Kinetics of Irreversible Thermal Denaturation of Horseradish Peroxidase

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(Received 21st November 2005, revised 13th June 2006)

Summary: Horseradish peroxidase (HRP) isolated from roots of horseradish was purified by a combination of ammonium sulfate, ion-exchange and gel filtration. The specific activity of the purified enzyme was 14.92 fold higher than the crude extract. Purified HRP was then used for kinetic and thermodynamic characterization. Irreversible thermal denaturation followed first order kinetics. The half-life ($t_{1/2}$) of peroxidase was 11.55 min at 65 °C. The enthalpy (ΔH^*) and free energy (ΔG^*) of thermal denaturation of HRP were 92.22 and 102.54 k $Jmol^{-1}$ respectively, at 65 °C. It was suggested that horseradish peroxidase was quite thermostable and could be used for industrial applications.

Introduction

Horseradish peroxidase (HRP; EC 1.11.1.7), an extracellular heme enzyme isolated from horseradish roots, oxidizes a variety of hydrogen donors, such as aromatic substrates, certain heterocyclic compounds and some inorganic ions, particularly the iodide anion, using hydrogen peroxide as oxidant [1]. Although peroxidases are widely prevalent in the plant kingdom, at present, the peroxidase isolated from the roots of horseradish (*Armoracia rusticana*) is the most studied heme peroxidases. The native enzyme consists of a single polypeptide chain with 308 amino acid residues, a heme prosthetic group and two Ca^{2+} ions maintaining enzyme conformation. Its molecular mass is 44 kDa [2].

Peroxidase has attracted industrial attention because of its usefulness as a catalyst in clinical biochemistry, immunoassays, in histochemical staining and in biosensors [3]. HRP applications as an indicator of food processing [4] and for removal of phenols and aromatic amines from wastewater [5], have also been proposed. Some modern applications of peroxidases include the synthesis of different aromatic chemicals and polymeric materials [6]. These diverse possibilities reflect the broad substrate specificity and high specific activity of the natural enzyme. However, actual commercial uses have been limited to the diagnostic arena due to the enzyme's limited stability at elevated temperatures.

The knowledge and understanding of the horseradish peroxidase thermal denaturation and its related mechanisms would offer new possibilities in improving the potential and effective use of this enzyme in such diverse and broad areas. Thus, the aim of this work is to investigate the kinetics and thermodynamics of thermostability for the irreversible denaturation of this enzyme in order to add information to the nature of denaturation of extracellular enzymic proteins as worked out for extracellular β-glucosidase from *Aspergillus* sp.

Results and Discussion

Crude horseradish peroxidase was partially purified after subjecting it to ammonium sulfate, anion-exchange chromatography and gel filtration. The specific activity of crude enzyme was 12 U mg^{-1}. The onset of HRP precipitation occurred at 30 %, while complete precipitation was observed at 85 % of ammonium sulfate at 0°C. The % recovery of HRP was 83.89 after ammonium sulfate precipitation (Table-1). Peroxidases from *Glycine max* var NH_2 [7] were found to be precipitated at 80% ammonium sulfate saturation.

Partially purified enzyme after ammonium sulfate precipitation was loaded on to anion-exchange column. Purification of HRP after anion-exchange column was 4.47 fold and 43.22 % recovery. Purified HRP, from anion-exchange column, was further purified by applying on gel filtration column. The percentage recovery of enzyme after gel filtration was 18.22 while its purification became 14.52 fold with respect to crude enzyme. The specific activity of the purified HRP was 179 U mg^{-1}. The results of purification are summarized in Table-1.

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Table-1: Purification of horseradish peroxidase

Treatment	Total Activity (U)	Total protein (mg)	Specific activity (U mg^{-1})	Purification factor	Recovery (%)
Crude	236	19	12	1.00	100
85 % $(NH_4)_2SO_4$ precipitation	198	12	16.50	1.37	83.89
Anion-exchange chromatography	102	1.8	53.68	4.47	43.22
Gel filtration	43	0.24	179	14.92	18.22

Thermostability is the ability of enzyme to resist against thermal unfolding in the absence of substrates, while thermophilicity is the capability of enzymes to work at elevated temperatures in the presence of substrate [8]. The thermal denaturation of enzymes is accompanied by the disruption of non-covalent linkages, including hydrophobic interactions with concomitant increase in the enthalpy of activation. The opening up of enzyme structure is accompanied by an increase in disorder, randomness or entropy of activation [9]. The protein melting temperature(T_m) of HRP was 69 °C (Fig. 1). Melting temperatures of 65 °C and 53.3 °C were reported for glucoamylase from *Arachniotus* sp. [10] and a wild-type nuclease [11], respectively.

