Asian Journal of Chemistry; Vol. 27, No. 8 (2015), 3057-3062

ASIAN JOURNAL OF CHEMISTRY

http://dx.doi.org/10.14233/ajchem.2015.18863

Influence of Temperature and pH on Spectral Characteristics of Horseradish Peroxidase in Aqueous Buffer as well as in Nonionic Reverse Micellar System

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Received: 18 November 2014; *Accepted*: 27 December 2014; *Published online*: 27 April 2015; AJC-17192

In this article, temperature dependent spectral characteristics of peroxidase dissolved in aqueous buffer as well as in nonionic reverse micellar systems are reported at different pH values over a wide range of temperature. It is found that the soret band of peroxidase remains unchanged in reverse micellar media as compared to aqueous buffer solution at lower temperature ($T \leq 20$ °C) and then gradually changes as the temperature is increased. At 35 °C the total shape of the spectra changes and at 50 °C it is much more in a distorted form. As the temperature is gradually increased, the total shape of the soret band changes into somewhat random type. This change of the spectral behaviour of horseradish peroxidase at different temperature can be attributed to the structural changes of the enzyme molecule encapsulated inside the aqueous core of the reverse micelles. The absorption spectra of peroxidase with temperature have been studied in reverse micellar media at various W_o. Two types of behaviour of the enzymes are found inside the micellar aqueous core; (i) in the temperature range of 10-30 °C and (ii) in the temperature range 30-50 °C. Again for $W_o \leq 15$ the enzyme behaves in the similar fashion and $W > 15$ and in the aqueous system, the enzyme behaves in a different fashion. The absorption values slowly increases for $W_o \leq 15$ and attains constant value after that. For $W_o = 18$ and 20, the absorption value remains constant till the temperature is 30 °C and above that it slowly decreases. The change in absorption spectra of peroxidase molecule with temperature is due to some structural perturbation of the enzyme molecule in reverse micellar media.

Keywords: 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[1-4]. The incorporation of enzymes and proteins into reverse micelles has opened up a new approach to the modeling of biochemical processes of the cell. The enzymes, the active biocatalysts for all kinds of biochemical transformation in living cells, most commonly remain active either on the surface of the biological membranes or inside them. However, the water around the interface, which is considered to be a crucial parameter in regulating enzyme activity, differs enormously in all aspects from "bulk" water[5]. Thus to have a better understanding of the enzyme action, reverse micellar enzymology has been evolved to provide a more realistic biomimetic model for living cells[6,7].

It is known that the biological membrane is based on a planner lipid bilayer[8,9]. The biomembrane also contains non-bilayer 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 intramem-braneous lipid particles[8]. It has been found that cytoplasmic phospholipase A1 preferentially attacks phospholipids within non-bilayer structures, while the bilayer portions of the membrane are not subject to enzymatic hydrolysis[10]. 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 *e.g.*, 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 main aim of the present study is to investigate temperature dependent spectral characteristics of an interfacial enzyme, horseradish peroxidase (HRP) in aqueous buffer solution as well as in nonionic reverse micellar systems.

EXPERIMENTAL

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 $R_z = A_{403}/A_{279} = 0.6$) 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 surfactant (Triton X-100) is represented by W_o.

UV spectroscopic studies: A nonionic reverse micellar solution composed of 0.05 M Triton X-100 in cyclohexane was prepared and used throughout the studies. 1-hexanol was used as a co-surfactant which was present 1:1 volume ratio with respect to Triton X-100 in cyclohexane. UV spectra were recorded 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].

RESULTS AND DISCUSSION

Spectral characteristics of peroxidase dissolved in aqueous buffer as well as in Triton X-100 reverse micelles at different pH at T = 25 °C: The spectral characteristics of peroxidase in aqueous buffer systems at three different pH at constant temperature (T = 25 °C) was recorded and are shown in Fig. 1a and 1b. It is 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). In the acidic medium at pH 5.60, the λ_{max} shifts from the original peak position which is at 403 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[12] has discussed the spectral characteristics of the heme protein in different pH solutions. He had shown that the acid splitting of the aqueous solution of horseradish peroxidase 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 behaviour 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[12]. The acidic form of the enzyme shows a peak at about 401 nm at pH 5.6 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 horseradish peroxidase in the alkaline range is remarkable for the protein molecule. The colour of horseradish peroxidase 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].

