Asian Journal of Chemistry Vol. 22, No. 10 (2010), 7883-7890

Kinetics and Thermodynamics of Thermal Inactivation of Peroxidase from Cauliflower (*Brassica oleracea* L. var. botrytis) Leaves

UMME KALSOOM, HAQ NAWAZ BHATTI*, IJAZ AHMAD BHATTI and MUHAMMAD ASGHER
Department of Chemistry & Biochemistry, University of Agriculture, Faisalabad-38040 Pakistan
Fax: (92)(41)9200764; E-mail: hnbhatti2005@yahoo.com

Peroxidase from leaves of cauliflower (*Brassica oleracea* L. var. botrytis) was isolated and partially purified by means of ammonium sulfate precipitation. The enzyme exhibited maximum activity at pH 5.5 and temperature 50 °C. The values of K_m and V_{max} for guaiacol oxidation were 7.14 mM and 204.1 μmol/min, respectively. Thermal inactivation of the peroxidase was carried out in aqueous solution at temperatures ranging from 60 to 80 °C. The enthalpy (ΔH^o) and free energy (ΔG^o) of thermal denaturation of cauliflower peroxidase were 89.60 and 109.73 k J/mol, respectively at 80 °C. Metals like Hg^{2+} slightly inhibited the peroxidase activity while Zn^{2+}, Mn^{2+}, Cr^{3+}, Ni^{2+}, Co^{2+} and Ca^{2+} have no effect on enzyme activity. The effects of some surfactants and commercial detergents have also been investigated. The thermal stability makes cauliflower peroxidase an alternative to horseradish peroxidase in commercial applications.

Key Words: Cauliflower peroxidase, Thermal inactivation, Kinetics, Thermostability, Free energy.

INTRODUCTION

Peroxidase (EC1.11.1.7; donor: hydrogen-peroxide oxidoreductase) is the heme containing glycoprotein that catalyzes the oxidation of different electron donor substrates such as aromatic amines, phenols, *etc.* by hydrogen peroxide[1]. Peroxidase is one of the important enzyme controlling plant growth and development. It is well established fact that this enzyme is involved in construction and rigidification of plant cell walls, in the biosynthesis of hydrogen peroxide, in the protection of plant tissue from damage and infection from pathogens, in wound healing and response to air pollutant stress and in auxin metabolism[2-5]. This enzyme is commonly used in microanalysis and construction of enzyme electrode[6,7]. Currently peroxidases are also used in the treatment of waste waters containing phenols and aromatic amines, organic synthesis and removal of peroxide food stuffs and industrial wastes[8-10]. This wide range of applications of peroxidases reflects their broad substrate specificity.

Although peroxidases are widely distributed in the plant kingdom, at present the major source of commercially available peroxidase is horseradish roots[11]. However, peroxidases with comparable as well as higher stabilities and different substrate specificities have been isolated from different plant sources[6,12-18]. Therefore, the

availability of thermally stable peroxidase from sources other than horseradish roots would go a long way towards the development of a biocatalyst with broad analytical and commercial applications.

In the present paper we describe the isolation, partial purification and thermal characterization of a peroxidase as a part of the efforts being made to discover new sources of thermostable biocatalyst for commercial applications.

EXPERIMENTAL

All the chemicals and reagents used in the present study were of analytical grade and mainly purchased from Sigma, Chemical Company, USA, unless otherwise mentioned.

Cauliflower (*Brassica oleracea* L. var. botrytis) leaves of a local cultivar, were collected from a local vegetable form and stored at 4 °C.

Peroxidase extraction: Cauliflower (*Brassica oleracea* L. var. botrytis) leaves were thoroughly washed with distilled water to remove surface impurities. The leaves were homogenized by blending in extraction buffer (0.1 M phosphate buffer, pH 7.0) at a ratio of 20 mL extraction per gram of tissue. The homogenate was filtered through Whatman filter paper No. 1 and the filtrate centrifuged at 10,000 x g for 15 min. After the centrifugates were pooled, the remaining residues were reextracted with extraction buffer by centrifuging the residue as above. All the centrifugates were pooled and assayed for peroxidase activity and protein contents.

