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Identification of an Independent Measurement Method for Denaturation Studies of Cytochrome C

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Developing some substitutionary ways such as electrochemical methods might be useful in order to follow structural changes of proteins. In this study, the effect of denaturants such as Urea and SDS (sodium dodecyl sulfate) was investigated on Cytochrome C structure, a metalloproteinase with an oxide/redox property. Structural alterations were studied with potentiometer and different spectrometer techniques. Cytochrome C was prepared in 20mM phosphate buffer with pH=7.0. The protein was treated with different concentrations of Urea and SDS. Related absorbance of native and denatured protein were recorded with Cary 50 (UV-Visible) and AVIV-215 (Circular Dichroism) spectrophotometers in range of 200-700 nm. Electrochemical measurements were also recorded with an acetate/Galvan acetate potentiometer device. Cytochrome C was reported to be structurally stabile in 5-8 and 3-9.5mM concentrations of Urea, which were monitored by voltammetry and spectroscopy methods, respectively. The protein was active in high concentration (26mM) of SDS. The concentration of SDS 20mM seems to be sufficient for proper folding of 0.5mM Cytochrome C. It has been concluded that, 40 µM Cytochrome C, crudity, and etc do not impact voltammetry results and it is an independent method for detection of structural changes of proteins. Voltammetry method is more sensitive to small changes in protein structure compared with spectroscopy methods.

Keywords: Cytochrome C, Electrochemistry, SDS, Urea.

1. INTRODUCTION

Different methods and techniques are being used to study the interaction of drugs and effectors with proteins. These techniques are classified as spectroscopic and voltammetric [1]. Accomplishing studies under specific circumstances in which proteins are stable, native or active and molecules can interact with, is the major priority of methods. Most protein denaturation studies have been taken out by spectroscopic methods such as optical, UV-Visible, Fluorescence spectroscopy and etc. Some methods use various light wavelengths in order to study structure and behavior of proteins [2,3,4]. Despite of easy handling and independency to high-tech equipments of spectroscopic methods, complete clearance, non contamination, and high purity of samples are in concern. Most Spectroscopic methods are not highly efficient dealing with samples [5,6]. Finding a way to follow the structural changes of proteins without mentioned restrictions seems to be necessary. According to some reports, voltammetry is supposed to be a method which lacks such limitations. In this method, presence of other proteins do not generally interfere measurement process because standard potential of each protein is different. Possible contaminations do not create noise, because same redox potentials of sample and contamination are improbable. However, oxygen within the electrolyte can cause unlikely results[7,8].Also, Buffers used in molecular biology do not interfere in voltammetry process.

Folding and structural changes are the most important aspects of native structural integrity of any protein. Protein activities and regulations can be interfered by different effectors molecules. Biological activities of redox proteins are results of ability to exchange electrons which strictly depends on folding process and three-dimensional structures of such molecules [9]. In this article, structural alterations of Cytochrome C under impacts of some denaturing molecules are studied based on ability of redox proteins to exchange electrons with different oxidation states.

Cytochrome C is not only an electron carrier in mitochondrial respiratory chain between Cytochrome C reductase and Cytochrome C oxidase complexes, but also is a main initiator of programmed cell death. Extensive researches on this protein have revealed 104 amino acids with four alpha helixes but not beta sheets. It has also a non-protein prosthetic group called hem[7,9].

The electro-chemical methods are new in biological sciences. Ability to study the thermodynamic and kinetic states is the main cause of widespread use of such methods. A simple cyclic voltammetry differential includes information such as formal restoration potential of electroactive materials, number of exchanged electrons and the rate constants in electrode reactions, chemical stabilities and mechanisms of information of processes such as catalytic reactions[10].

In this study, some aspects of spectrophotometric and electrochemical methods are compared based on structural changes of Cytochrome C. However, results determined and highlighted higher efficiencies of electrochemical methods as new born procedure. In spite of the fact that much works have been performed such fields, still more optimized method should be developed to undermine different limitations.

