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Effects of sodium dodecyl sulphate on enhancement of lipoxygenase activity of hemoglobin

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Lipoxygenases comprise a family of non-heme iron-containing enzymes that catalyze the stereospecific dioxygenation of polyunsaturated fatty acids with 1, 4-cis-cis-pentadiene structure. Hemoglobin, a heme iron-containing protein has been reported to have lipoxygenase activity but the assay conditions that could enhance the activity remain obscure. Therefore, establishment of optimum assay conditions for lipoxygenase activity of hemoglobin could allow modeling of hemoglobin as lipoxygenase. Hemoglobin was extracted from blood of an identified individual of genotype AA. The hemoglobin was dialyzed at 4 ^{o}C for 24 h against 50 mM Tris-HCl buffers (*p*H 8.5 and 7.2) and effects of sodium dodecyl sulphate (SDS) and linoleic studied at *p*H 5.0 and 7.2 with UV–VIS Titration Spectrophotometry. The results show that 3.3, 8.6 and 88.1% concentrations of met-hemoglobin were found in presence of 0.0 mM SDS at *p*H 5.0 and 7.2, 1.043 mM SDS at *p*H 7.2 and 0.404 mM SDS at *p*H 5.0 respectively. Also, the difference spectra of hemoglobin in presence of linoleic acid showed positive peak at 285 nm which suggest the presence of oxodienes–a reaction product of hydroperoxidase activity of lipoxygenase. Formation of met-hemoglobin/met-myoglobin is highly correlated with lipid oxidation. Since highest concentration of met-hemoglobin (88.1%) was observed in presence of 0.404 mM SDS at *p*H 5.0, lipoxygenase activity of hemoglobin was enhanced in presence of SDS under these conditions.

Keywords: Hemoglobin, Linoleic acid, Lipoxygenase, Met-hemoglobin, Oxodienes, Sodium dodecyl sulphate

Lipoxygenase (Linoleate: Oxygen oxidoreductase, EC 1.3.11.12) is a dioxygenase that catalyzes the oxygenation of polyunsaturated fatty acid containing a cis, cis-1, 4-pentadiene system to hydroperoxide (fatty acid hydroperoxide). Gata *et al*[1] observed that lipoxygenase from pig muscle has optimum *p*H of 5.5 and more affinity for linoleic acid than linolenic acid and arachidonic acid. In mammals, fatty acid hydroperoxides are substrates for pathways that lead to leuketrienes or lipoxins, the potent messengers that are involved in inflammatory response[2]. Consequently, lipoxygenase inhibitors are major targets of the pharmaceutical industries as potential drugs against conditions such as arthritis and asthma[3]. The fatty acid hydroperoxides themselves may play a role in a variety of phenomena, including cell maturation and development of atherosclerosis[4]. Hemoglobin—an oxygen transport iron metalloprotein found in the red blood cells of vertebrates[5] and tissue of some invertebrates has been shown to have some enzymatic activities[6] of which lipoxygenase is one of them. Hemoglobin catalyzes at low concentrations, a qausi-lipoxygenase reaction with remarkably high substrate specificity. The activity of hemoglobin-catalyzed oxygenation of linoleate is comparable with those of true lipoxygenase[7].

Hemoglobin has two transition states namely native and denatured states. External influence (chemical or physical agents) such as sodium dodecyl sulphate (SDS) can affect the force of interaction within a protein and unfold the protein slightly until a certain point is reached where the protein is denatured. The unfolded or denatured state may have significant implication in the enhancement of lipoxygenase activity of hemoglobin. Although the functioning of protein whether *in vitro* and *in vivo*, is associated with their denaturation which could be understood as the loss of the three-dimensional structure[8] of the protein but there is a dearth of information on whether this can enhance enzyme activity or not. Some ligands such as SDS are detergents that disrupt protein folding[9]. It is used more often than any other detergent as an excellent denaturing or 'unfolding' detergent[10] of proteins..ybbg. Therefore, the aim of the present study is to investigate the potential of lipoxygenase activity of hemoglobin enhancement in

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unfolded and or denatured state which can pave way for modeling of hemoglobin as lipoxygenase for identification of lipoxygenase inhibitors which can serve as potential anti-inflammatory drugs.

Material and Methods

Materials—Sodium dodecyl sulphate (SDS), linoleic acid and other chemicals used were obtained from BDH, England and Sigma, Germany and were of analytical grade. All reagents were freshly prepared unless otherwise stated.