Protein and peroxidase assay: Total proteins were estimated by Bradford micro assay using bovine serum albumin (BSA) as the standard[19]. Peroxidase activity was determined at 30 °C with a spectrophotometer (Hitachi model U-2001) following the formation of tetraguaiacol (A_{max} = 470 nm, ε = 26.6 mM^{-1} cm^{-1}) with slight modification[12]. The reaction mixture contained 1 mL of 0.1 M acetate buffer (pH 5.0), 1 mL of 15 mM guaiacol, 1 mL of 1.6 mM H_2O_2 and 60 µL of enzyme extract. One unit of peroxidase activity (U) was defined the amount of enzyme catalyzing the oxidation of 1 µmol of guaiacol in 1 min.

Partial purification of peroxidase: Solid ammonium sulfate (168 g) was added to *ca.* 300 mL of crude extract of cauliflower leaves to get 80 % saturation. It was left overnight at 4 °C overnight. After centrifugation at 10,000 x g for 15 min at 4 °C, the precipitate was dispensed in 0.1 M phosphate buffer of pH 7.0 and dialyzed extensively against four-five changes of distilled water to remove the salts. The eluates containing peroxidase activity were pooled and assessed for kinetic and thermal characterization.

Effect of pH and temperature: The effect of pH on cauliflower leaves peroxidase activity was determined by assaying the enzyme as mentioned before with the difference that the activity was determined at different pHs ranging from 2-10 at 30 °C using acetate, phosphate, glycine/HCl and Tris/HCl buffer. The temperature optima of the enzyme was determined using an appropriate dilution of the enzyme in 0.1M acetate buffer (pH 5.0) and incubating it at different temperatures (30- 70 °C)[16].

Effect of substrate concentration: Cauliflower leaves peroxidase was assayed in the reaction mixtures containing variable amounts of guaiacol [1.0-30 mM)] as substrate using 0.1 M acetate buffer (pH 5.0).The data were plotted to determine the values of kinetic constants (V_{max} and K_m)[20].

Kinetics of thermal inactivation: Kinetics and thermodynamics of irreversible thermal inactivation of cauliflower peroxidase was investigated in 0.1 M acetate buffer (pH 5.0) at a particular temperature. Aliquots were taken at different time intervals, cooled on ice for 2-3 h[21] and assayed for enzyme activity at 30 °C as described above. This procedure was repeated at five different temperatures ranging from 60 to 80 °C. The data were fitted to first order plots (Fig. 1) and analyzed as described earlier[22].

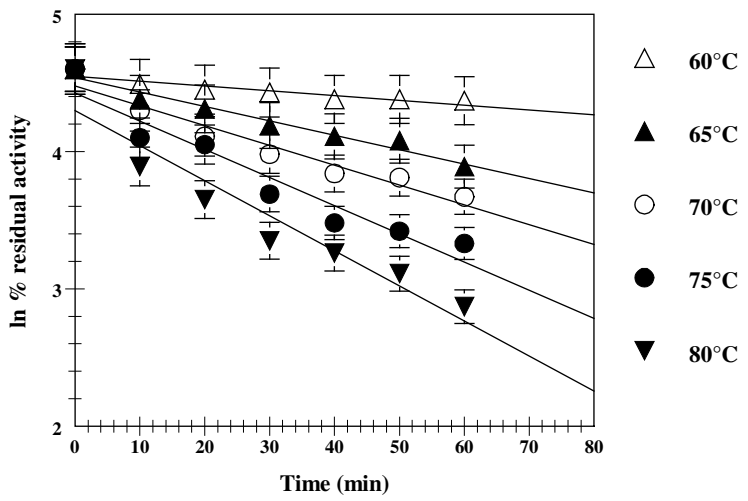

Fig. 1. Pseudo-first order plots of irreversible thermal inactivation of cauliflower peroxidase at different temperatures

The thermodynamic parameters for thermostability were calculated by rearranging the Eyring's absolute rate equation derived from the transition state theory[23].

