking activity at temperature-optimum as control (100%). In another set of experiment, BGP and HRP (0.4 U/mL) were incubated at 60 °C for varying time intervals in 100 mM sodium acetate buffer, pH 5.6. After each incubation period the enzyme was quickly chilled in crushed ice for 5 min. The enzyme was brought to room temperature and then the peroxidase activity was determined.

2.4. Effect of pH

Activity of BGP and HRP (0.4 U/mL) was determined in the buffers of different pH values. The buffers used were glycine–HCl (pH 2.0 and 3.0), sodium acetate (pH 4.0 and 5.0), sodium phosphate (pH 6.0, 7.0, and 8.0) and Tris–HCl (pH 9.0 and 10.0). The percent remaining enzyme activity was calculated by taking activity at pH-optimum as control (100%).

2.5. Effect of urea

BGP and HRP (0.4 U/mL) were incubated with increasing concentration of urea (2.0–8.0 M) for 2 h in 100 mM sodium acetate buffer, pH 5.6 at 37 °C. In another set of experiment, BGP and HRP preparations (0.4 U/mL) were incubated with 4.0 M urea for varying time intervals. Peroxidase activity was determined after each incubation period. The activity of the untreated enzyme was considered as control (100%) for calculating percent activity.

2.6. Effect of SDS (detergent)

BGP and HRP (0.4 U/mL) were incubated with increasing concentration of SDS (0.1–1.0%, w/v) in 100 mM sodium acetate buffer, pH 5.6 at 37 °C for 1 h. Peroxidase activity was determined after the incubation period. The activity of the untreated enzyme was considered as control (100%) for calculating percent activity.

2.7. Effect of water-miscible organic solvent

BGP and HRP (0.4 U/mL) were incubated with varying concentrations of water-miscible organic solvent, DMF (10–60%, v/v) in 100 mM sodium acetate buffer, pH 5.6 at 37 °C for 1 h. The activity of the untreated enzyme was considered as control (100%) for calculating percent activity.

2.8. Assay of peroxidase activity

Peroxidase activity was estimated from the change in the optical density ($\lambda_{460\,nm}$) at 37 °C by measuring the initial rate of oxidation of *o*-dianisidine–HCl by hydrogen peroxide using the two substrates in saturating concentrations [13].

One unit of peroxidase activity is defined as the amount of enzyme protein that catalyzes the oxidation of 1.0 μmol of *o*-dianisidine–HCl per min at 37 °C into colored product (ε_m at 460 = 30,000 M^{-1} cm^{-1}) [13]. Each value in the activity analysis represents the mean for three independent experiments performed in duplicate.

2.9. Protein assay

Protein concentration was determined by using the Bradford method [21]. Bovine serum albumin was used as standard for making a calibration curve.

2.10. CD spectroscopy

Circular dichroism measurements were made on a JASCO J-720 spectropolarimeter calibrated with D-10-camphorsulphonic acid. The instrument was equipped with a water-jacketed, thermostatically controlled cell holder, Neslab RTE circulating water bath and a microcomputer. The path length of the cell and protein concentration was chosen to optimize the measuring conditions.

3. Results and discussion

3.1. Thermal stability

The temperature-activity profiles of BGP and HRP are depicted in Fig. 1. Temperature-activity profiles of BGP and HRP exhibited similar temperature-optima. However, temperature-activity profile of BGP showed more broadening as compared to HRP. BGP retained nearly 81% of the initial activity after incubation at 60 °C whereas HRP retained only 39% activity. Peroxidase has been reported to be one of the most thermo-stable enzymes in plants [22]. BGP emerged out to be a more thermo-stable enzyme than HRP and retained a significantly high fraction of catalytic activity at 60 and 80 °C.

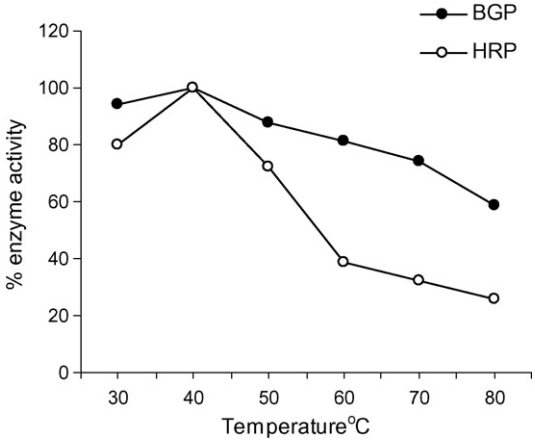

Fig. 1. Temperature-activity profiles for BGP and HRP. BGP and HRP (0.4 U/mL) were assayed at various temperatures (30–80 °C) in 100 mM sodium acetate buffer, pH 5.6. Activity expressed at 40 °C was taken as control (100%) for calculating percent activity.

