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Enzyme–polysaccharide interaction: A method for improved stability of horseradish peroxidase

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ABSTRACT

With the advent of green technology, use of enzymes as biocatalyst has become increasingly popular. However, in doing so, enzymes can lose their structure and catalytic activity under conditions that might be necessary for other components of processes. Compared to other strategies, chemical modification is a simple and effective technique for generating stable enzyme. Horseradish peroxidase (HRP; EC 1.11.1.7) was chemically modified by conjugating with 10 different polysaccharides. All polysaccharides were found to increase the thermal and pH stability of HRP with starch being most promising. Further, different parameters were evaluated for effective conjugation and thus stability of HRP conjugate. The degradation kinetics and storage stability of HRP proved the conjugate to be 6.4 times more stable than free enzyme. The starch conjugated HRP and free HRP were further evaluated for its application in decolorization of bromophenol blue dye. Both the enzymes were able to efficiently (>90%) decolorize the dye within minutes.

1. Introduction

Proteins and enzymes have become an integral part of many industrial processes as well as being applied both in cosmetics and in therapeutics. Due to advances in biotechnology and genetic engineering, mass production of protein and enzymes has become feasible. Use of enzymes is environmentally friendly as they catalyze reactions under mild conditions against polluting inorganic catalysts which works in the extremes of pH and temperature. However, this can be disadvantageous as enzymes can lose their structure and catalytic activity at process conditions that might be necessary for other components. This often becomes a constraint in using them commercially [1]. Increase in stability is therefore of great importance and can be accomplished by various methods such as protein engineering, chemical modification, use of stabilizing additives, and immobilization [2].

Horseradish peroxidase (HRP; EC 1.11.1.7) is a heme-containing oxidoreductase produced from the roots of a perennial plant *Armoracia rusticana* (horseradish), belonging to the Brassicaceae family. It is produced on commercial scale for variety of applications including waste water treatment, biodegradation, organic synthesis, chemiluminescence, immunodetection and biosensors due to high turnover number [3]. HRP isoenzyme C (44.1 kDa) is the most abundant of the seven isoenzymes of HRP. Two calcium ions present in enzyme's structure play a crucial part in its catalytic activity and stability [4]. Stability of HRP has been discussed by many authors and it varies with temperature and pH. At 65 °C, the relative activity of HRP was 35% and 10% after incubation for 10 and 30 min respectively at pH 7.0 while relative activity of acetylated HRP was 50% [5]. Hassani et al. [6] reported HRP to retain 10% of initial activity when incubated for 30 min at 70 °C while HRP modified with citraconic anhydride in 20% sorbitol retained 80% of its activity. Mogharrab et al. [7] reported that incubation of HRP in 0.2 M phosphate buffer at 70 °C for 10 min resulted in complete inactivation of the enzyme. Thus, there exists a need to improve the stability of HRP for varied applications.

The molecular structure of an enzyme can be easily altered by external factors. In nature, enzymes are present in the cytosol of living cells and are surrounded by proteins, nucleic acids, lipids and mono/polysaccharides. The interactions between these ambient biomacromolecules and the enzyme play crucial roles in stabilizing the structure and function of enzymes [8]. Covalent modification of an enzyme with hydrophilic polymer (polysaccharides) could form a shell around the enzyme and provide rigidity [9] and hydration [10] to enzyme and increase their thermal and pH stability. Hence, this is one of the approach encompassing basic principles of chemical modification, stabilization by additives and immobilization. Covalent binding of β-glucuronidase to sodium carboxymethyl cellulose has shown improved thermal and pH stability [11]. Binding

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of horseradish peroxidase, glucose oxidase [12] and amylase [13] to dextran has been reported to be successful.

Polysaccharides differ in structure and chemical nature and provide different levels of thermal and pH stability to the enzyme by binding in a unique and specific way. Molecular crowding and hydration are believed to be the factors pertaining to the effect of polysaccharides on thermal stability. Molecular crowding caused by addition of polysaccharides not only maintains the native structure but also inhibits enzyme aggregation. Hydrophilic polysaccharide will preferentially build up more stable water layer around enzyme surface while simultaneously strengthening hydrophobic interactions among non-polar amino acid residues [11,14].

This paper explores the effect of conjugation of an enzyme HRP with different polysaccharides and its effect on pH and thermal stability. This paper also discusses the optimization of various factors that will influence the activity and stability of HRP. Further, the free enzyme and its conjugate were evaluated for its application in dye decolorization of bromophenol blue, a triphenylmethane dye.

2. Materials and methods

2.1. Materials

All chemical and reagents used were of AR grade and were purchased from Sigma–Aldrich, Himedia and s.d. Fine-Chem. Ltd., Mumbai, India. HRP (RZ ≥ 2; 1750 U/mg (*o*-dianisidine)) was purchased from Hunan Jiahao Chemicals Co. Ltd., China.