2. MATERIALS AND METHODS

2.1. Preparation of buffer solution

All experiments on Cytochrome C was performed in 20mM phosphate buffer with pH=0.7. To prepare 1 liter of buffer solution, 3.5 mg of K_2HPO_4 and 1.3 mg of KH_2PO obtained from Sigma-Aldrich (USA) are dissolved in 1 liter DDW (deuterium-depleted water). If any pH adjustments were needed, 2M NaOH and HCl would be utilized.

2.2. Preparation of protein samples

Preparation of protein samples in denaturation experiments could be either as batches of determined concentrations of protein dissolved in different concentrations of denaturant compounds or as a fixed concentration of protein with gradual addition of effector solutions ,which is known as titration, but this method is less accurate because of little equilibration time of samples. This study is based on the first method along with 1 hour delay for proper equilibration of reactants.

Soluble Cytochrome C has specific light absorbance spectrum in visible region with a maximum at 409nm. Molar absorption coefficient of horse heart Cytochrome C is $1/06\times10^5$M/cm at this wavelength.

Concentration of Cytochrome C was calculated using the Beer-Lambert equation (Absorbance of protein solution in 409nm). Usually, commercially purchased Cytochrome C is a mixture of both oxidized (98%) and reduced (2%) forms. In order to fully oxidize, potassium cyanide obtained from Merck was added into the sample with 10% protein content. Cytochrome C is a stable protein and its solution can to be used for several days to weeks stored at room temperature or refrigerator, respectively; but in the present work all samples were prepared fresh.

2.3. Spectroscopy studies

Native and totally denatured (with high concentrations of urea) spectra of protein were recorded at 200-700nm by spectrophotometer *Cary* 50 (Shimadzu UV-1601PC). CD spectra were recorded at far UV for protein samples (0.2 mg/ml) by *Aviv 215* spectropolarimeter (Aviv Associates, Lakewood, NJ).

2.4. Preparation of Sensing Electrodes

To investigate direct electron transfer reaction between electrode and Cytochrome C, Gold was chosen as working electrode and was prepared as below:

1. Surface of the Gold electrode polished by suspension of aluminum oxide 0.6 μ.
2. To remove alumina particles, DDW along with ultrasonication for 10 min were utilized.
3. Washing electrode with Sulfuric acid for 10 min.

4. Clean electrode was calibrated within 0.1 M Sulfuric acid and 0.01 M Potassium Chloride solution for 10 Cycles. Calibrations were performed between 0.2 and 0.75 V at 100 mV/s (All connected to the reference electrode of Silver-Silver Chloride).
5. 3 more cycles were performed with upper potential limits of 1, 1.25 and 1.5 V.
6. Now the electrode is ready for experiments.

2.5. Electrochemical Measurements

The electrochemical measurements were performed with a Potassium Acetate/Acetate Galvan A263 model (EG & GC, USA). All experiments were accomplished using a three electrode system where Silver/Silver Chloride electrode was used as reference. A Platinum electrode was used as counter electrode. In this study, gold electrode modified with mercaptoethanol was used as sensing one. Electrochemical measurements were performed using a cyclic voltammetry method. Also, experiments were conducted in the Faraday cage to minimize noise levels. Before each experiment, each sample was deoxygenized for 30 min with pure Nitrogen.

3. RESULTS

Most studies about denaturants and their effects on protein structures have been done with spectroscopy methods, such as visible, UV, fluorescence emission or absorption spectroscopy and colorimetery methods. In this study, cyclic voltammetry method was used to follow structural changes of Cytochrome C in presence of two denaturant compounds of Urea and SDS. Results were compared with spectroscopic methods.