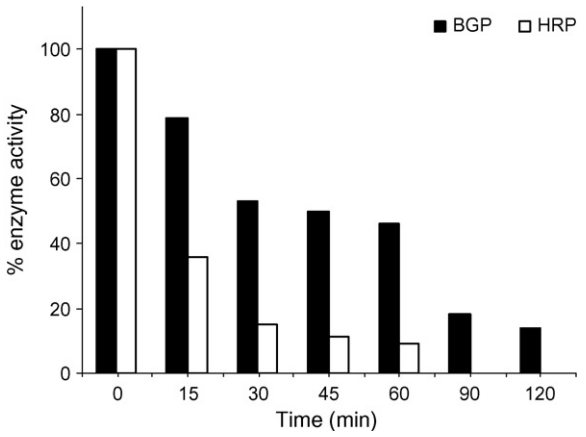

Fig. 2. Thermal denaturation of BGP and HRP. BGP and HRP (0.4 U/mL) were incubated at 60 °C for varying times in 100 mM sodium acetate buffer pH 5.6. Aliquots of each preparation were taken out at indicated time intervals and enzyme activity was determined as described in the text. Unincubated samples at 60 °C were taken as control (100%) for the calculation of percent activity.

However, HRP retained relatively less activity at the same temperatures. Earlier investigators have already shown a significant decrease in the catalytic activity of HRP with increase in temperatures [23].

BGP incubated at 60 °C retained nearly 50% of the initial activity after 1 h of incubation while HRP retained only 9% activity under similar experimental conditions (Fig. 2). The far-UV CD spectra of BGP at 80 °C (curve 1), 60 °C (curve 2) and 30 °C (curve 3) are depicted in Fig. 3. There was a greater loss of helicity at 80 °C as compared to 30 and 60 °C. It has already been shown that there is a decrease in CD signal intensity of HRP above 60 °C [23]. It has also been demonstrated that HRP lost its full catalytic activity after 1 h of incubation at 54.7 °C in a buffer of pH 3.0 [24].

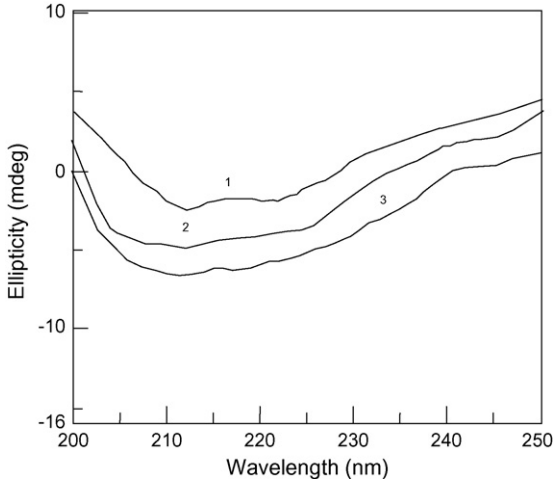

Fig. 3. Far UV-CD spectra of BGP at different temperatures. Spectra were taken in the wavelength region of 200–250 nm at three different temperatures. The CD experiment was performed in 100 mM, sodium acetate buffer, pH 5.6. The path length of the cell and protein concentration was chosen to optimize the measuring conditions. The spectra show at: (1) 80 °C, (2) 30 °C, and (3) 60 °C.

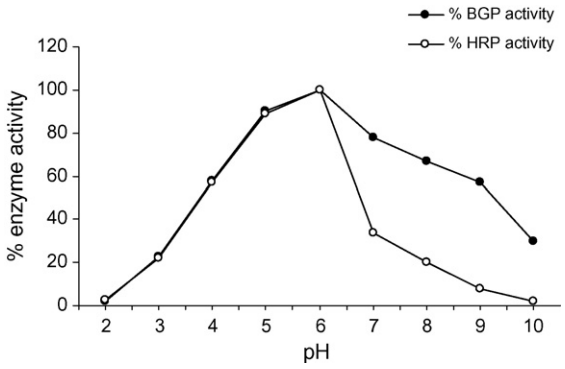

Fig. 4. pH-activity profiles for BGP and HRP. BGP and HRP (0.4 U/mL) were incubated with buffers of different pH values. The buffers used were glycine–HCl (pH 2.0 and 3.0), sodium acetate (pH 4.0 and 5.0), sodium phosphate (pH 6.0–8.0) and Tris–HCl (pH 9.0 and 10.0). The molarity of each buffer was 100 mM. Activity expressed at pH 6.0 was taken as control (100%) for calculating percent remaining activity.