Collection of blood sample—Blood sample (4 mL) was collected from an identified individual of genotype AA after informed consent, having obtained an approval for the study protocol from the Ethics Committee of the University of Nigeria. The blood sample was collected with an ethylene diamine tetracetic acid (EDTA) vail.

Isolation and purification of haemoglobin—Blood was combined with cold normal saline in 50 m*M* Tris-HCl, *p*H 8.5 (wash buffer) in the ratio of 2:3 and kept at 8 °C for 10 min. The resulting solution was centrifuged for 10 min at 4000 rpm[11]. Thereafter, the supernatant was removed by aspiration. The same amount of wash buffer was introduced into the pellet and kept at 8 °C for 20 min. The above steps were repeated for 2-4 times until a clear supernatant was gotten. The clear supernatant was removed and the resulting pellet was made up to 5 mL using 50 m*M* Tris-HCl buffer, *p*H 8.5. The red cells were lysed and, 5% NaCl was added to the resulting volume and centrifuged for 10 min at 4000 rpm. The crude hemoglobin was collected into separate vial and dialyzed at 4 ^{o}C for 12 h against 50 m*M* Tris–HCl buffer, *p*H 8.5. Same dialysis was repeated as above but at a *p*H of 7.2 using a freshly prepared hemoglobin. The dialyzed hemoglobin was collected and stored at -20 ^{o}C for further experiments.

UV–visible titration—The hemoglobin (100 µL of 0.01 m*M*) calculated on heme basis using ε_{415} = $1.25\times10^{5}M^{-1}cm^{-1}$ (ref. 12) was scanned from 250 to 650 nm using JENWAY 6405 UV-VIS Spectrophotometer in absence and presence of different concentrations of ligands (sodium dodecyl sulphate, SDS and linoleic acid) in 50 m*M* buffers of *p*H 5.0 and 7.2 after appropriate buffer baselines. The titrations were done by addition of the hemoglobin (0.1 mL) in 4 mL cuvette containing a known volume of the buffer (2.1 mL for SDS and 2.3 mL for linoleic acid) then various volumes of the ligands (0 to 0.6 mL) corresponding to different *in situ* concentrations (0 to 1.043 mM for SDS and 0 to 0.400 m*M* for linoleic acid) were added in stepwise manner from stock concentration of the ligand (5 mM for SDS and 2 mM for linoleic acid). This was rapidly mixed and scanned from 250 to 650 nm. Spectrum readings were recorded at each titration point (after each addition of the ligand solution). The results were analyzed by monitoring changes in absorbance at different wavelengths (275, 340, 415, 542, 560, 576, and 630 nm) and concentrations of oxy-, deoxy- and met-hemoglobin were calculated according to equation 1-3 as reported by Reza *et al*[13].

$$[Oxy]=(1.0154\ A_{576}-0.2772A_{630}-0.742A_{560})\times10^{-4}\ \text{mol} \quad \ldots(1)$$

$$[Deoxy]=(1.335A_{560}-0.7356A_{576}-0.6254A_{630}\times10^{-4}\ \text{mol} \quad \ldots(2)$$

$$[Met]=(2.6828A_{630}+0.174A_{576}-0.3414_{560})\times10^{-4}\ \text{mol} \quad \ldots(3)$$

Results and Discussion

Interaction of hemoglobin with ligands—Interaction of SDS or linoleic acid with hemoglobin causes change on the spectrum of hemoglobin. These changes involve increase or decrease in absorbance at wavelength(s) corresponding to soret band (415 nm), oxyhemoglobin bands (542 and 576 nm), aromatic band (275 nm), 630 nm, 340 nm; disappearance of the delta band (δ-band) (345-360 nm) including spectral shift (Figs 1 and 2). SDS caused spectral shift of the soret band from 415 nm to between 417 to 420 nm with disappearance of the δ-band. The spectral shift indicates the formation of low-spin derivative of hemoglobin[14]. According to Matsui *et al*[15], spectral shift or decrease in the peak absorbance signifies structural transformation of the hemproteins. The increase in the peak absorbance of the aromatic band suggests that the protein is unfolding[16]. Increase in absorbance observed at 340 nm when SDS or linoleic acid was used as ligand can be referred to as hardness of the non-covalent bond between histidine of globin and heme iron of hemoglobin. This is because decrease in absorbance at this wavelength (340 nm) refers to the stretching or weakness of the non-covalent bond between histidine of globin and heme iron[17]. The decrease in absorbance observed from SDS concentration of 0.404 mM might be due to breakage of the non-covalent bond leading to its weakness (Fig. 1b). Usually, hemoglobin has three conformational forms: Oxy-, deoxy- and met-hemoglobin conformations. Interaction of hemoglobin with ligand can lead to conversion of hemoglobin from one conformational form to another which can

