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p^H Dependent Spectral Characteristics of Horseradish Peroxidase dissolved in Aqueous Buffer as well as in Nonionic Reverse Micellar Systems

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Abstract

In this article, the spectral characteristics of horseradish peroxidase in aqueous buffer as well as nonionic reverse micelles is reported at various pH values. It is found that the soret band of HRP slightly shifted in acidic as well as in alkaline media with respect to the neutral p^H(pH 7.00). The shifting of λ_{max} could be related with the conformational change of the enzyme molecule at different pH values. Further, the absorption spectra of the peroxidase in aqueous buffer as well as in nonionic micellar media at Wo = 15, pH = 7.00, is found virtually unchanged. The molar extinction coefficient (ε) of peroxidase at λ = 403 nm remains more or less constant till p^H 11.8 inside the aqueous pool of the reverse micellar system and above this it falls rapidly. The fall of εvalue indicates that there is a change of conformation of the enzyme molecule after pH 11.8.

Key words: Horseradish peroxidase, molar extinction coefficient, Triton X-100, reverse micelles, micellar enzymology.

Introduction

Reverses micelles with solubilized enzymes and proteins find applications in various technological fields[1-7]. It is known that the biological membrane is based on a planner lipid bilayer[8-9].The bio-membrane also contains non-layer lipid structures, in particular "lipid particles"[9]. These are associates of lipid molecules constructed by reverse micelle type and contain between the monolayers of the bilayer membrane. Some proteins, for example cytochrome C and methemoglobin, are capable of inducing the formation of reverse micelles in bilayer membrane, simultaneously inserting into their inner cavity[8]. ATPase, a key bioenergetics enzyme, shows maximum catalytic activity specifically under conditions of the

formation of intramembraneous lipid particles[10]. It has been found[10] that cytoplasmic phospholipase A_1 preferentially attacks phospholipids within non-bilayer structures, while the bilayer portions of the membrane are not subject to enzymatic hydrolysis. It is not excluded that many other membrane enzymes function in vivo in a complex with lipids in the form of reverse micelles[1].

These data in fact present molecular enzymology with new problem: to study the structure and function of enzymes incorporated in reverse micelles. A problem can be solved using the protein-surfactant-water-organic solvent model system. Keeping these ideas in mind the major goal of the present study was to investigate pH dependent spectral characteristics of an interfacial enzyme, horseradish peroxidase (HRP) in an aqueous buffer as well as nonionic reverse micellar systems comprising Triton X-100 - cyclohexane.

Experimental Section

Materials

Triton X-100 (t-octyl-C_6H_4-$(OCH_2CH_2)_x$-OH, where x = 9-10), 1-hexanol, cyclohexane and horseradish peroxidase (with spectral index *Rz* = A_{403}/A_{279} = 0.6) were procured from SRL, India and were used without any further purification. Double distilled water was used in preparing the buffer solutions. Molar ratio of water to Triton X-100 is represented by Wo.

UV spectroscopic studies :

A nonionic reverse micellar solution composed of 0.05 M Triton X-100+1-hexanol (1:5 molar ratio) in cyclohexane was prepared and used throughout the studies. All measurement were done in a Shimadzu (Japan) UV 3000 spectrophotometer in which cells were thermostatically maintained at constant temperature by circulating water from a thermostat. The measured temperature was within ± 0.01°C. The detailed experimental procedure has been described elsewhere[11].

Result and Discussion

pH dependent spectral characteristics of peroxidase dissolved in aqueous buffer as well as Triton X-100 Reverse Micelles :

The spectral characteristics of peroxidase in aqueous buffer systems at three different pH have been shown in figure1a &1 b. It has been observed that in acidic as well as in alkaline media the peak of the soret band of peroxidase slightly shifts from that of enzyme in the neutral pH (pH 7.00). In the acidic medium at pH 5.90, the λ_{max} shifts from the original peak position which is at 403 nm to 401 nm and in alkaline medium it shifts from 403 to 417 nm. This shifting of λ_{max} in an aqueous buffer system can be correlated with the conformational change of the enzyme molecule at different pH values. Maehly *et al.*[12] has discussed the spectral characteristics of the heme protein in different pH solutions. He has shown that the acid splitting of the aqueous solution of HRP proceeds with the formation of number of intermediate compounds, formed and decomposed spontaneously once the acid is added. This splitting reaction highlighted the nature of bonds between hemin and protein and gives a better understanding

of the chemical and eventually enzymatic behavior of peroxidase. The spectral change which occurs when the pH of the enzyme is lowered can be described in the light of Maehly's works. The acidic form of the enzyme shows a peak at about 401nm at pH 5.9 which is due to the formation of protohemin. Due to the instability of the compound, the value of the maximum optical density is maintained only for a short period of time, a time interval which decreases with decreasing pH. The stability of HRP in the alkaline range is remarkable for the protein molecule. The colour of HRP changes from brown to red upon addition of alkali. The maximum absorption in alkaline pH was found at about 417 nm which is in good agreement to that reported by Maehly[12].