3.2. pH stability

The pH-activity profiles of BGP and HRP are demonstrated in Fig. 4. In comparison to HRP the stability of BGP was much higher in the alkaline range. In fact, HRP lost 92% of its original activity at pH 9.0 while BGP retained nearly half of its activity at the same pH value. A sharp decline in the stability of BGP and HRP occurred as the pH was lowered below 5.0. The pH dependence of the enzyme activity (measured at 460 nm) and ellipticity at 222 nm of BGP are shown in Fig. 5. There was an increase in the negative ellipticity from pH 2.0 onwards which reached a maximum at around pH 6.0. Further increase in pH led to the continuous decrease in the CD signal value, which reached a conspicuous minimum at pH 10.0. BGP lost all secondary contacts at pH 10.0 and assumed a completely unfolded structure. A similar pattern was observed when the BGP activity was measured at the different pH values. The far UV-CD spectra of BGP at pH 6.0 and 10.0 have been overlaid for comparison of

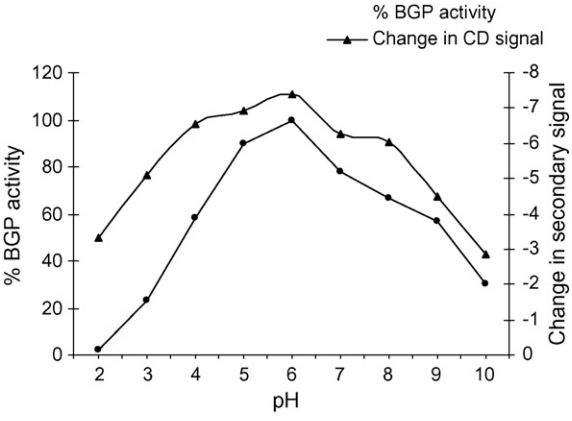

Fig. 5. Effect of pH on the activity and secondary structure (222 nm) of BGP. The buffers used were glycine–HCl (pH 2.0 and 3.0), sodium acetate (pH 4.0, 5.0 and 6.0), sodium phosphate (pH 8.0) and Tris–HCl (pH 9.0 and 10.0). The molarity of each buffer was 100 mM. Activity expressed at pH 6.0 was taken as control (100%) for calculating percent remaining activity. The CD experiment was carried out at 25 °C. The protein concentration and path length of the cell were chosen to optimize the measuring conditions.

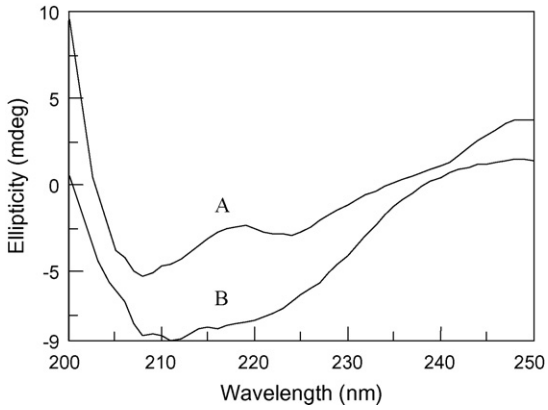

Fig. 6. CD spectra of BGP at two different pH values. Secondary structure change of BGP was analyzed at pH 10.0 (A) and pH 6.0 (B). CD experiment was carried out at 25 °C. Spectra were taken in the wavelength region, 200–250 nm. Sodium acetate buffer (pH 6.0) and Tris–HCl buffer (pH 10.0), 100 mM were used. The protein concentration and path length of the cell were chosen to optimize the measuring conditions.

changes in the spectral features (Fig. 6). There was a loss of secondary structure as shown by the decrease in the negative value of the spectrum at pH 10.0 (Fig. 6, curve A). BGP retained more secondary structure at pH 6.0 than at pH 10.0 as manifested by a more negative signal and hence a higher helicity at pH 6.0.