Fig. 1c shows the spectral characteristics of peroxidase in an aqueous medium (pH 7) and in a medium of nonionic reverse micelles composed of Triton X-100 + 1-hexanol (1:1 volume ratio) – cyclohexane with $W_o = 15$ (pH 7). It is evident from the figure that the character of the peroxidase spectrum

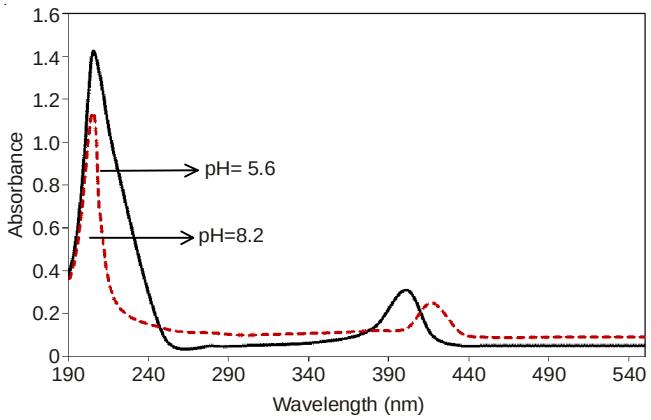

Fig. 1. (a) Spectral characteristics of peroxidase in aqueous buffer at pH 5.6 and pH 8.2, [E] = 1.9 µM at pH 5.6, [E] = 1.5 µM at pH 8.2, T = 25 °C

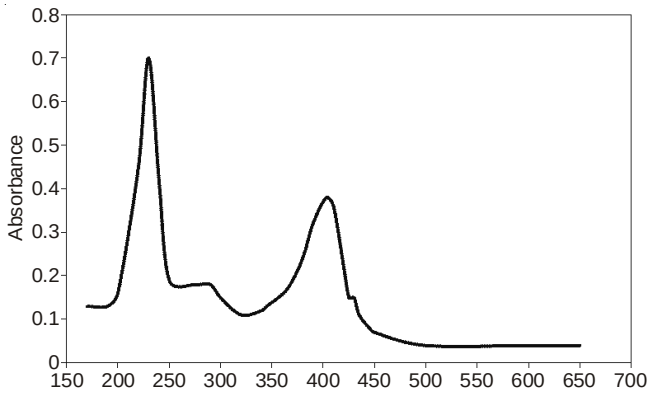

Fig. 1. (b) Spectral characteristics of peroxidase in aqueous buffer at pH 7, [E] = 5 µM, T = 25 °C

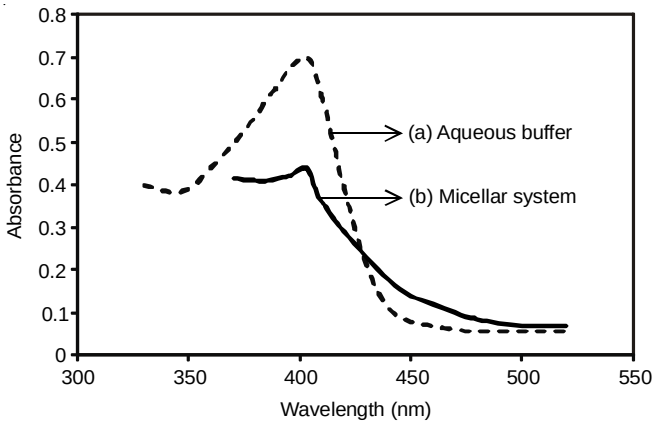

Fig. 1. (c) Spectral characteristics of peroxidase (a) in aqueous buffer [E] = 6.52 µM and (b) in Triton X-100 reverse micellar system at W_o = 15, [E] = 4.5 µM, pH = 7, T = 25 °C

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 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 microenvironment of the enzyme molecule. Further, it is apparent from the Fig. 1c that the solubilization of hemoprotein in reverse micelles does not shift the original soret