3.1. Effects of urea on structure of Cytochrome C

About 30 μM Cytochrome C along with 2-mercaptoethanol modified gold electrode were used to study impacts of urea upon structure of protein. Figure 1 demonstrates the cyclic voltammetry curves of the protein in different concentrations (0-8M) of urea. Increasing urea concentration causes decrease in current peaks. Reduction of rate constant is consistent with decrease of current peaks. In Figure 2, effects of various concentrations of urea on cycle voltammetry of Cytochrome C are visualized. Cathode and anode current peaks are reduced and changed into a sigmoid pattern which is a common behavior in denaturation processes.

Furthermore, Cytochrome C denaturation was studied with spectroscopic methods. Figure 3 shows absorption spectrum of 30μM the protein in UV region. Typical peaks are at 230, 280, 400, and 520nm which are demonstrating peptide bonds, aromatic rings, hem dye, and beta absorbance, respectively. In order to fully denature the protein, urea concentration was increased up to 10M.

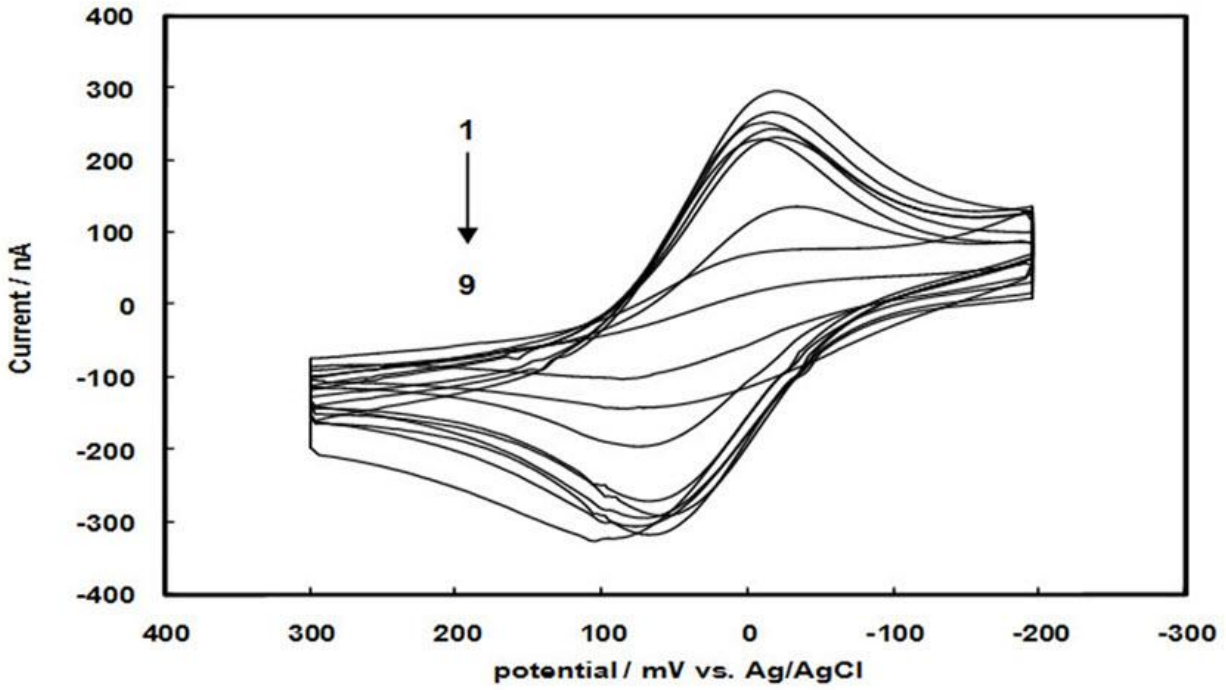

Figure 1. Voltammogram cycle of Cytochrome C. Voltammogram is sketched for 0 to 9 M concentrations of urea. 20 mM phosphate buffer and 100 mV/s probe speed.