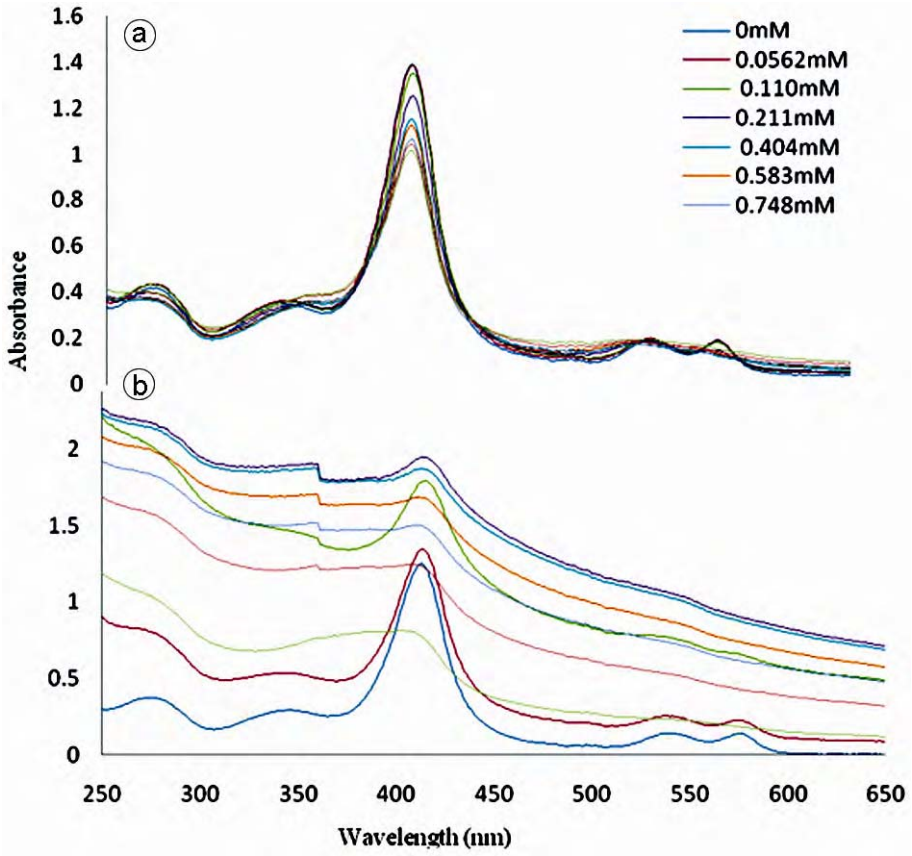

Fig. 1—Absorption spectra of HbA in presence of varying concentrations (0-1.043 mM) of SDS at (a) *p*H 7.2 and (b) *p*H 5.0.