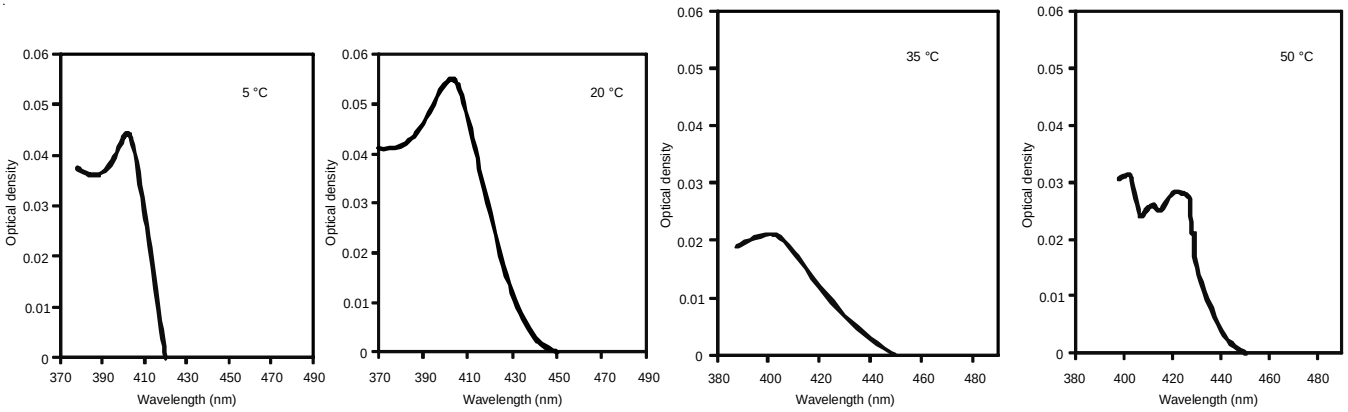

Fig. 2. Spectral characteristics of peroxidase in reverse micellar media at various temperatures, W_o = 18, pH = 7

band position which is at 403 nm. The above result is in consistence with the results reported by Martinek *et al.*[13] according to whom 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.

Temperature dependent spectral characteristics of peroxidase in Triton X-100 reverse micelles: To know the influence of temperature on the spectral characteristics of peroxidase molecule hosted in Triton X-100 reverse micellar system at W_o = 18, the spectral behaviour of peroxidase has been recorded in the soret band at different temperature are shown in Fig. 2. It is found that at lower temperature range of 5-20 °C the spectral behaviour remains unchanged as compared to aqueous buffer solution and then gradually changes as the temperature is increased. At 35 °C the total shapes of the spectra changes and at 50 °C it is much more in a distorted form. As the temperature is gradually increased, the total shape of the soret band changes into somewhat random type. This change of the spectral behaviour of peroxidase at different temperature can be attributed to the structural changes of the enzyme molecule hosted in a large water pool of size corresponding to W_o = 18. As the enzyme is perfectly hosted[14] at W_o = 15 so at W_o = 18, it has got lot of space to move about. So the enzyme gets enough space to unfold due to the unfavorable temperature. One more interesting thing is that when the enzyme molecule after being exposed to 50 °C for 15-20 min is brought back gradually to 5 °C, it is found that the intensity of the soret band obviously is decreased compared to the initial value but the spectral characteristic is found to be more or less same as that of original spectra. By studying these entire characteristics one can say that the enzyme molecule is brought back to its native state when the effect of temperature is no more there. That is the whole process of denaturation of the enzyme may be reversible in nature.

pH Dependent molar extinction coefficient (ε) of peroxidase in Triton X-100 reverse micellar system: Fig. 3 shows pH dependent molar extinction coefficient of peroxidase in Triton X-100 reverse micelles in cyclohexane at W_o = 18, T = 25 °C. It is clear from the figure that an increase in 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} value remains more or less constant till pH 11.8 and above which it falls rapidly. The fall of ε_{403} value indicates that there is a change of conformation of the enzyme molecule at that particular wave length after pH 11.8.

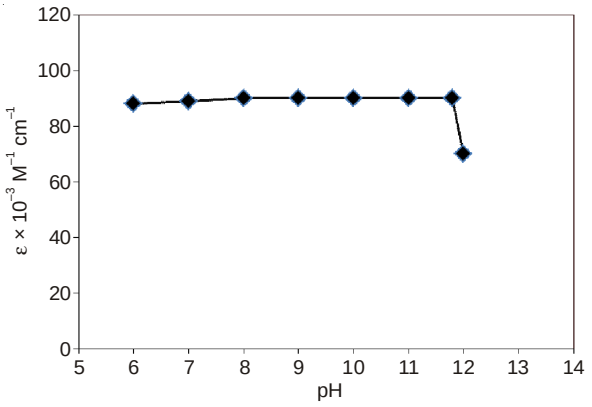

Fig. 3. Variation of molar extinction coefficient of peroxidase with pH in Triton X-100 reverse micellar system at W_o = 18, [E] = 6.2 μM, T = 25 °C