Figure 1cshows the spectral characteristics of peroxidase in an aqueous medium (pH 7.00) and in a medium of nonionic reverse micelles composed of Triton X-100+1-hexanol (1:5 molar ratio) – cyclohexane with Wo = 15 (pH 7.00). It is evident from the figure that the character of the peroxidase spectrum is virtually unchanged when the enzyme is transferred from an aqueous medium to a medium of strongly hydrated Triton X-100 reverse micellar system. Upon comparison of the peroxidase spectra in an aqueous buffer solution (curve a) and peroxidase solubilized in nonionic reverse micellar media (curve b); the fact can be made clear. The small difference of the ε value of course exists, which may be due to the change of the microenviron-ment of the enzyme molecule. Further, it is apparent from the figure that the solubilization of hemoprotein in reverse micelles does not shift the original soret band position which is at 403 nm. The above result is in consistence with the results reported by Martinek[13] according to whom this is apparent upon comparison of the spectra of peroxidase in an aqueous buffer solution and peroxidase solubilized in octane by AOT reverse micelles with a 26.1 degree of hydration. Both the spectra have the identical position of the characteristics bands *i.e.* at 640, 498 and 403 nm. The small difference of the molar absorption of the compared samples is obviously there, which is due to the influence of the microenvironment *i.e.* the transfer of protein molecule from as aqueous environment to the hydrocarbon phase. The soret band intensity at 403 nm however, is changed when the enzyme is dissolved into aqueous core of the reverse micelles. The small difference in the absorption of the compared samples is apparently due to the influence of the microenvironment.

pH dependent molar extinction coefficient (ε) of peroxidase in TritonX-100 reverse micellar system :

Figure 2 shows pH dependent molar extinction coefficient of peroxidasein Triton X-100 reverse micelles in cyclohexane at Wo = 18. It is clear from the figure that an increase in the pH of the solubilizing peroxidase solution also results in the appearance of the alkaline form of the peroxidase as in the aqueous solution but the difference is that they are observed only at pH above 11.8. Unlike buffer system

the value of ε_{403} nm remains more or less constant till pH 11.8 inside the aqueous pool of the reverse micellar system and above which it falls rapidly. The fall of ε_{403} value indicates that there is a change of conformation of the molecule at that particular wave length after pH 11.8.

It has been established by Maehly that peroxidase in an aqueous medium changes its spectral characteristics in a wide a wide range of pH values[12]. The spectral changes occur when the pH of the enzyme solution is lowered by the addition of HCl. Its spectrum shows a peak at 407.5 nm in the soret band which is increasingly unstable with decreasing pH; it is converted into an intermediate compound 'B' at wavelength of 398.5 nm. Then this compound 'B' is immediately transferred into an intermediate compound 'C' with a spectrum very similar to that of protohemin called as protohemin I having λ_{max} value at 375 nm. Then gradually compound 'C' is converted into compound 'D' with a significant different spectrum. These are the facts that we are observing a sharp change of ε value at 403 nm in an acidic medium[12].

In an alkaline medium the stability of HRP is remarkable for a protein molecule. Kelin and Mann[14] have found that the colour of HRP changes from brown to red upon addition of alkali. They found the pK of the change to be 10.90 as measured spectroscopically. The peak of the soret band is shifted from 403 nm to 417. Upon a pH about 12.00 no measurable instability is observed and undamaged HRP can be obtained by lowering the pH again. At pH 12.5 splitting sets in, leading to alkaline to protohemin (hematin). The spectrum of the latter has a flat maximum at about 395 nm. But the product of this alkaline protohemin could not be recombined to the original enzyme by lowering the pH again under the same conditions. Even the titration curve is uninfluenced by the anion concentration is a measure of pH only. So it seems reasonable to conclude that either a protein enters a hem-linked group of the HRP molecule, or that a OH- group leaves the Fe-atom when compound 'B' is formed. Therefore it is an acid form of HRP which may be the factor for the low catalytic activity of HRP in an acid medium as compound 'B' is unable to form any H_2O_2 complexes. Then the final splitting of the reaction leading from 'B' to protohemin probably represent the splitting of two linkages, one may be the breaking of carbonyl iron bond and the other may be propionyl-protein linkage. The final spectral changes of protohemin I $\rightarrow$ protohemin II is possibly a rearrangement of the hemin molecules after the loss of protein. Theorell *et al*.[15] found in the differential titration experiments a difference of two equivalents per mole between the free protein and recombined HRP in the range of pH 5.50–9.00.