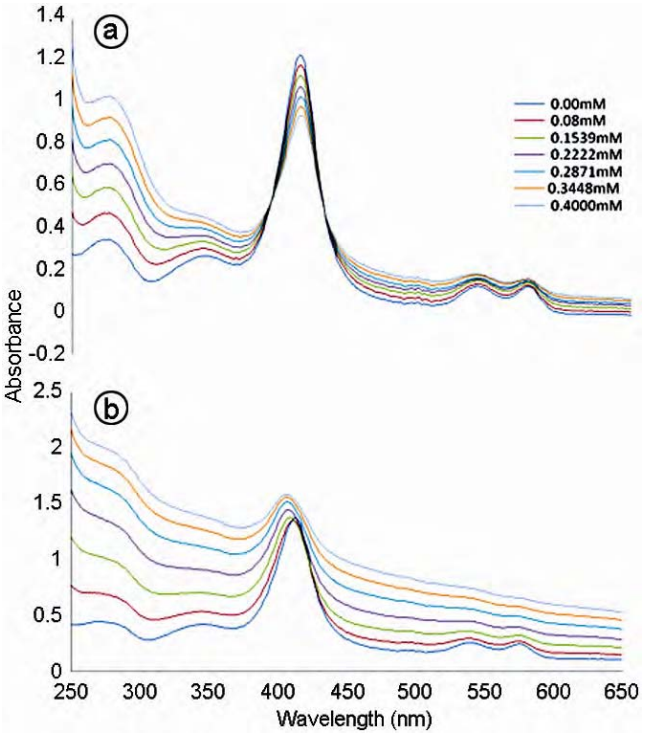

Fig. 2—Absorption spectra of HbA in presence of varying concentrations (0-0.4000 mM) of linoleic acid at (a) *p*H 7.2 and (b) *p*H 5.0.

be monitored from the spectra of hemoglobin. For instance, the degree of conversion of oxy-hemoglobin to met-hemoglobin depends on the degree of unfolding of the protein which could lead to existence of a hybrid of low spin and high spin states as it appears from shift toward shorter wavelength of the soret band[17].

At a concentration of 0–0.1539 mM linoleic acid at *p*H 5.0, there was an observed shift in the soret band from 415–412 without the disappearance of the δ-band and the oxy-hemoglobin bands but at a concentration of 0.2222 mM, the band shifts to between 408-410 nm with disappearance of the δ-band. Observation of specie with maximum soret peak at 412 nm without disappearance of the δ-band suggests the presence of alkaline low spin ferryl derivative of hemoglobin while detection of specie with maximum soret peak between 408-410 nm with disappearance of the δ-band suggests the presence of acidic high–spin ferryl derivative of hemoglobin[14,18]. Also, decrease in the peak absorbance of the soret band were observed in 0–0.1539 mM linoleic acid but as from 0.2222 mM linoleic acid, the absorbance of the soret band increased in a concentration dependent

manner. Decrease in absorbance in the soret region occurs in parallel to formation of reaction products (conjugated diene hydroperoxide at 234 nm and oxodiene at 285 nm)[7] while the increase in absorbance at 275 nm refers to dynamic motion of the studied protein and its deviation from normal structure and function[17]. Increase in the peak absorbance of the soret band in presence of linoleic acid *p*H 5.0 suggests that the heme moiety of the studied protein is being exposed at high lipophilic environment while increase in absorbance at 630 nm suggests presence of met-hemoglobin[17] which is formed probably by oxidation of ferrous iron (Fe^{2+}) of hemoglobin to ferric iron (Fe^{3+}) as a result of the exposure of heme moiety.

The difference spectra of hemoglobin in the presence of linoleic acid (Fig. 3) showed positive peak at 285 nm which suggests the presence of oxodienes- a reaction product of hydroperoxidase activity of lipoxygenase[7]. The difference spectra also showed negative trough at the soret band region of the hemoglobin in presence of linoleic acid at *p*H 7.2 (Fig. 3a). This could be due to perturbation of the heme moiety which suggests that linoleic acid reacts with the hemoglobin at the heme region. Positive troughs observed at the soret region in the presence of linoleic acid, *p*H 5.0 (Fig. 3b) suggests the exposure of the heme moiety of the studied protein which can facilitate lipid oxidation.

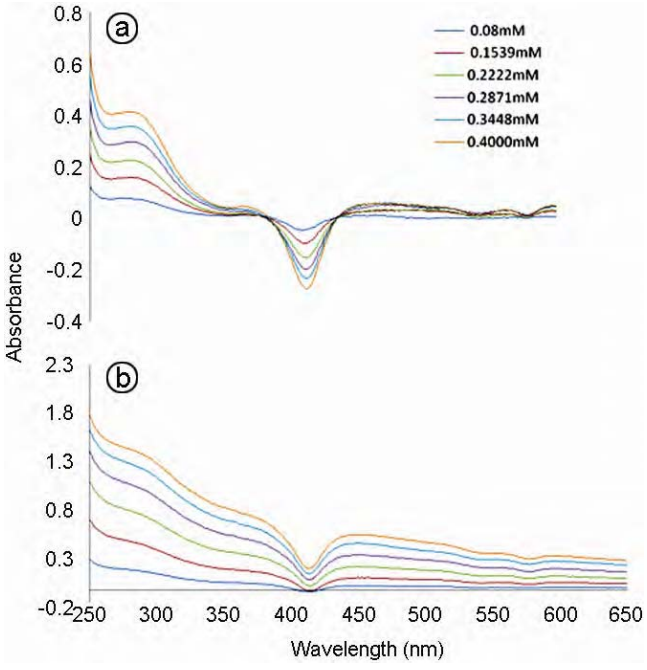

Fig. 3—Absorption difference spectra of HbA in presence of varying concentrations (0.08–0.4000 mM) of linoleic acid at (a) *p*H 7.2 and (b) *p*H 5.0.