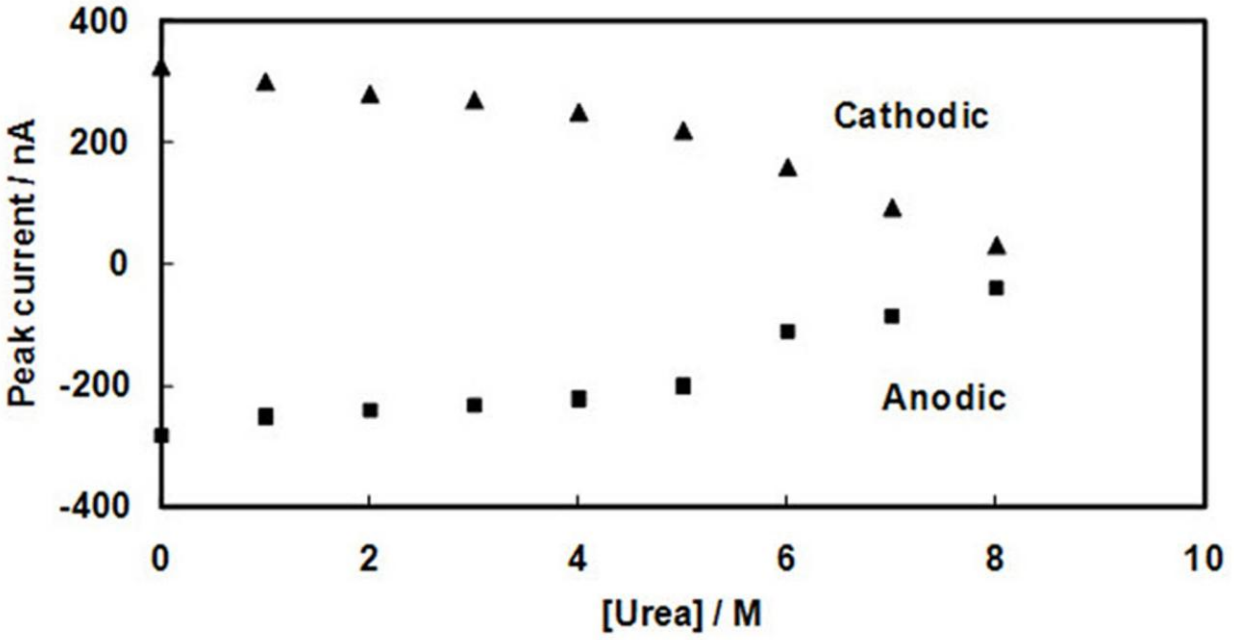

Figure 2. The cathode and the anode current peaks of influences of different concentrations of urea, Cyclic Voltammetry of Cytochrome C.