It has been established by Maehly[12] that peroxidase in an aqueous medium changes its spectral characteristics in a wide range of pH values. 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 alkaline medium, the stability of horseradish peroxidase is remarkable for a protein molecule. Kelin and Mann[15] have found that the colour of horseradish peroxidase changes from brown to red upon addition of alkali. They found that pK value of the change to be 10.90 as measured spectroscopically. The peak of the soret band is shifted from 403 nm to 417 nm. At pH about 12 no measurable instability is observed and undamaged horseradish peroxidase 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 horseradish peroxidase molecule, or that a OH- group leaves the Fe-atom when compound 'B' is formed. Therefore it is an acid form of horseradish peroxidase which may be the factor for the low catalytic activity of horseradish peroxidase 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 → protohemin II is possibly a rearrangement of the hemin molecules after the loss of protein. Theorell *et al*.[16] found in the differential titration experiments a difference of two equivalents per mole between the free protein and recombined horseradish peroxidase 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 at low pH values also. But the most interesting fact is that a Triton X-100 cyclohexane reverse micellar system at $W_o = 18$, 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. 3). 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[17,18]. This shift of the pH profile in the micellar system made it possible to measure the catalytic activity of horseradish peroxidase by using pyrogallol as the substrate even at pH 8 in the micellar media, which is impossible in the aqueous system.

Temperature dependent absorption spectra of peroxidase in aqueous buffer as well as in reverse micellar system at various W_o: Fig. 4 shows the absorption spectra of horseradish peroxidase in aqueous medium with wide range of temperature 10-50 °C and the same absorption spectra of horseradish peroxidase in a homogeneous micellar solution at different surfactant hydration (W_o) with the temperature range of 10-50 °C. In both cases each sample has been exposed to that particular temperature of about 15-20 min. It is found

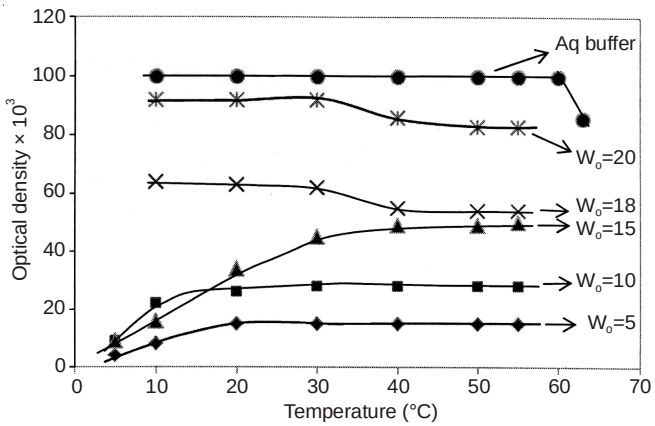

Fig. 4. Absorption spectra of peroxidase in aqueous buffer as well as in Triton X-100 reverse micellar system with temperature at various W_o. [E] = 3.5 μM

that the variation of temperature in the wide range has got some effects on the absorption both in the aqueous as well as in the micellar media. The concentration of the enzyme in the micellar core is maintained same in every W_o values. Two types of behaviour of the enzymes are found inside the micellar aqueous core; (a) in the temperature range of 10-30 °C and (ii) in the temperature range 30-50 °C. Again for $W_o \leq 15$ the enzyme behaves in the similar fashion and $W > 15$ and in the aqueous system, the enzyme behaves in a different fashion. The absorption values slowly increases for $W_o \leq 15$ and attains constant value after that. For $W_o = 18$ and 20, the absorption value remains constant till the temperature is 30 °C and above that it decreases slowly. In aqueous buffer system, absorption value remains constant up to 60 °C and a sudden fall of absorption is observed above 60 °C. The change of absorption is certainly reflecting some structural perturbation of the enzyme molecule. To know exactly, as to what is happening inside the enzyme molecule, molar absorption coefficient (ε) of the enzyme has been calculated[19] at room temperature *i.e.* T = 25 °C is taken 9.1×10^4 $M^{-1}cm^{-1}$.