Several earlier investigators have used CD techniques to study the relationship between the structure and functional stability of proteins [18,23,25]. Ellipticity at 222 nm was used to monitor the unfolding of a helical protein [26]. It has already been shown that there was a decrease in the stability of HRP with decrease in pH [24]. A significant loss in catalytic activity of HRP was further confirmed by low relative activity of HRP at pH 8.0, 9.0 and 10.0 [27]. The catalytic activity of HRP was closely related to its structural changes [23,28]. The change in the catalytic activity of BGP over a wide range of pH values also corresponds to the change in the secondary structure as manifested at 222 nm. The retention of higher secondary structure of BGP was further supported by far UV-CD analysis at pH 6.0 and 10.0.

3.3. Effect of chaotropic agent, urea

The inactivation of BGP and HRP at different concentrations of urea has been demonstrated in Fig. 7. There was no significant change in the activity of BGP and HRP after their incubation with 2.0 M urea for 2 h. However, the change in catalytic activity became more pronounced from 4.0 M urea concentration onwards. BGP retained a significantly high activity, 90% after exposure with 6.0 M urea for 2 h whereas HRP lost nearly 50% original activity. Further incubation of BGP and HRP with 8.0 M urea resulted in the retention of only 30% HRP activity whereas BGP exhibited 88% of the initial activity. The stability of both BGP and HRP against the urea (4.0 M) induced inactivation at various time intervals has been investigated (Fig. 8). BGP was more resistant to inactivation mediated by 4.0 M urea compared to HRP. HRP retained 64% activity after 2 h exposure with 4.0 M urea whereas BGP retained 90% of the initial enzyme activity under similar treatment. BGP appeared to be more resistant

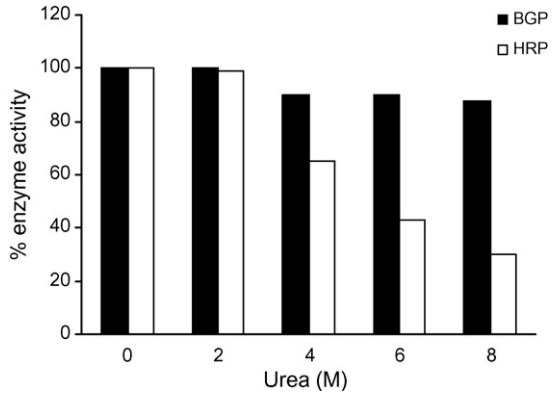

Fig. 7. Effect of increasing concentration of urea on BGP and HRP. BGP and HRP (0.4 U/mL) were incubated in 2.0, 4.0, 6.0 and 8.0 M urea in 100 mM, sodium acetate buffer, pH 5.6 at 37 °C for 2 h. Enzyme activity was determined as mentioned in the text. For calculating the percent activity untreated samples were considered as control (100%).

than HRP when both the peroxidases were incubated in different concentration of urea. The catalytic activity of HRP remained unaltered in the presence of 2.0 M urea. It has already been reported that HRP exposed to 8.0 M urea for 1 h lost 50% of its activity [29].

3.4. Effect of SDS

The effect of increasing concentration of SDS, an anionic detergent (0.1–1.0%, w/v) on the activity of BGP and HRP has been shown in Fig. 9. There was activation in both preparations of peroxidase till 0.3% (w/v) SDS. The percent activity of BGP was remarkably enhanced to 790% whereas HRP exhibited a percent activity of 111 at 0.3% (w/v) SDS. The percent enzyme activity of BGP was higher than HRP at all the SDS concentrations and then it leveled off at 0.4% showing a constant value till 1% (w/v) SDS. Preincubation of BGP and HRP with 1.0% (w/v) SDS for 1 h resulted in a significant loss of 83% of the original activity of HRP whereas the activity of BGP was enhanced up to 200% of the initial activity. Some enzymes can show enhanced activity in water/detergent media owing to the positive interactions between enzyme and detergent [30]. The enhancement in enzyme activity even at high concentration of SDS suggested

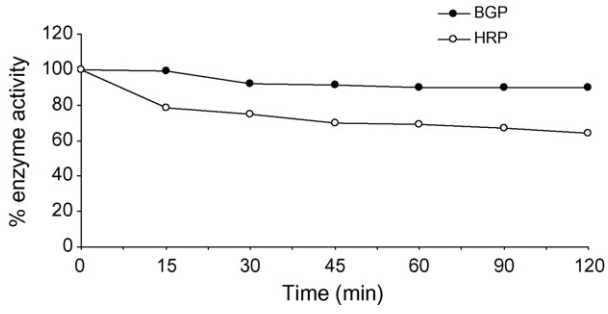

Fig. 8. Effect of 4.0 M urea on BGP and HRP. BGP and HRP preparations (0.4 U/mL) were incubated in 4.0 M urea in 100 mM sodium acetate buffer, pH 5.6 at 37 °C. Enzyme activity was determined at different time intervals under conditions mentioned in the text. For calculating the percent activity untreated samples were considered as control (100%).