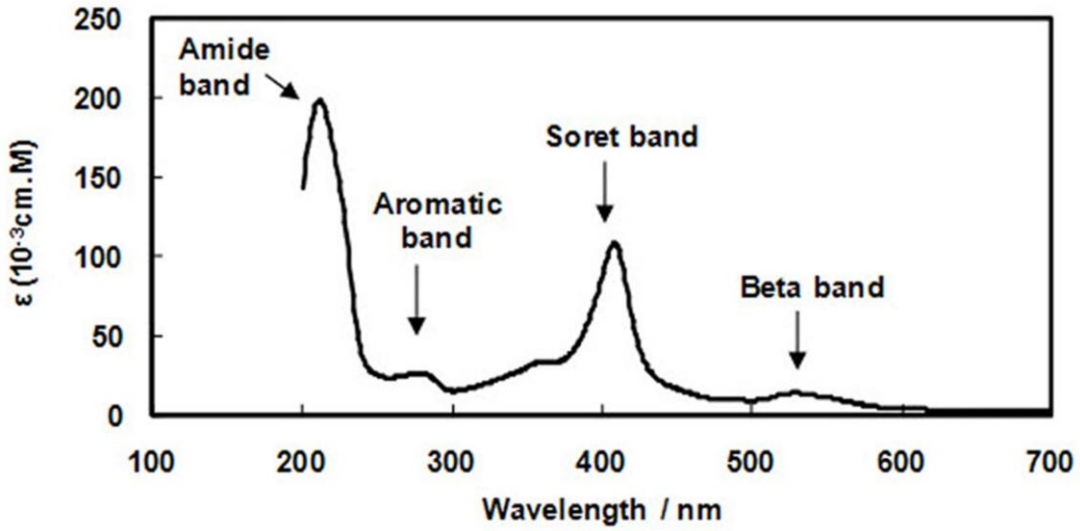

Figure 3. Uv-Vis spectrum of 30 µM Cytochrome C in 100 mM phosphate buffer.

Figure 4a, shows an increase in the protein stability. Maximum absorbance experiences a blue shift. Figure 4b demonstrates same shift of aromatic absorbance which is due to usual thermodynamic reaction of aromatic groups of the protein core against solvent and effector molecules.

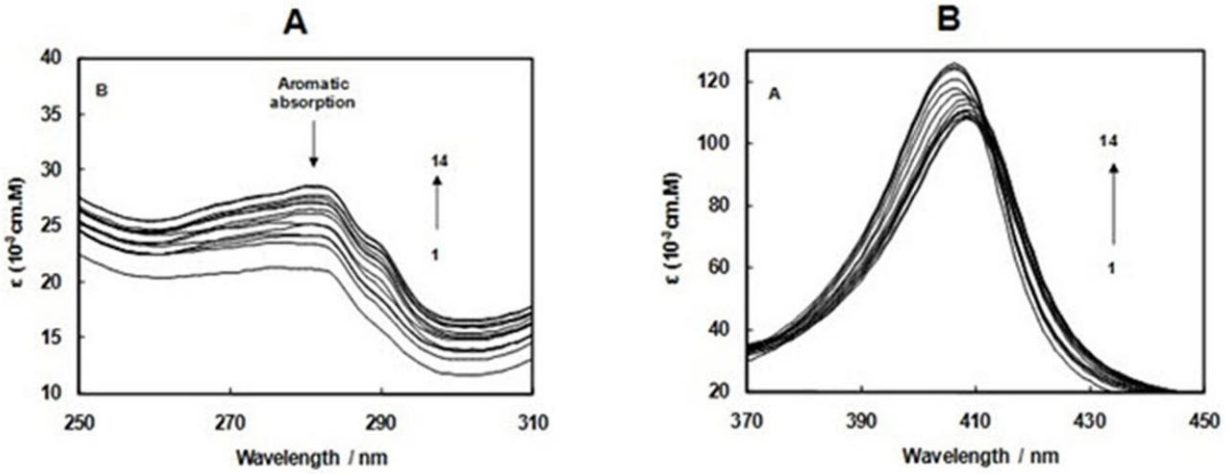

Figure 4. Effect of urea on (A) surat absorbance spectrum, (B) aromatic absorbance of 30 µM Cytochrome C in 100 mM phosphate buffer along with different concentration of urea (0 to 10 M).

Figure 5a shows CD spectrum of Cytochrome C at far UV. This spectrum provides information about secondary structure of the protein. In this chart, two down falls at 208 and 222nm are important. Figure 5b illustrates the impacts of different concentrations of urea on 222nm area. Increasing concentration of urea to 9M changes absorbance pattern at 22nm.