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

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.

$$\Delta H^o \text{ (enthalpy of activation)} = Ea - RT \quad (2)$$

$$\Delta G^o \text{ (free energy of activation)} = -RT \ln (k_d \cdot h/k_b \cdot T) \quad (3)$$

$$\Delta S^o \text{ (entropy of activation)} = (\Delta H^o - \Delta G^o)/T \quad (4)$$

Energy of activation (Ea) for thermal inactivation was determined from Arrhenius plot (Fig. 2).

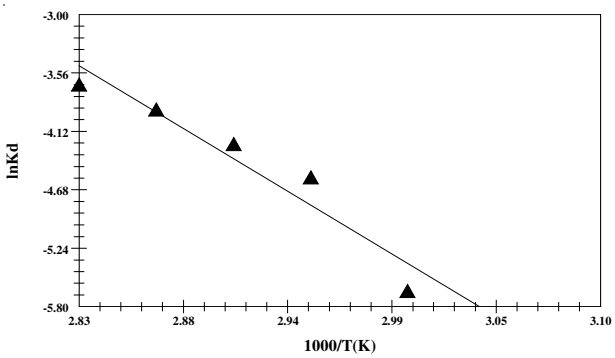

Fig. 2. Arrhenius plot for the determination of energy of activation (Ea) for irreversible thermal inactivation of cauliflower peroxidase

Effect of metal ions and surfactants on activity: The effect of metal ions and surfactants on peroxidase activity was determined in a normal manner in the presence of 0.5 mM and 1 % solutions of different metal ions and surfactants respectively.

RESULTS AND DISCUSSION

Cauliflower (*Brassica oleracea* L. var. botrytis) peroxidase was extracted with 0.1 M phosphate buffer by blending the leaves in a blender with short intermission. The enzyme was then partially purified using ammonium sulphate (80 %) precipitation. The partially purified peroxidase was then used to determine kinetic and thermodynamic parameters. The effect of pH on the activity of cauliflower peroxidase was determined by incubating the enzyme at different pH using appropriate buffers and results are shown in Fig. 3. The optimum pH of the enzyme was found to be 5.5. The change in pH affects the dissociation of amino acid involved in substrate binding and catalysis. Therefore, an optimum pH is necessary for proper working of a biocatalyst. An optimum pH of 5.5 has also been reported for peroxidase isozyme isolated from turnip (*Brassicanapus* L. var. purple top white globe) root[24] and chick pea leaves[16].

The effect of temperature on the activity of peroxidase was investigated by incubating the enzyme at different temperatures (30-70 °C) for 3 min. The results regarding the effect of temperature are presented in Fig. 4. The graph indicated that the enzyme showed maximum activity at 50 °C as compared to low or high temperature. The decrease in activity was more pronounced above 60 °C. Peroxidase was thought to be more heat stable enzyme in plants. An optimum temperature in the range of 40 to 55 °C has been reported for turnip peroxidases[25]. The kinetic parameters (K_m and V_{max}) of cauliflower peroxidase were determined by direct linear method of Lineweaver-Burk plot of initial guaiacol oxidation rates from experimental data. The Michaelis constants K_m and V_{max} for the enzyme estimated from double reciprocal plot were 7.14 mM and 204.1 μmol/min respectively (Fig. 5).

