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

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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.
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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

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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].

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