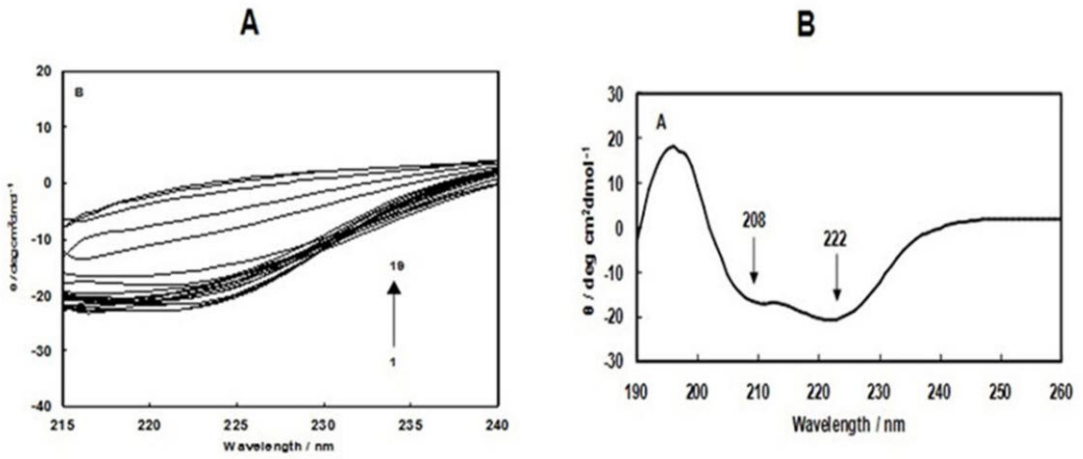

Figure 5. (a) CD absorbance spectrum of 30 µM Cytochrome C; (b) Impacts of different concentrations of urea (0-9 M) on the spectrum of Cytochrome C at 222 nm

3.2. Effects of SDS on structure of Cytochrome C

Figure 6 shows impacts of different concentrations from 0 to 26mM of SDS on voltammetry peaks of protein. In spite of very high concentrations of SDS, voltammetry peaks are considerable which determines native structure of the protein.

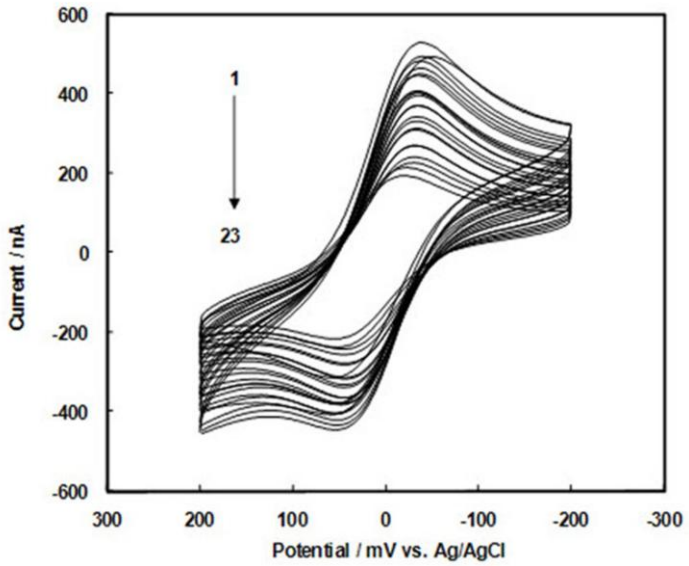

Figure 6. Effect of different concentrations of SDS on the electrochemical response of Cytochrome C; respectively, from top to bottom: 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9, 0, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 12, 14, 16, 18, 20, 22, 24 and 26 mM. Protein concentration is 100 mM in 20 mM of buffer, gold electrode modified with 2-mercaptoethanol.

Figure 7 determines consistency of spectroscopy and electrochemical results of SDS effects.