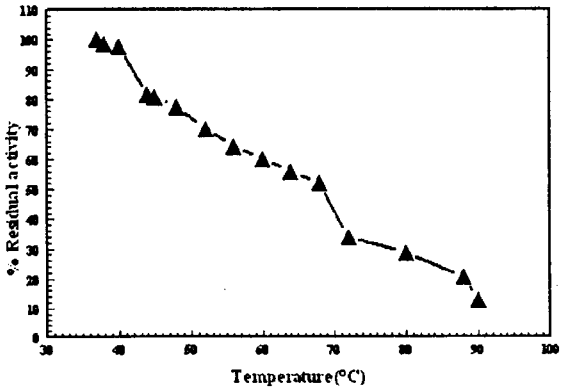

Fig. 1 Melting temperature of HRP.

The kinetics and thermodynamics of irreversible thermal denaturation of HRP were studied at different temperatures *i.e.*, from 45 to 65 °C. The half-life of the enzyme was 99 min at 45 °C and 11.55 min at 65 °C (Table-2). The thermodynamic parameters were determined from Fig. 3. The values of enthalpy of denaturation (ΔH^*) and free energy of denaturation (ΔG^*) were 92.39 $kJmol^{-1}$ and 101.99 $kJmol^{-1}$ while that of entropy of deactivation (ΔS^*) was -30.18 $Jmol^{-1}$ K^{-1} at 45 °C.

Table-2: Kinetics and thermodynamics of irreversible thermal denaturation of horseradish peroxidase

Temp (K)	k_d (min^{-1})	$t_{½}$ (min)	ΔH^* $kJmol^{-1}$	ΔG^* $kJmol^{-1}$	ΔS^* $Jmol^{-1}K^{-1}$
318	0.007	99.00	92.39	101.99	-30.18
323	0.016	43.31	92.35	101.42	-28.08
328	0.021	33.00	92.30	102.29	-30.45
333	0.037	18.73	92.26	102.32	-30.31
338	0.060	11.55	92.22	102.54	-30.53

k_d (first order rate constant of denaturation) are determined from Fig. 2.
$t_{1/2}$ (half-life) = 0.693/ k_d
$\Delta H^* = E_a$ (95.03 kJ mol^{-1}) – RT
Ea (activation energy of denaturation) is calculated from Fig. 3.
$\Delta G^* = -RT \ln (k_d h/k_b.T)$
$\Delta S^* = (\Delta H^* - \Delta G^*)/T$

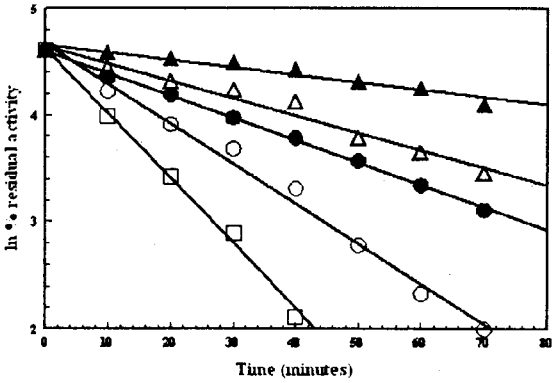

Fig. 2: Irreversible thermal inactivation of HRP. Closed triangle (45 °C), open (50 °C), closed circle (55 °C), open circle (60 °C) and open square (65 °C).

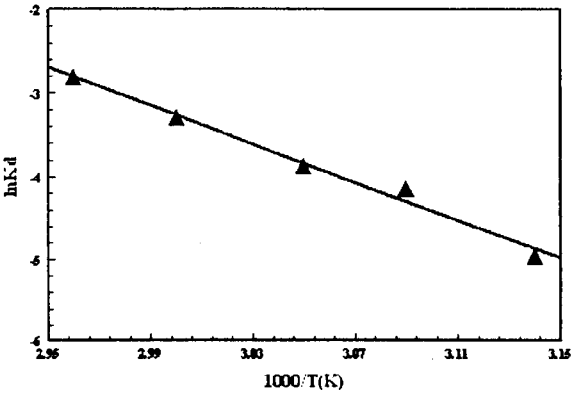

Fig. 3 Arrhenius plots for the determination of energy of activation for irreversible thermal inactivation of HRP

With an increase in temperature, a decrease in half-life was observed (Table-2). At 65 °C, the value of

(ΔG^*) was 102.54 kJmol^{-1} indicating that the HRP showed high resistance to thermal unfolding. Thermodynamically, the enzyme molecule with high (ΔG^*) is considered to be more stable. Moreover, the value of entropy of deactivation (ΔS^*) is less at higher temperature (-30.53 Jmol^{-1} K^{-1}) suggesting that there was negligible disorder in the configuration of HRP at higher temperature. These results are in accord with those of β-glucosidase from *A. wentii* or the transition state of α-amylase from *Bacillus licheniformis,* which were found to be more ordered at a higher temperature as revealed by their negative ΔS^* [12].