2.2. Estimation of HRP activity

The activity of HRP was estimated according to method described by Altikatoglu et al. [12] with modifications. Ten μl of enzyme solution was mixed with 110 μl of 50 mM phosphate buffer (pH 5.8), 20 μl of 10 mM *o*-dianisidine and 10 μl of 0.3% H_2O_2 solution. The reaction mixture was placed immediately in the spectrophotometer and an absorbance (460 nm) was recorded at an interval of 1 s for 30 s. The difference in absorbance (in linear region) was taken for total activity estimation. The total activity was calculated as

$$\frac{Y}{\text{mg}} = \frac{\Delta A_{460}/\text{min} \times 106}{\varepsilon \times \text{volume} \times \text{cHRP}}$$

where ε, molar absorption coefficient of *o*-dianisidine, *t*, incubation time (min), cHRP, HRP concentration (mg/ml), and ΔA_{460}/min, change on absorbance at 460 nm/min

One unit of enzyme activity is defined as the amount of enzyme which oxidizes 1 μmol of substrate per unit.

2.3. Preparation of HRP–polysaccharide conjugates

Sodium metaperiodate (0.1 M) solution was prepared in 0.1 M sodium acetate buffer of pH 5.0 and used as the oxidizing solution. Polysaccharides viz. dextran 20, 40, and 70 kDa, carrageenan, sodium carboxymethyl cellulose (NaCMC), methyl cellulose, gellan, pectin, dextrin and starch, 100 mg each was oxidized in 10 ml of oxidizing solution in dark for 90 min after which the oxidation was stopped by adding 0.3 ml of ethylene glycol, and kept in dark for 60 min. Oxidized polysaccharide solutions were dialyzed against 0.1 M sodium acetate buffer of pH 5.0 at 4 °C overnight. The oxidized polysaccharide solution was estimated for reducing sites generated. HRP solution (1 mg/ml) was prepared in buffer of pH 5.0 and mixed with equal volume of each oxidized polysaccharide solution and kept for conjugate formation for 20 h at ambient temperature (~28 ± 2 °C). Sodium borohydrate (20 mg) was then added to 10 ml of conjugate mixture to reduce remaining oxidized sites of polysaccharide and kept for 4 h. Finally, all the prepared conjugate solutions were dialyzed against 20 mM of sodium citrate buffer of pH 5.0 at 4 °C overnight [10,13]. These conjugates were used for analyzing the activity and stability of HRP.

2.3.1. Effect of polysaccharides on HRP stability

Ten different polysaccharides were conjugated with HRP to evaluate its effect on thermal and pH stability. The thermal stability was evaluated by incubating the free enzyme and its conjugates at different temperatures (40, 50 and 60 °C) in 50 mM phosphate buffer (pH 5.8) for 60 min followed by immediate chilling on ice. The enzymatic activity and percent residual activity based on the initial activity were estimated after appropriate dilutions. The original activity is referred to as the activity at time $t = 0$.

The pH stability of the enzyme was evaluated by incubating the free enzyme and its conjugate at ambient temperature (28 ± 2 °C) with 50 mM citric acid/sodium citrate buffer of pH 3.0–6.0, 50 mM NaH_2PO_4/Na_2HPO_4 buffer of pH 7.0–8.0, and 50 mM glycine/NaOH buffer of pH 9.0–10.0. The samples were incubated for 60 min, diluted appropriately, and estimated for enzymatic activity and percent residual activity.

The polysaccharide which resulted in higher thermal and wider pH stability was selected for further studies.

2.3.2. Effect of ratio of starch to HRP concentration

The ratio of starch to HRP concentration was varied from 01:01 to 30:01 in order to optimize and determine the ratio which supports the maximum thermal and pH stability. These different conjugates were prepared by varying the concentration of starch from 1 to 30 mg/ml while keeping the concentration of enzyme (1 mg/ml) constant. Equal volume of oxidized starch and free enzyme were mixed in order to form conjugates. The thermal and pH stability of the conjugates were determined as described above.

2.3.3. Effect of conjugation on kinetics of thermal degradation of HRP

Free and conjugated HRP were incubated at 40, 50, and 60 °C in 50 mM phosphate buffer of pH 5.8. The samples were withdrawn periodically, chilled quickly, and then analyzed for residual enzymatic activity. A semi-log plot of percent residual activity vs. time was plotted from which the inactivation rate constant, *k*, was calculated as the slope, and $t_{1/2}$, the time required for the activity to decrease to half its original activity was calculated as 0.693/*k*.

2.4. Fluorescence spectrophotometry analysis of free and conjugated HRP

Intrinsic fluorescence intensity of both (free and conjugated) enzymes incubated for 60 min at 50 °C were measured using spectrofluorometer (Jasco FP6500, Japan) and structural changes in the free and conjugated HRP were investigated. The samples were excited at an excitation wavelength of 234 nm and the emission spectrum was recorded from 300 to 450 nm. Fluorescence measurements were carried out in 50 mM phosphate buffer of pH 5.8.

2.5. Characterization of free and conjugated HRP

In order to determine the optimum pH and temperature of the free and conjugated HRP, their enzymatic activities were determined at different pH (3–10) and temperature (4–50 °C). Further, the enzyme and its conjugate were characterized by determining their kinetics using Michaelis–Menten model (K_m and V_{max}).