Temperature dependent molar extinction coefficient (ε) of peroxidase in aqueous buffer as well as in reverse micellar system at various W_o: Fig. 5 shows the variation of molar extinction coefficient (ε) of peroxidase with temperature in aqueous buffer as well as in Triton X-100 reverse micelles at different W_o values. Different trends of variation of ε are observed in Fig. 5. In the micellar system having low degree of surfactant hydration, *i.e*. $W_o = 5$ and 10, ε gradually increases with temperature up to about 30 °C and above which it more or less attains a constant value but less than 9.1×10^4 M^{-1} cm^{-1} above certain temperature. On the contrary at $W_o = 15$, a little change in ε was observed in the entire range of temperature. Further at $W_o = 20$ and in aqueous buffer solution ε values remains more or less constant at 9.1×10^4 M^{-1} cm^{-1} up to about 30 °C and above this temperature it decreases with increases in temperature. These facts can be very well explained on the basis of Fig. 1c and 4 also.

In the aqueous buffer system with a broad temperature range from 10-50 °C though the enzyme functions, but it undergoes temperature dependent conformational changes. Possibility remains that at each value of temperature, the enzyme is in a "specific conformation", characteristic only at

that temperature. Such structural rearrangements also encompass the region of the active sites. This is clearly found in the case of peroxidase in Fig. 5 where ε value is least change in the temperature range of 10-30 °C and then gradually decreases when the temperature changes from 30-50 °C and falls suddenly. The lower ε value of the protein in the aqueous core of reverse micelles at $W_o = 5$ and 10 in the lower temperature range of 10-30 °C is due to the separation of heme outside the protein. As the temperature increased, the size of aqueous core increases due to partial coalescence of the droplets. So the heme part which is located outside the protein gradually gets space to penetrate into it. Therefore, the rate of change of ε in the temperature range of 10-30 °C is maximum for the lowest W_o value ($W_o = 5$) and minimum for $W_o = 15$. These facts is due to the coincidence of the size of the protein to the internal cavity of the reverse micelles at $W_o = 15$, where the peroxidase molecule gets sufficient space without a significant change in the conformation of the protein and without the emergence of heme from its active center. But when the enzyme is incorporated into the micelles whose size is less than the globule *i.e.* $W_o < 15$, the solubilization of the protein requires the reorganization of the micelles, their enlargement under such a mechanism of solubilization of peroxidase changes conformation and/or there occurs a loss of heme, as it also evidenced from the data presented in Fig. 1c and 5.

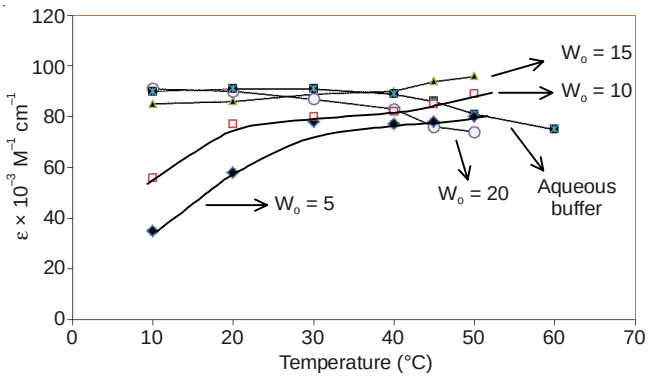

Fig. 5. Variation of molar extinction coefficient of peroxidase with temperature in aqueous buffer as well as in Triton X-100 reverse micellar system at various W_o

Alternatively, due to the small micellar size corresponding to $W_o = 5$ and 10, diameter of the aqueous core are smaller[20,21] as compared to that of horseradish peroxidase molecule[22] which is 50 Å. As a result, the protrusion of the heme part of peroxidase molecule is expected, so the hydrophilic polypeptide chains are hosted in the aqueous core leaving the hydrophobic heme outside and perhaps in the hydrocarbon region of the surfactant aggregates or in the bulk organic solvent. With increase in temperature, the size of the aqueous core increases due to partial coalescence of the droplets and become sufficient to accommodate, fully or partially or entire enzyme molecule. So the heme part of the protein can come back gradually within the protein core. The broad peaks observed for peroxidase near the soret band region which is due to the separation of the heme from the protein come closer. This mechanism of unfolding of protein is hindered when enzyme is bonded at multiple points of the inner interface of Triton X-100 reverse micelles in the present case at $W_o = 15$. Further at $W_o = 15$, the diameter of the aqueous core of the parent micellar droplet is 50 Å[21] which is surprisingly close to that of horseradish peroxidase molecule. The molar extinction coefficient (ε) value close to 9.1×10^4 M^{-1} cm^{-1} at temperature even above 50 °C in the micellar system indicates that the enzyme remains in the micellar aqueous core without a significant change in the conformation of the active form of the protein.

