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Short communication

Kinetic stabilities of soybean and horseradish peroxidases

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Abstract

Peroxidases have attractive biocatalytic properties and are used in biosensing and immunoassays. Among various peroxidases, isoenzyme C of horseradish peroxidase (HRP-C) is the most studied, and is also the most commercially used due to its high structural stability. Soybean peroxidase (SBP) and horseradish peroxidase share strikingly similar three-dimensional structures with ∼60% sequence homology. We reported previously, that the conformational and thermal stabilities of SBP are substantially higher than HRP-C. In the present study, we show that the kinetic stability of SBP is much higher than HRP-C as obtained by measuring their unfolding rates at various guanidine hydrochloride (GdnHCl) concentrations. In contrast, the heme-free forms of SBP and HRP-C showed similar kinetic stabilities. We conclude that the higher structural stability of SBP compared to HRP-C stems from the heme binding to the apo protein. Commercial interest of these results is twofold. A cheaper, abundant, better active, and more stable SBP could replace HRP-C. The stability and hence the biocatalytic property of a peroxidase can be improved by suitably engineering the heme active-site that enhances the heme-apo-protein interaction.

Keywords: Soybean peroxidase; Horseradish peroxidase; Homologous proteins; Unfolding rate; Kinetic stability; Heme binding

1. Introduction

Peroxidases (donor: H_2O_2, oxidoreductase: EC 1.11.1.7) are heme enzymes catalyzing oxidative reactions that use hydrogen peroxide as an electron acceptor [1]. They have been extensively studied and show many attractive properties for biocatalysis such as wide specificity, high stability in solution and easy accessibility from plant materials. They show potentially interesting applications in a number of fields. The most important application so far is in analytical diagnosis, where they are utilized as a key component of biosensors and immunoassays [2–4].

Among several plant peroxidases, isoenzyme C of the horseradish perxidase (HRP-C) has been the most widely studied and is the most commercially used peroxidase [5–8]. It was thought to be the highest stable peroxidase until McEldoon and Dordick [9] showed a higher thermal stability for soybean seed coat peroxidase (SBP); melting temperature (T_m) reported is 90.5 °C for SBP whereas it is 81.5 °C for HRP-C, at pH 8.0 and in 1 mM calcium chloride. Later, we reported a T_m of 86 °C for SBP in absence of any added calcium chloride in the buffer at pH 7.0 [10], which is again much higher than the value reported for HRP-C (74 °C) at identical conditions [11]. Also, we reported for the first time [10] a higher conformational stability for SBP over HRP-C; equilibrium free energy of unfolding [$\Delta G(H_2O)$] of SBP is ∼43 kJ mol^{-1} [10] as opposed to ∼17 kJ mol^{-1} of HRP-C [12]. We also found a ∼20fold higher catalytic efficiency for SBP over HRP-C at their pH optima [13]. Both the proteins belong to the same subfamily (Class III) of the plant peroxidase super family and share very similar three-dimensional structures (Fig. 1), amino acid sequence (homology is ∼60%), and catalytic mechanism. They have many structural stabilizing factors in common [1,14,15]. They are heme prosthetic group (Fe (III) protoporphyrin IX), four disulfide bonds, two Ca^{2+} ions, and eight glycans.

In any industrial process involving use of an enzyme, it is subjected to varying chemical and physical environments [16]. Choice of the enzyme is therefore dictated by its integrity and functional stability besides operational requirements and economic considerations. Therefore, besides high activity, high

Abbreviations: SBP, soybean peroxidase; HRP-C, horseradish peroxidase isoenzyme C; GdnHCl, Guanidine hydrochloride (enclosing square brackets indicate concentration); $\Delta G(H_2O)$, equilibrium free energy of unfolding in water; k_U (H_2O), unfolding rate constant in water; $t_{1/2}$ (H_2O), half-life of unfolding in water; $\Delta G^*(H_2O)$, activation free energy of unfolding in water.

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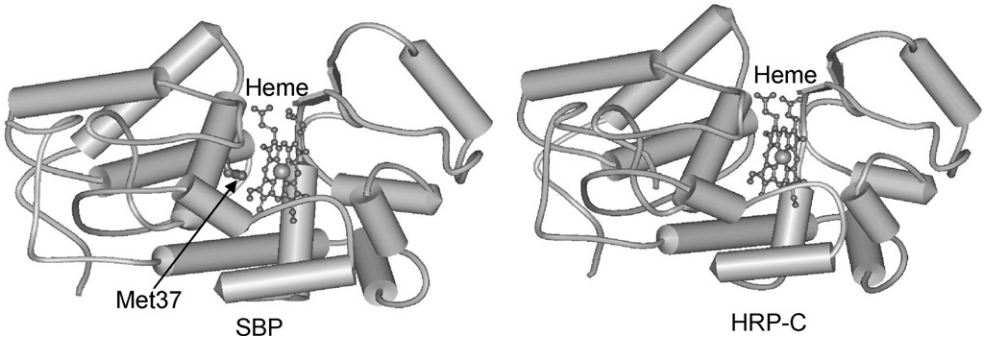