These facts confirm the fact that in an aqueous medium the enzyme acquires different conformations; which is directly responsible for the loss of enzymatic activity

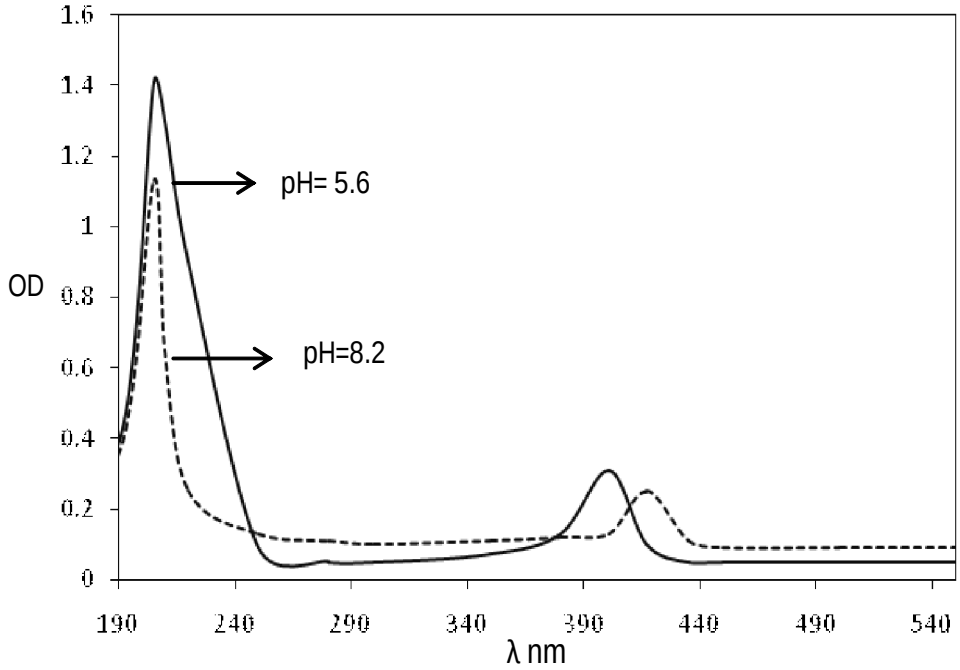

Figure.1a. Spectral characteristics of peroxidase in aqueous buffer at pH 5.6 and pH 8.2, [E] = 1.9 µM at pH 5.6, [E] = 1.5 µM at pH 8.2, T = 25ºC.

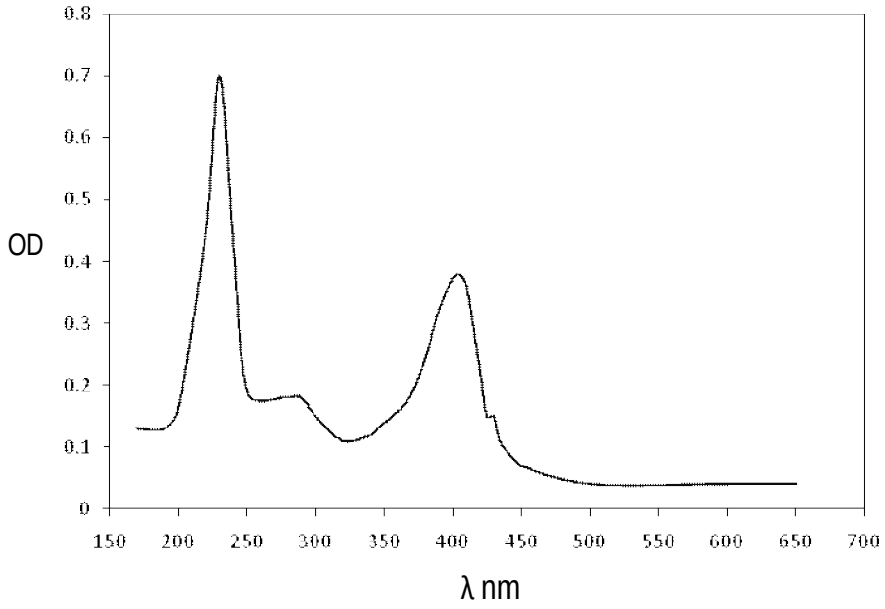

Figure1b. Spectral characteristics of peroxidase in aqueous buffer at pH 7.00, [E] = 5.00 µM, T = 25 ºC.