It was concluded that HRP isolated from local horseradish possessed high thermal stability and could be used in industrial applications.

Experimental

Enzyme Isolation

Horseradish was purchased from the local vegetable market and stored at 4 °C. Clean legumes of horseradish were sliced into small bits and homogenized by thorough blending in extraction buffer (20 mM phosphate buffer, pH 6.8) at a ratio of 20 cm^3 extraction per gram of tissue. The homogenate was filtered through Whatman filter paper and the filtrate centrifuged (10000 x *g*, 15 min). After the centrifugates were pooled, the remaining residues were re-extracted with five volumes of extraction buffer by centrifuging the residue as above. All the centrifugates were pooled and assayed for peroxidase activity and enzyme contents.

Protein Estimation

Total proteins were estimated by Bradford micro assay [13] using bovine serum albumin (BSA) as the standard.

Peroxidase Assay

Peroxidase activity was determined at 25 °C with a spectrophotometer (Hitachi model U-2001) following the formation of tetraguaiacol as reported earlier [7] with slight modification in pH. The reaction mixture contained 0.95 ml phosphate buffer pH 6.5, 1 ml of 15 mM guaiacol, 1 ml of 1.6 mM H_2O_2 and 50 μl of enzyme extract. One unit of peroxidase activity (U) represented the amount of enzyme catalyzing the oxidation of 1 µmol of guaiacol in 1 min.

Enzyme Purification

Solid ammonium sulfate was added to the crude extract of horseradish peroxidase to get 85 % saturation. It was kept at 4 °C overnight. After 24 h, the resulting precipitate was collected by centrifugation at 10000 x *g* for 30 min. The precipitate was dispersed in phosphate buffer pH 6.8 and dialyzed extensively against three changes of same buffer to remove the salts. The dialyzed sample was applied on DEAE-cellulose column (2.4 x 26 cm) equilibrated with Tris/ HCl buffer pH 7.5. The linear gradient of NaCl (0 – 1.0 M) in 20 mM Tris/HCl pH 7.5 was used as elution buffer. Two ml size fractions were collected. The active fractions were pooled and dialyzed against distilled water. The sample from DEAE- cellulose column was then applied to Sephadex G-100 column (2.4 x 26 cm) previously equilibrated with phosphate buffer pH 6.8 and eluted with the same buffer as described earlier [7]. The eluates containing peroxidase activity were pooled and assessed for irreversible thermal denaturation.

Thermal Stability

Thermal stability of the purified peroxidase was determined by incubating the enzyme in 50 mM MES monohydrate buffer (pH 5.5) at different temperatures for 5 min. At the end of incubation, the enzyme samples were cooled on ice before performing assay as described above [7].

Kinetics of Thermal Denaturation

Kinetic and thermodynamic parameters for irreversible thermal denaturation of HRP were determined by incubating the enzyme in 50 mM MES monohydrate buffer (pH 5.5) at a particular temperature. Aliquots were withdrawn at different times, cooled on ice for 3 h [14] and assayed for enzyme activity at 25 °C as described above. This procedure was repeated at five different temperatures ranging from 45 to 65 °C. The data were fitted to first order plots (Fig. 1) and analyzed as described earlier [15, 16].

The thermodynamic parameters for thermal stability were calculated by rearranging the Eyring's absolute rate equation derived from the transition state theory [17] as described by [18].

$k_d = (k_b T/h)\, e^{(-\Delta H^*/RT)} .e^{(\Delta S^*/R)}$ ..... (1)

Where,

h = Planck's constant = 6.63×10^{-34} Js

k_b = Boltzman's constant (R/N) = 1.38×10^{-23} JK^{-1}

R = gas constant = 8.314 JK^{-1} mol^{-1}

N = Avogadro's No. = 6.02×10^{23} mol^{-1}

T = Absolute temperature

ΔH^* (enthalpy of activation) = $Ea - RT$ ..... (2)

ΔG^*(free energy of activation) =- RT ln (k_d $h/k_b.T$)..... (3)

ΔS^*(entropy of activation) = $(\Delta H^* - \Delta G^*)/T$..... (4)

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