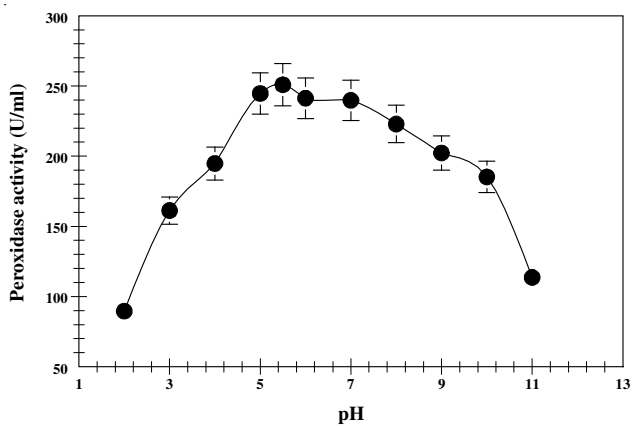

Fig. 3. Effect of pH on the activity of cauliflower peroxidase

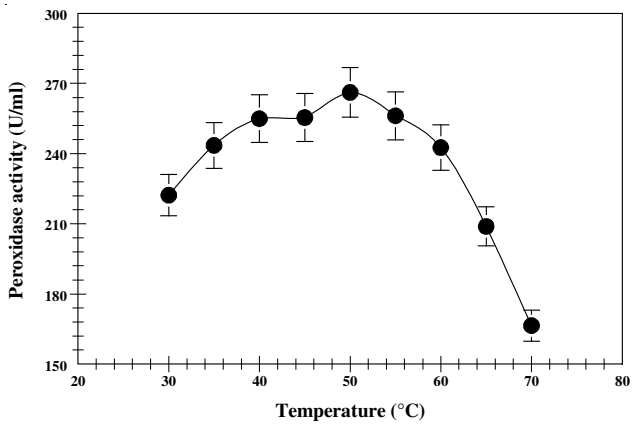

Fig. 4. Effect of temperature on the activity of cauliflower peroxidase

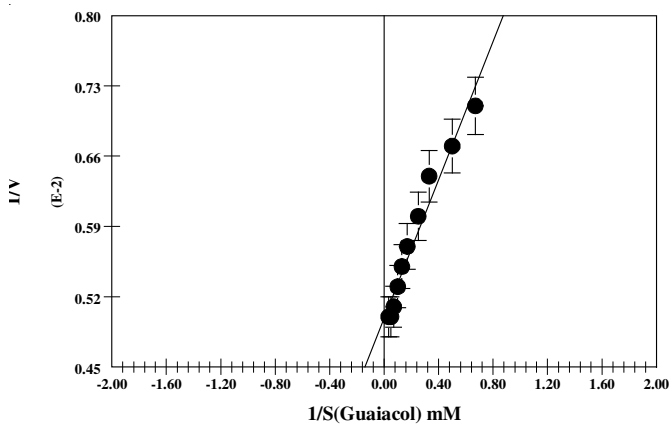

Fig. 5. Effect of guaiacol concentration on the activity of cauliflower peroxidase

The effect of metal ions on cauliflower peroxidase activity was studied and no remarkable increase or decrease in peroxidase activity was observed (Table-1). However Hg^{2+} slightly decreased the peroxidase activity while all other metal ions caused a little bit increase in cauliflower peroxidase activity. The decrease in enzyme activity with 0.1 mM $HgCl_2$ has also been reported for soluble turnip peroxidase[26]. In a previous study it was reported that Zn^{2+}, Mn^{2+}, Co^{2+}, Al^{3+} and Hg^{2+} ions slightly inhibited the chick pea peroxidase activity at a concentration of 1 mM[16]. Industrial wastewater is also contaminated with detergents so effect of surfactants and various commercially available detergents on cauliflower peroxidase activity was also studied and it was found that the peroxidase activity was decreased with Tween-80 and Triton-X-100 and a remarkable decrease in peroxidase activity was observed in the presence of SDS. In commercial detergents Lemon Max bar and Sofi washing powder enhanced the peroxidase activity while other detergents decreased the cauliflower peroxidase activity (Table-2).

TABLE-1
EFFECT OF METAL IONS ON
CAULIFLOWER PEROXIDASE ACTIVITY

Metal ions	Relative activity (%)
Control (H_2O)	100
Zn^{2+}	103
Mn^{2+}	103
Cr^{3+}	103
Ca^{2+}	102
Hg^{2+}	97
Ni^{2+}	101
Co^{2+}	102

*0.5 mM

TABLE-2
EFFECT OF SURFACTANTS ON
CAULIFLOWER PEROXIDASE ACTIVITY

Compound	Relative activity (%)
Control	100
Tween-80	79
SDS	15
Triton-X-100	89
Brite	38
Ariel	35
Bonus	82
Surf excel	54
Sofi	126
Vim	99
Lemon max	182

*1 %.