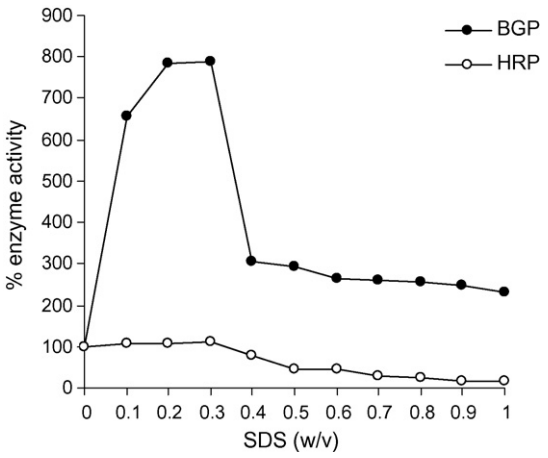

Fig. 9. Effect of SDS concentration on enzyme activity of BGP and HRP. BGP and HRP (0.4 U/mL) were incubated with increasing concentration of SDS (0.1–1.0%, w/v) in 100 mM sodium acetate buffer, pH 5.6 at 37 °C for 1 h. The enzyme activity was determined as described in the text. Untreated samples were taken as control (100%) for calculating percent remaining activity.

that BGP could be effectively exploited for various uses in the presence of such detergents for example in reverse micelle bioprocessing applications and for preparation of media for hosting enzyme reactions [30]. It can also be used in various studies of electrochemistry [31].

3.5. Effect of organic solvent

Organic solvents have been used most extensively as solvents for the polymerization of phenols by HRP. Organic solvents are needed for an increase in the solubility of the monomers and for obtaining polyphenols of high molecular weight [23]. For synthetic reactions catalyzed by enzymes, stability of the enzymes in the reaction media is important, especially when organic solvents are used. At present, a large body of multienzymatic amperometric biosensors is realized by entrapping peroxidases into a polymer matrix. The polymers used for entrapment are soluble in organic solvents [32]. Due to various potential applications of peroxidases in organic solvents, it became important to investigate the changes in the catalytic activity of both BGP and HRP at different concentrations of DMF.

Table 1 summarizes the effect of increasing concentration of water-miscible organic solvent (DMF, 10–60%, v/v) on the activity of BGP and HRP. There was a conspicuous activation in enzyme activity when treated with increasing concentration of DMF. However, the activation was more pronounced in case of BGP. BGP was activated to 143% after 1 h incubation in 60% (v/v) DMF at 37 °C whereas HRP was activated to 106% under similar conditions. Both the peroxidases are activated by exposure to 20–60% (v/v) of DMF. It has been reported earlier that HRP retained a high catalytic activity in 20–60% (v/v) of DMF [23]. The catalytic efficiency of enzymatic reactions can be higher in organic media [33]. These observations indicated that although the activation of HRP took place in 20–60% (v/v) DMF, this activation was low as compared to that of BGP. Earlier workers have shown that HRP was activated by those nitrogenous compounds, which have a lone pair on nitrogen

Table 1
Effect of DMF on BGP and HRP

Organic solvent (%, v/v)	Percent remaining activity	
	BGP	HRP
10	89	36
20	135	103
30	140	110
40	143	118
50	143	106
60	143	106

BGP and HRP (0.4 U/mL) were incubated with DMF (10–60%, v/v) in 100 mM sodium acetate buffer, pH 5.6 at 37 °C for 1 h. The enzyme activity was determined as described in the text. Each value represents the mean for three independent experiments performed in duplicate, with average standard deviation <5%.

[27]. This could be a reason for the activation of BGP and HRP by DMF, a nitrogenous compound with a lone pair on nitrogen.

In this work, we have compared the structural and functional aspects of BGP and HRP for their stability against the denaturation induced by heat, pH, urea, SDS and water-miscible organic solvent, DMF. Interestingly, BGP exhibited a remarkably high stability as compared to HRP against all the stress conditions taken in this study. Measuring enzyme activity and structural analyses has made this comparison in stability. In view of extremely high stability of BGP compared to HRP against various tested parameters, BGP can be easily substituted in place of HRP in various clinical, biochemical, environmental and biotechnological applications.

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