In case of $W_o = 20$, in the lower temperature range of 10-30 °C the enzyme has got enough space to be accommodated in the aqueous core of the reverse micelles so a least change of conformation is noted in that region. However, the minor change of ε is due to the change of mobility of the protein globules at different temperatures. The tendency of the structural organization becomes prominent at $W_o = 20$ and in aqueous buffer system in the higher range of temperature (30-50 °C). A sudden fall of ε value is noted in the higher temperature range T > 30 °C in the above cases.

Fig. 6 shows the variation of the molar extinction coefficient (ε) of peroxidase *versus* hydration of surfactant (W_o) at 20 °C, pH 7. The boundary between the region of sufficiently high degree of surfactant hydration, where there occurs the partial separation of heme from protein, corresponds approximately to $W_o = 15$ *i.e.* the breaks corresponds to $W_o = 15$ (Fig. 6). It is known that the degree of hydration of surfactant determines the size of their inner cavity[20,21]. At $W_o = 15$, the diameter of the inner cavity[21] is approximately 50 Å. The obtained value is close to the molecular size of peroxidase; its globule can be approximated by a sphere of 50Å diameter[22]. This surprising coincidence between the size of the protein molecule and that of the "critical" internal cavity of the micelles can be interpreted such that the incorporation of peroxidase into a sufficiently large micelles (at $W_o = 15$) occurs without a significant change in the conformation of the protein and without the emergence of heme from the active center. However, the enzyme is incorporated into micelles whose size is less than the globule when $W_o < 15$, the solubilization of protein evidently requires the reorganization of the micelles, their enlargement under such a mechanism of solubilization of peroxidase changes conformation and/or there occurs a loss of heme, as is also evident from the data presented in Fig. 6.

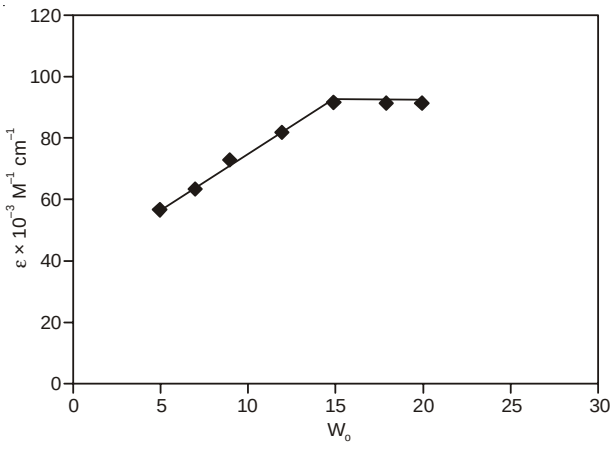

Fig. 6. Variation of molar extinction co-efficient of peroxidase with W_o in Triton X-100 reverse micellar system at pH = 7, T = 20 °C

Conclusion

In this article, the spectral characteristics of horseradish peroxidase in aqueous buffer as well as in nonionic reverse micellar system have been reported at various pH values over a wide range of temperature. It is found that the soret band of horseradish peroxidase is slightly shifted in acidic as well as in alkaline media with respect to the neutral pH (pH 7) at 25 °C. 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 $W_o = 15$, pH = 7, is found virtually unchanged. The pH dependent molar extinction coefficient (ε) of peroxidase in reverse micelles shows that ε_{403} value remains more or less constant upto pH 11.8 and thereafter with increase in pH, ε_{403} value falls rapidly. The fall of ε_{403} value indicates that there is a change of conformation of the peroxidase after pH 11.8.

Temperature dependent spectral characteristics of peroxidase in nonionic micellar media shows that the absorption spectra of horseradish peroxidase remains unchanged as compared to aqueous buffer solution at lower temperature (T = 20 °C) and then gradually changes as the temperature is increased. At 35 °C the total shape of the spectra changes and at 50 °C it is much more in a distorted form. As the temperature is gradually increased, the total shape of the soret band changes into somewhat random type. This change of the spectral behaviour of horseradish peroxidase at different temperature can be attributed to the structural changes of the enzyme molecule encapsulated inside the aqueous core of the reverse micelles. One more interesting thing is that when the enzyme molecule after being exposed to 50 °C for 15-20 min is brought back gradually to 5 °C, it was found that the intensity of the soret band was decreased compared to the initial value but the spectral characteristic was found to be more or less same as that of original spectra. By studying these entire characteristics one can say that the enzyme molecule is brought back to its native state when the effect of temperature is no more there. That is the whole process of denaturation of the enzyme may be reversible in nature.

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