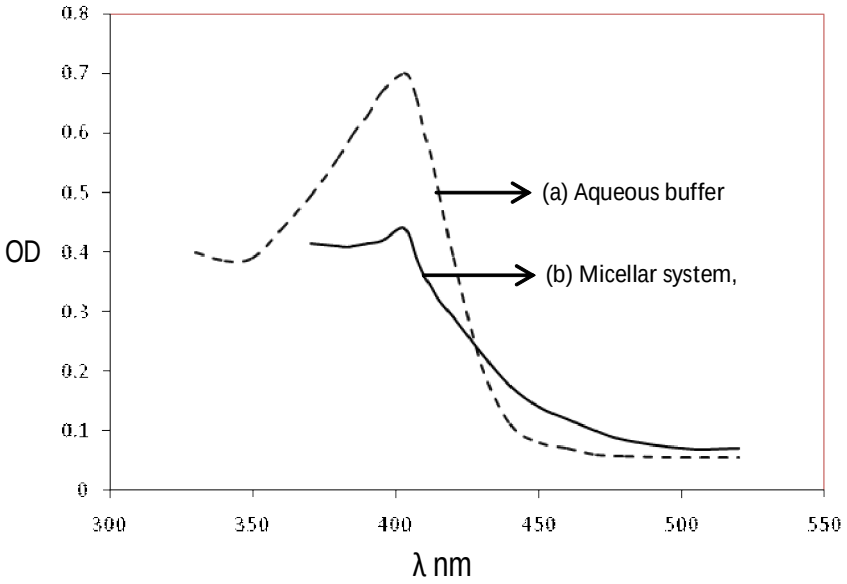

Figure 1 C. Spectral characteristics of peroxidase (a) in aqueous buffer [E] = 6.52 µM and (b) in Triton X-100 reverse micellar media at Wo =15. [E] = 4.5 µM, pH= 7.00, T=25ºC.

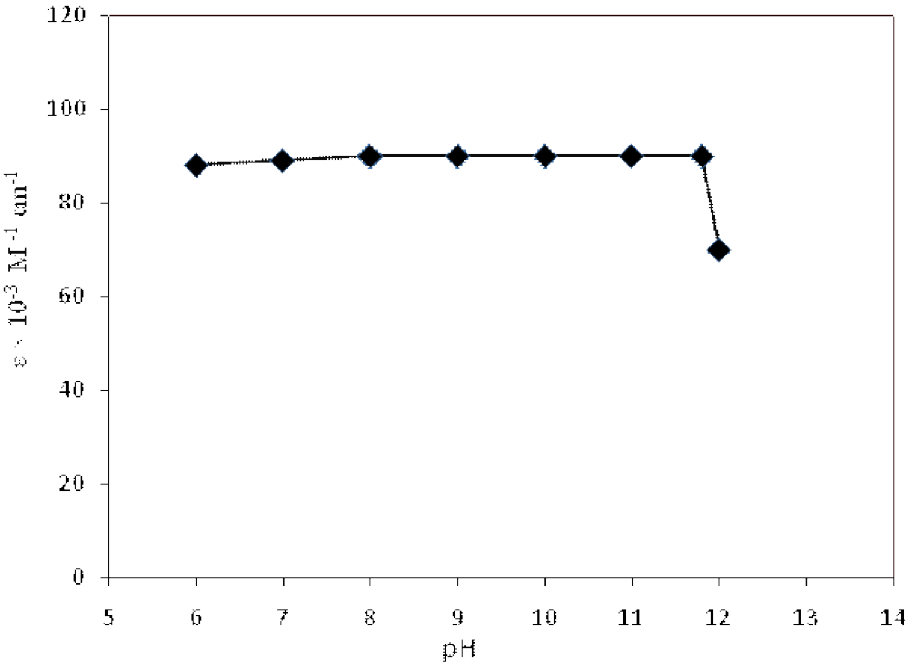

Figure 2. Variation of molar extinction coefficient of peroxidase with pH inTriton X-100 reverse micellar media at Wo = 18, [E] = 6.2 µ M, T= 25 ºC.

at low pH values also. But the most interesting fact is that a Triton X 100 - cyclohexane reverse micellar system at Wo = 18, an increase in the pH of the stock peroxidase solution results in the appearance of new absorption bands "alkaline form" of peroxidase; characterized by the same spectral characteristics as in an aqueous solution but with the difference only at pH above 11.8 (Fig. 2). But that transition in an aqueous solution is in fact characterized by a pK of 11.1. Thus a pH, a higher value in micellar media than that in an aqueous solution is observed. The reason for the observed effect is, apparently, a local shift in pH in the internal cavity of the enriched micelles due to nonionic nature of the surfactant used[16-17]. This shift of the pH profile in the micellar system made it possible to measure the catalytic activity of HRP by using pyrogallol as the substrate even at pH > 8 in the micellar media, which is impossible in the aqueous system.