Fig. 1. Schematic structures of SBP (PDB entry 1fhf) and HRP-C (PDB entry 1atj) obtained from crystallographic results. Sketches were made using WebLab ViewerLite (MSI).

conformational and thermal stability requirements, a very high kinetic stability of the enzyme are also essential for their large-scale use. While conformational (thermodynamic) stability is the free energy difference between the unfolded and native states ($\Delta G(H_2O)$) that provides a measure of how much energetically stable the folded state is with respect to unfolded states, kinetic stability on the other hand is the free energy difference between the transition state and native state (activation free energy of unfolding, $\Delta G^*(H_2O)$) and hence it is a measure of how much resistant the native state is against external perturbations. In other words, kinetic stability is a measure of the lifetime of an enzyme in its functional state. Kinetic stability can be estimated by measuring the unfolding rate constants at varying concentrations of a denaturant followed by linear extrapolation of the data to obtain the parameters in water [17]. In this paper, we present results on the measurements of the kinetic stabilities of holo and apo forms, of SBP and HRP-C. The kinetic stability of SBP is found to be substantially higher than HRP-C; the activation free energy of unfolding obtained for SBP is $\sim$96 kJ mol^{-1} and that obtained for HRP-C is $\sim$78 kJ mol^{-1} assuming a rate value of 10^{-6} s^{-1} for the transition state conversion to unfolded state. We further show that the key structural element responsible for the stability as well as differential stabilities of SBP and HRP-C is the heme prosthetic group.

These superior biochemical properties together with the low cost and high abundance of SBP as compared to an expensive and low abundant HRP-C [18] render SBP an ideal candidate to replace HRP-C in industrial and medical applications. Recent research has shown that soluble SBP can be efficiently used as a biocatalyst in the processes such as bio-bleaching of paper dyes [19] and removal of phenol and other aromatic pollutants from waste water [20].

2. Experimental

SBP ($R_Z = A_{403}/A_{280} \approx 0.5$), HRP-C ($R_Z \geq 3.0$), and GdnHCl were purchased from Sigma Co. SBP was purified by DEAE-Sepharose chromatography [10] to $R_Z = 2.8$–3.0. Apo-SBP and apo-HRP-C were prepared by the acid-butanone procedure [21]. Concentration of the protein samples prepared in 50 mM phosphate buffer pH 7.0 were determined spectrophotometrically using $\varepsilon_{403} = 94.6$ mM^{-1} cm^{-1} for SBP [10], $\varepsilon_{403} = 102$ mM^{-1} cm^{-1} for HRP-C [22], $\varepsilon_{280} = 24$ mM^{-1} cm^{-1} for apo-SBP [10], and $\varepsilon_{280} = 20$ mM^{-1} cm^{-1} for apo-HRP-C [23]. The guanidine hydrochloride (GdnHCl) concentration in the stock solution was determined using refractometer according to the reported method [24]. Deionized water (PURITE RO 50) was used in all the experiments. All other chemicals were of analytical grade.

The time course of the unfolding of SBP and HRP-C ($\sim$5 μM) was followed by observing the decrease in absorbance of the Soret band (A_{403}) in Shimadzu UV-2100 spectrophotometer using a cell of 10 mm path length. The reaction was initiated by adding the proteins to the cuvette containing GdnHCl, EDTA, and buffer at pH 7.0, 25 °C. Excess of EDTA (15 mM) was maintained in the solutions of holo proteins so that the unfolding proceeds irreversibly [25]. Unfolding kinetics of apo proteins (10 μM) was followed by monitoring the increase in the tryptophan fluorescence in Hi-Tech SF61MX stopped-flow spectrometer at pH 7.0, 22 °C. The reaction was initiated by rapidly mixing (dead time of the instrument is <2 ms) two buffered solutions of protein and GdnHCl in 1:1 or 1:2 ratios. Tryptophan was excited at 295 nm, where the contribution from tyrosine residues is minimum [26]. A 320 nm cut off filter was used to prevent scattering. The kinetic traces were analyzed by the non-linear least squares algorithm for a first order reaction provided with the stopped-flow instrument and with the *Origin* (*MicroCal*) software.

$$Y = A \exp(-k_U t) + Y_0 \quad (1)$$

where Y, is the observed change of the physical parameter (absorbance or fluorescence) with time t, A the amplitude of the change in the physical parameter corresponding to the unfolding rate constant k_U and Y_0 is the final offset. Goodness of the fit was determined from the randomness of residual distribution.

3. Results

Both SBP and HRP-C are resistant to urea [12,27] and therefore we monitored unfolding in GdnHCl. Fig. 2 represents typical unfolding kinetics of SBP and HRP-C in 7.6 M GdnHCl. All kinetic unfolding curves were fitted to single exponential function in the GdnHCl concentration range of 6.5–8.0 M for SBP, 5.5–8.0 M for HRP-C and 3.4–4.5 M for apo proteins giving rise to the rate constant, k_U, for the unfolding process from