Thermostability is the ability of enzymes to resist against thermal unfolding in the absence of substrate while thermophilicity is the capability of enzymes to work at elevated temperatures in the presence of substrate[27]. The results of thermal inactivation of cauliflower peroxidase are shown in Table-3. It is obvious from the results that the half-life of the cauliflower peroxidase is 198 min at 60 °C and decreases with increase in temperature. At 80 °C, the half-life decreases to 27.18 min indicating denaturation of enzyme at higher temperature. The enthalpy of thermal unfolding (ΔH^o) decreases with increase in the temperature indicating that the less energy is required for thermal unfolding at higher temperature. The free energy (ΔG^o) of thermal unfolding was 108.85 kJ mol^{-1} at 60 °C. With an increase in temperature, a decreasing trend is observed in the value of ΔG^o indicating thermal inactivation of the enzyme. However, at 80 °C an increase in ΔG^o value (109.73 kJ mol^{-1}) is observed which indicated that cauliflower peroxidase shows resistance against

TABLE-3
KINETICS AND THERMODYNAMICS OF IRREVERSIBLE
THERMAL INACTIVATION OF CAULIFLOWER PEROXIDASE

Temp. (K)	k_d (min^{-1})	$t_{½}$ (min)	ΔH^o (kJ mol^{-1})	ΔG^o (kJ mol^{-1})	ΔS^o (J mol^{-1} K^{-1})
333	0.0035	198.00	89.76	108.85	-0.057
338	0.0105	66.00	89.72	107.44	-0.052
343	0.0144	48.13	89.68	108.17	-0.054
348	0.0200	34.65	89.64	108.84	-0.055
353	0.0255	27.18	89.60	109.73	-0.057

k_d (first order rate constant of denaturation) are determined from Fig. 4; $t_{1/2}$ (half-life) = $0.693/k_d$; $\Delta H^o = E_a$ (104.17 kJ mol^{-1}) – RT; Ea (activation energy of denaturation) is calculated from Fig. 5; ΔG^o = -RT In (k_d h/k_b.T); $\Delta S^o = (\Delta H^o - \Delta G^o)/T$.

thermal unfolding/denaturation at higher temperature. This value is comparable to chick pea peroxidase (103.9 kJ mol^{-1})[16] and horseradish peroxidase (102.54 kJ mol^{-1})[21]. Thermodynamically, the enzyme with high ΔG^o value is considered to be more stable. The entropy of inactivation (ΔS^o) exhibits a fairly constant value at all temperatures at 333 K (-0.057 J mol^{-1} K^{-1}) which indicates that cauliflower peroxidase active site is remains fairly in the ordered state the studied temperatures.

Catalytic proteins, like all other proteins, are only marginally stable at higher temperatures[28]. The thermal unfolding of enzymes is accompanied by the disruption of non-covalent linkages, accompanied by an increase in disorder, randomness or entropy of activation[29]. The apparent kinetics for a soybean hull peroxidase previously[30] indicated that irreversible deactivation is comprised primarily of enthalpic contributions, with ΔH^o_{deact} = 22.4 kcal/mol and $T\Delta S^o_{deact}$ = 0.2 kcal/mol at 95 °C. Heme transfer studies from the peroxidases to apomyoglobin has indicated that soybean hull peroxidase holds onto its heme much more tightly than does horseradish peroxidase and this is consistent with a thermodynamically more stable enzyme[30]. However, the loss of heme from a peroxidase to form the apoenzyme is well-known to occur at extremely elevated temperatures[31]. This apoenzyme is expected to be less stable than the native enzyme.

Conclusion

The present investigation revealed that peroxidase isolated from cauliflower leaves has high specific activity and exhibited considerable thermal stability at higher temperature. This enzyme could be used for commercial applications.

ACKNOWLEDGEMENTS

This work is a part of Ph.D. degree of Miss Umme Kalsoom. The authors are thankful to Higher Education Commission (HEC) of Pakistan for providing funds to accomplish this work.