Effects of SDS on haemoglobin—SDS has increasing effect on the soret and aromatic bands of the studied protein at *p*H 5.0. The increasing effect of SDS at *p*H 5.0 on the absorbance peak of these bands reached maximum at 0.211 mM SDS and started decreasing at 0.404 mM SDS. Also observed were decreasing effects on these bands by SDS at *p*H 7.2 (Figs 4 and 5). The increase in the absorbance peak of the aromatic band refers to dynamic motion of the molecule and its deviation from normal structure and function[17] or unfolding of the studied protein[16,15]. This unfolding exposes the heme moiety and buried aromatic amino acids of the protein which explains the increasing effect of SDS on soret and aromatic bands of the studied protein. The ferrous iron (Fe^{2+}) of

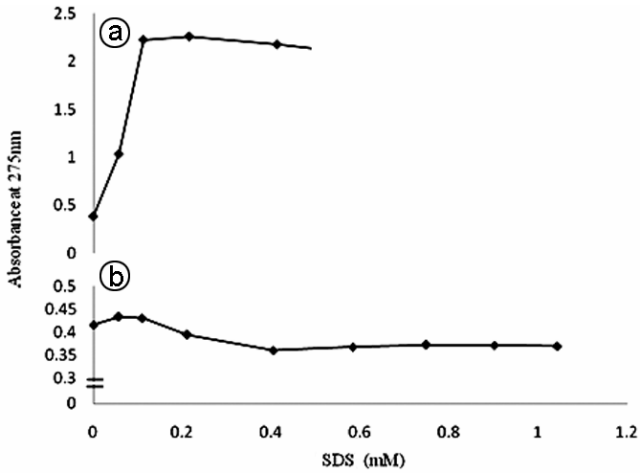

Fig. 4—Changes in peak absorbance (275 nm) of aromatic band of hemoglobin in presence of varying concentrations (0-1,043 mM) of SDS at (a) *p*H 5.0 and (b) *p*H 7.2.

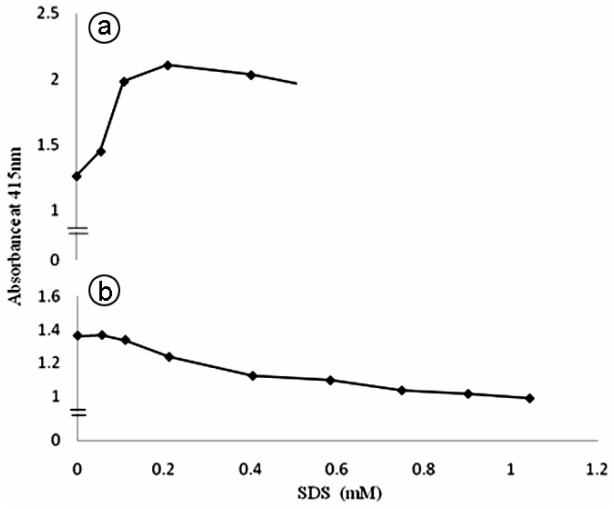

Fig. 5—Changes in peak absorbance (415nm) of soret band of hemoglobin in presence of varying concentrations (0–1.043 mM) of SDS at (a) *p*H 5.0 and (b) *p*H 7.2.

the exposed heme moiety can oxidize to ferric iron (Fe^{3+}) to form met-hemoglobin[19] and formation of met-hemoglobin is associated with lipid oxidation[20].