Conclusions

In this paper,the spectral characteristics of horseradish peroxidase in aqueous buffer as well as nonionic reverse micelles have been reported at various pH values. It was found that the soret band of HRP slightly shifted in acidic as well as in alkaline media with respect to the neutral pH (pH 7.00). The shifting of λ_{max} could be related with the conformational change of the enzyme molecule at different pH values. The small difference of the ε value of course exists, which may be due to the change of the microenvironment of the enzyme molecule**.** Further, the absorption spectra of the peroxidase in aqueous buffer as well as in nonionic micellar media at Wo = 15, pH = 7.00, is found virtually unchanged. The pH dependent molar extinction coefficient (ε) of peroxidase in reverse micelles shows thatε values at λ 403nmremains more or less constant upto pH 11.8 and thereafter with increase in pH, it falls rapidly**.** The fall of εvalue indicates that there is a change of conformation of the peroxidase after pH 11.8.

References

1. (i). S. Roy, A. Dasgupta, P.K. Das. *Langmuir*, 22, 4567-4573 (2006) and references therein. (ii). R. Biswas, A.R. Das, T. Pradhan, D. Touraund, W. Kunz, and S. Mahiddin., *J. Phy. Chem. B,*112, 6620 – 6628 (2008).
2. K. Holmberg, *Adv.Colloid. Interface Sci, 51,* 137 (1994).
3. M. Moniruzzaman,,N. Kamiya, M. Goto, *Langmuir, 25,* 977-982 (2009) and references therein.
4. A. S. Bommarius, T. A. Hatton, D.I.C. Wang, *J. Am. Chem. Soc. 117,* 4515 (1995).
5. F. Franks, Water A Comprehensive Treatise. Plenum, New York (1975).
6. C.M.L. Carvalho, J.M.S. Cabral, *Biochimie, 82,* 1063 (2000).
7. P.L. Luisi, L. Magid**,** *CRC Critical Rev. Biochem***,** *20,* 409 (1986).
8. (i) K. Martinek, A.V. Levashov, Y.L. Khmelnitsky, N. L. Klyachko and I.V. Berezin, *Science, 218,* 889 (1982). (ii) N. L. Klyachko, A.V. Levashov and K.

Martinek, *J. Mol.Bol*, *18(4)*, 830-840 (1984) and references therein.

9. R. P. Cullis, B. de kruijff, M.H. Hope, R. Nayar, and S.L Schmid, *Cad. J. Biochem*, *58*, 1091-1100 (1980).
10. (i). S.W. Huk, T.P. Stewart, P.L.Yeagle and A.D. Albert, *Arch. Biochem. Biophys*, *207*, 227-240 (1981).
    (ii). R. M.C. Dawson, *J. Am. Oil. chem. Soc*., *50*, 401-416 (1982).
11. S. Sarkar, T.K. Jain and A. N. Maitra, *Biotechnol Bioeng***,** 39, 474 (1992).
12. A. C. Maehly, *Biochemics Biophysics Acta*, *4*, 1-17 (1952).
13. (i). K. Martinek, A.V. Levashov and N. L. Klyachko, *Molecular Biology (Russ)*, *18(4)*, 1019-1031 (1984).
    (ii). Yu. L. Khel'nitskii, A.V. Levashov, N. L. Klyachko, V. Ya. Chernyak and K. Martinek, *Biockimiya*, *47*, 86-99 (1981).
14. D. Kelin and T. Mann, *Proc. Roy. Soc*. (London), B1. 22, 118 (1937).
15. H. Theorell, *Arkiv Kemi. Mineral Geol*, 16A, No 3 (1942).
16. F.M. Menger, G. Saito, *J. Am. Chem. Soc.*, *100*, 4376-4379 (1978).
17. A.V. Levashov, N. L. Klyachko and K. Martinek, *Bioorg. Kemi*, *7*, 670-679 (1981).