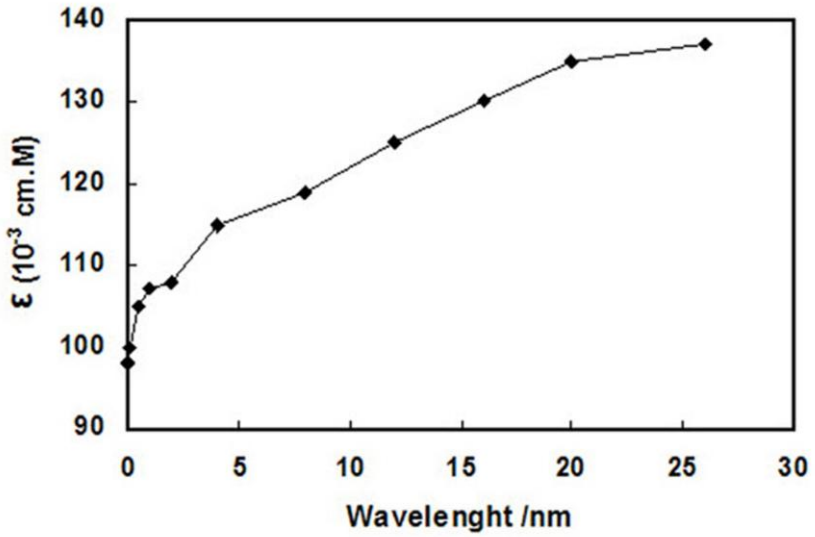

Figure 7. Effect of different concentrations of SDS absorbance line for 100 μM of Cytochrome C in 100 mM phosphate buffer.

4. DISCUSSION

Denaturing molecules such as urea and SDS directly interact with protein and disintegrate tertiary structure of it. In fact, these substances increase solubility of hydrophobic parts of proteins. Since interactions of such molecules have definite stoichiometry, concentrations of denaturants depend on concentrations of proteins [11].As protein concentration in electrochemical studies is higher than usual, it causes subsequent unpredictable increase in concentrations of denaturants. Increases in concentrations of urea lead to reduction in current peak suggesting reduced electrochemical activity of immobilized proteins [12,13].Most likely to reduce the flow-induced is changes in protein structure. This proposes that minor changes are happened in the structure of protein. According to previous studies [14],low concentrations of urea (5M) probably bring about instabilities in some part of the protein structure (especially residues surrounding Hem). Structural instabilities of Cytochrome C in voltammetry method are characterized by decreasing of peak heights. Excessive reduction of peak heights at above 5M concentrations of urea (Figure 1) is due to total denaturation of tertiary structure of Cytochrome C. Along with denaturation of protein, hem group (which is responsible for voltammetry responding) exposes solvent and due to environmental polarity changes the electron transfer rate constant decreases.

Different concentrations of urea cause changes in the protein spectrum, the most significant alterations are in folding and aromatic absorbance bands. Totally, addition of chemical agents such as urea increases absorbance at 280nm which might be cause of aromatic residues located on the protein surface. Higher concentrations of urea over 8M, decrease absorbance of the protein at 232nm. It also causes loss of secondary structure of the protein[14]

SDS is a surfactant molecule which has different special arrangements in different concentrations such as single layer molecules, micelle, and mono or double layer vesicles. This molecule has different behaviors interacting with proteins. At low concentrations, it helps integrity but at higher concentrations destroys protein structure. Based on previous reports, in lower concentration ranges of proteins (20 to 50mM) SDS to protein ratio should be 40 for complete denaturation. 20mM SDS would be enough to denature 0.5mM of Cytochrome C; electrochemical signals were still traceable at even 26mM of SDS (Figure 5) [15,16]

5. CONCLUSION

In this study, structural changes of Cytochrome C were studied with voltammetry and spectroscopic methods in presence of SDS and urea. It is realized that voltammetry is an independent method to detect structural changes of the protein. Denaturation curves from voltammetry are different from the one obtained from spectroscopy methods. The transition region in voltammetry (3-8M urea) is broader than spectroscopy methods (5-8M urea). This reflects that electrochemical method is more sensitive to small changes of protein structure. Spectroscopy method is based on a static process (the interaction of light with molecules) but electrochemical algorithms are dynamic (active electron transfer from reducing elements into). Therefore, this method can be used as new equipment in on protein structure denaturation studies.

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