Figure 6 shows that interaction of SDS with hemoglobin decreased the concentration of oxy-hemoglobin and increased the concentrations of deoxy-hemoglobin and met-hemoglobin. However, concentrations of the met-hemoglobin and deoxy-hemoglobin decreased at SDS concentration of 0.583 mM at pH 5.0. Decrease in oxy-hemoglobin concentration and increase in deoxy- and met-hemoglobin concentrations indicate that the oxy-hemoglobin is being converted to deoxy-hemoglobin which its iron (Fe^{2+}) can oxidize to Fe^{3+} to form met-hemoglobin (Fig. 6) while the decrease in the concentration of met-hemoglobin and deoxy-hemoglobin at SDS concentration of 0.583 mM and above indicates dissociation of the iron in the hemoglobin or that the oxy-hemoglobin in the reaction system is diminishing. Also, it can be calculated from Fig. 6 that 3.3, 8.6 and 88.1% met-hemoglobin are present in 0 mM SDS (*p*H 5.0 and 7.2), 1.043 mM SDS (*p*H 7.2) and 0.404 mM SDS (*p*H 5.0) respectively. Chaijan[20] noted that formation of met-hemoglobin is highly correlated with lipid oxidation. *In vitro*, ferrous iron (Fe^{2+}) of Hb can undergo slow autooxidation to give Fe^{3+} Hb (met-hemoglobin) and superoxide. The fraction of met-hemoglobin which cannot transport oxygen in normal blood cells does not exceed 3%[12]. Therefore, the observed increase in met-hemoglobin concentration at 0.0 mM SDS could be associated with auto-oxidation of hemoglobin while 8.6 % met-hemoglobin in presence of 1.043mM SDS (*p*H 7.2) may be due to spatial contact of the heme moiety of hemoglobin with oxygen during the folding process. Since formation of met-hemoglobin is highly correlated with lipid oxidation[20,21] and highest concentration of met-hemoglobin (88.1 %) was observed in 0.404 mM SDS *p*H 5.0, therefore, lipoxygenase activity of hemoglobin could be enhanced under these conditions. Since products of lipoxgenase catalyzed reaction such as fatty acid hydroperoxides are substrates for pathways that lead to leukerienes or lipoxins, potent messengers involved in inflammatory response. It is possible that modeling of hemoglobin as lipoxygenase under these conditions can pave way for identification of lipoxygenase inhibitors by assaying for lipoxygenase activity of hemoglobin in presence of lipoxygenase inhibitors. The inhibitors when identified can be useful as potential anti-inflammatory drugs.

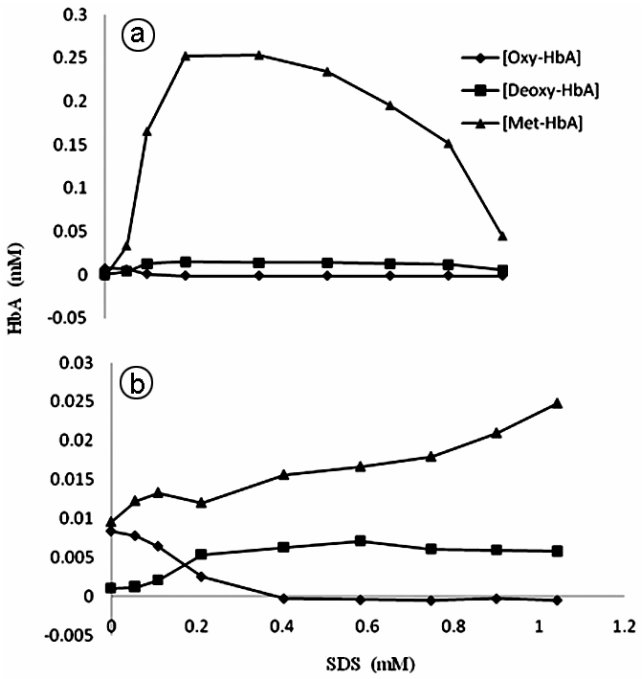

Fig. 6—Concentrations of oxy-, deoxy-, and met-conformations of hemoglobin in varying concentrations (0 -1.043 mM) of SDS at (a) *p*H 5.0 and (b) *p*H 7.2

Conclusion

The highest concentration of met-hemoglobin (88.1%) was observed in 0.404 mM SDS (*p*H 5.0) when compared with the concentration of met-hemoglobin of 8.6 and 3.3% in 1.043 mM (*p*H 7.2) and 0 mM SDS respectively. Formation of met-hemoglobin/met-myoglobin is highly correlated with lipid oxidation[20] and highest concentration of met-hemoglobin (88.1%) was observed in presence of 0.404 mM SDS (*p*H 5.0), therefore lipoxygenase activity of hemoglobin could be enhanced under these conditions. Thus, hemoglobin could be modeled as lipoxgenase under these conditions.

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