CHAPTER 2

CHARACTERIZATION

TECHNIQUES
2.1. CHARACTERIZATION TECHNIQUES

All the absorption spectral data were obtained by an UV-Vis diode array spectrophotometer (Agilent 8453). EPR spectra were obtained by a Jeol (JES FA200) spectrophotometer, samples were run at 77 K in a liquid nitrogen finger dewar. All electrochemical experiments were performed using a CH Instrument (model CHI710D Electrochemical Analyzer). Biopotentiostat, reference electrodes, Teflon® plate material evaluating cell (ALS Japan) were purchased from CH Instruments. The rotating ring disk electrochemical (RRDE) set up from Pine Research Instrumentation (E6 series ChangeDisk tips with AFE6M rotor) was used to obtain the RRDE data. The AFM data were obtained at room temperature in a Veeco dicip II (Model no: AP-0100) instrument bearing a phosphate doped Si cantilever (1-10 ohm.cm, thickness 3.5-4.5 μm, length 115-135 μm, width 30-40 μm, resonance frequency 245-287 KHz, elasticity 20-80 N/m). Solution and Surface Enhanced Resonance Raman data were collected using a Trivista 555 spectograph (Princeton Instruments) and using 413.1nm excitation from a Kr⁺ laser (Coherent, Sabre Innova SBRC-DBW-K).

2.2. PEROXIDASE ACTIVITY MEASUREMENT

3,3’,5,5’-tetramethylbenzidine (TMB) was used as the substrate for peroxidase activity measurement. 10 mg of TMB was dissolved in 0.5 ml glacial AcOH and 10 mL AcOH/NaOAc buffer (1 M, pH 4.5). The solution was diluted to 25 mL and 100 µL, 30 volume H₂O₂ was added, followed by 10 µL of 0.05mM of the protein sample. Kinetic traces were obtained by monitoring the increase of the 652 nm absorption band.

2.3. CATALASE ACTIVITY MEASUREMENT

Catalase activity was measured by following the rate of decay of H₂O₂ at 240 nm. 1.5 mL de-ionized water and 100 µL 0.05 M H₂O₂ were mixed in a cuvette. 20 µL, 0.5 mM protein was added to it. All spectra were recorded in a kinetic mode, with continuous stirring at 25 °C.
2.4. PROS MEASUREMENT

Xylenol orange assay was performed as follows. 4.9 mg of Mohr’s salt and 3.9 mg of xylenol orange were dissolved in 5 ml, 250 mM H$_2$SO$_4$ and stirred for 10 minutes. 200 µL of this solution was taken in 1.8 mL of nanopure water and a calibration curve for the quantitative estimation of H$_2$O$_2$ was obtained for 0.05 µM, 0.1 µM, 0.5 µM, 1 µM, 2.5 µM, 5 µM and 10 µM concentrations of H$_2$O$_2$ by recording their absorbance at 560 nm. The calibration curve was expressed as absorbance at a fixed wavelength of 560 nm vs. concentration of H$_2$O$_2$ in µM units for a 2 mL volume. For the detection of PROS of an unknown quantity a blank was obtained in the UV-Vis spectrophotometer with 1.8 mL nanopure water in a cuvette. 200 µL of the xylenol orange solution was added to this cuvette and the absorbance was recorded. This served as the control. The cofactor-Aβ complex was reduced by ascorbic acid (for Cu-Aβ) or dithionite (for heme-Aβ and heme-Cu-Aβ) under anaerobic conditions (observed by absorption and EPR spectroscopy), followed by their reoxidation by O$_2$ (followed by absorption and EPR spectroscopy). 200 µL of 0.025 mM reoxidized solution was added to the cuvette containing the control. Absorbance of this solution was recorded. The value of absorbance of the above solution (after subtracting the control) at 560 nm when plotted on the calibration curve yielded the corresponding H$_2$O$_2$ concentration.

2.5. GEL ELECTROPHORESIS

In native gel electrophoresis method, 10% polyacrylamide gel was used as the resolving gel. The samples were prepared by mixing 20 µL of each sample (containing 20 µg samples) with equal volume of Laemmli buffer. 4 % stacking gel was prepared on top of the resolving gel and the comb was inserted carefully. It was then allowed to stay for 1 hour. After formation of the defined wells the gel was loaded in the electrophoresis setup. It was then filled with 1x tank buffer and the samples were loaded in the wells. Electrophoresis run was monitored by the movement of bromophenol blue. Finally the gel was stained with Coomassie blue (G-250) stain to obtain the protein bands, and after complete staining, it was destained with a solution of 25 % methanol, 7 % acetic acid, and water overnight.