REFERENCES

1. H.B. Dunford, Heme Peroxidases, Willey-VCH, New York, p. 480 (1999).
2. R.L. Farrell, K.E. Muetagh, M. Tien, M.D. Mozuch and T.K. Kirk, *Enzyme Microb. Technol.*, **11**, 322 (1989).
3. K. Wakamatsu and U. Takahama, *Physiol. Plants*, **88**, 167 (1993).
4. J.A. Smith and R. Hammerschmidt, *Physiol. Mol. Plant Pathol.*, **33**, 255 (1988)
5. K.E. Espelie and P.E. Kolattukudy, *Arch. Biochem. Biophys.*, **240**, 539 (1985)
6. A. Rodriguez, D.G. Pina, P. Yelamos, J.C. Leon, G.G. Zhadan, E. Villar, F. Gavilanes, M.G. Roig, I.Y. Sakharov and V.L. Shnyrov, *Eur. J. Biochem.*, **269**, 2584 (2002).
7. L. Gorton, *Electroanalysis*, **7**, 23 (1995).
8. L.J. Blum, *Enzyme Microb. Technol.*, **15**, 407 (1993).
9. R. Batra and M.N. Gupta, *Biotechnol. Lett.*, **10**, 1059 (1994).
10. K. Ikehata, I.D. Buchanan, M.A. Pickard and D.W. Smith, *Bioresour. Technol.*, **96**, 1758 (2005).
11. I.Y. Sakharov and I.V. Sakharov, *Biochem. Biophys. Acta,* **1598**, 108 (2002).
12. W. Liu, J. Fang, W. Zhu and P. Gao, *J. Sci. Food Agric.*, **79**, 779 (1999).
13. I.G. Gazaryan and L.M. Lagrimini, *Phytochem.*, **41**, 1029 (1996).
14. P.M. Civello, G.A. Martinez, A.R. Chaves and M.C. Anon, *J. Agric. Food Chem.*, **43**, 2596 (1995).
15. T.L. Arnaldos, M.A. Ferrer, R. Munoz and A.A. Calderon, *Plant Physiol. Biochem.*, **39**, 479 (2001).
16. H.N. Bhatti, A. Najma, M. Asgher, M.A. Hanif and M.A. Zia, *Protein Pept. Lett.*, **13**, 799 (2006).
17. T. Thongsook and D.M. Barrett, *J. Agric. Food Chem.*, **53**, 3206 (2005).
18. E. Köksal and I. Gülçin, *Protein Pept. Lett.*, **15**, 320 (2008).
19. M.M. Bradford, *Anal. Biochem.*, **72**, 248 (1976).
20. H.N. Bhatti, M. Asgher, A. Abbas, R. Nawaz and M.A. Sheikh, *J. Agric. Food Chem.*, **54**, 4617 (2006).
21. H.N. Bhatti, M.N. Akbar and M.A. Zia, *J. Chem. Soc. Pak.*, **29**, 99 (2007).
22. F.J. Montes, E. Battanar, J. Catalan and M.A. Galan, *Proc. Biochem.*, **30**, 217 (1995).
23. H. Eyring and A.E. Stearn, *Chem. Rev.*, **24**, 253 (1939).
24. M.A. Duarte-Vazquez, B.E. Garcia-Almendarez, C. Regalado and J.R. Whitakar, *J. Agric Food. Chem.*, **49**, 4450 (2001).
25. M.A. Duarte-Vazquez, B.E. Garcia-Almendarez, C. Regalado and J.R. Whitakar, *J. Agric Food. Chem.*, **48**, 1574 (2000).
26. M. Matto and Q. Husain, *J. Mol. Catal. B*, **57**, 164 (2009).
27. J. Georis, F.L. Esteves, J.L. Brasseur, V. Bougnet, B. Devreese, F. Giannotta, B. Granier and J.M. Frere, *Protein Sci.*, **9**, 466 (2000).
28. R. Jaenicke, *Eur. J. Biochem.*, **202**, 715 (1991).
29. C. Vieille and J.G. Zeikus, *Trends Biotechnol.*, **14**, 183 (1996).
30. J.P. McEldoon and J.S. Dordick, *Biotechnol. Prog.*, **12**, 555 (1996).
31. Y. Tamaru and Y. Morita, *J. Biochem. (Tokyo)*, **78**, 561 (1975).

(*Received*: 3 March 2010; *Accepted*: 28 July 2010) AJC-8914