



Tyrosinase enzyme Langmuir monolayer: Surface chemistry and spectroscopic study

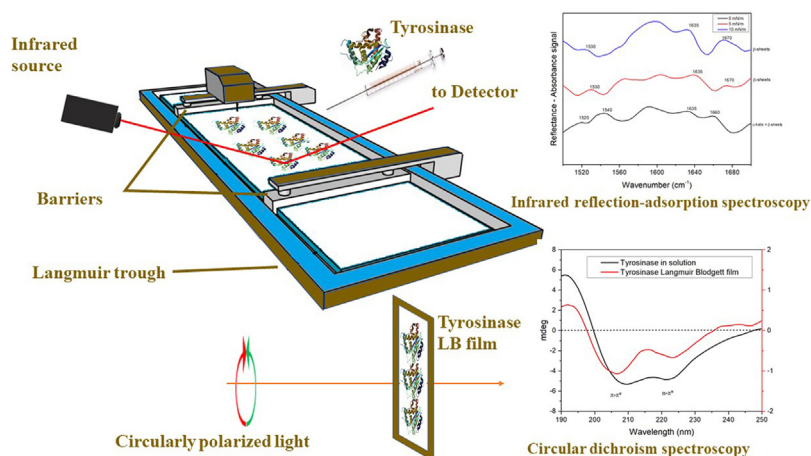
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GRAPHICAL ABSTRACT



ARTICLE INFO

Article history:

Received 21 November 2019

Revised 25 December 2019

Accepted 26 December 2019

Available online 28 December 2019

Keywords:

Tyrosinase

Surface chemistry

Langmuir monolayer

Infrared reflection-adsorption spectroscopy

Langmuir-Blodgett film

ABSTRACT

This study investigates the surface chemistry properties of the tyrosinase enzyme Langmuir monolayer at air-aqueous interface using sodium chloride in the subphase to induce the surface activity of the enzyme. Investigation of surface packing and stability of the tyrosinase Langmuir monolayer were performed using surface chemistry experiments while spectroscopic analysis was done to study enzyme conformation. It was found that the tyrosinase enzyme forms a fluid film at air-aqueous interface with good stability as shown by compression-decompression cycles experiments and stability measurements at various surface pressures. UV-vis absorption and fluorescence measurements at different surface pressures revealed that the Langmuir monolayer has good homogeneity with no evidence of aggregates during compression. To gain insight on the conformation of tyrosinase Langmuir monolayer p-polarized infrared-reflection absorption spectroscopy was used. It was found that at high surface pressures the predominant secondary structures were β -sheets while at lower surface pressure both α -helices and β -sheets were present. The circular dichroism spectra were obtained by transferring the Langmuir monolayer at 10 mN.m⁻¹ to a solid quartz support (Langmuir-Blodgett film, LB film), which showed that the major conformation present were α -helices. Images from the immobilized LB films were obtained using

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atomic force microscopy which showed homogenous and regular deposition with a mean thickness ranging from 3 to 4 nm.

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1. Introduction

Tyrosinase is a copper containing enzyme which catalyzes monophenols to their respective diphenols through o-hydroxylation and oxidation of diphenols to their respective o-quinones. In humans tyrosinase has an important role in the synthesis of melanin pigment. It catalyzes o-hydroxylation of tyrosine to DOPA (3,4-dihydroxyphenylalanine) and further oxidizes DOPA to dopaquinone. This process carries on through a series of enzymatic reactions to produce melanin pigments [1,2]. The monophenol hydroxylase and diphenol oxidase activities of the tyrosinase enzyme are the foundation for many biotechnological applications such as biosensors for detection of phenolic compounds [3], detection of oxygen consumption [4] and bioremediation of phenolic compounds from industrial wastewater [5]. Tyrosinase also has potential use in the pharmaceutical industry for production of L-DOPA (for treating Parkinson's disease) and has been tested as a potential marker for melanoma patients [6]. Considerable attention has been given for quantification of phenols in complex environmental matrices. Phenols are considered toxic pollutants in industrial wastewater from a number of industries such as coal conversion, petroleum refineries, textiles, dyes and steel. The use of tyrosinase enzyme for removal of phenols and its derivatives can be an effective method [7].

Researches on development of biosensors using enzymes film has grown considerably in past few years [8–10]. To use tyrosinase enzyme as biosensors, one needs to obtain important information about surface packing, dipole orientation and stability. Langmuir monolayer technique provides many information to understand how surface proteins interact at the interfaces by studying their air-subphase behavior [11–13]. This unique technique involves the generation of a one molecule thick monolayer of analyte whose behavior at the air-water interface can be studied. Measurement of the surface pressure, mean molecular area and surface potential can be employed to develop basic information like protein packing, conformation study and stability [14,15]. Furthermore, the detail information about secondary structures can be obtained by using circular dichroism (CD) and infrared reflection-absorption spectroscopy (IRRAS), which give information on the Langmuir monolayer properties at different surface pressures [16,17].

A Langmuir setup consists of a set of barriers, a Langmuir trough and a surface pressure sensor. The barriers, which are software controlled, are placed at the interface for the compression of the Langmuir monolayer. The trough is usually made up of hydrophobic material that holds subphase of interest where the Langmuir monolayer is developed. A sensor called Wilhelmy plate gives information about surface pressure of the Langmuir monolayer during compression. A surface pressure–area (π -A) isotherm is obtained, which gives information on the existence of different phase transitions at the air-subphase interface [18]. One has to consider that the dynamics of the Langmuir monolayer isotherm depends on the physical and chemical properties of the analyte and composition of the subphase [19].

In this work surface chemistry properties of the tyrosinase enzyme at air-subphase interface has been studied using Langmuir technique. In order to induce the surface activity of the enzyme, sodium chloride has been solubilized in the subphase. The stability of the Langmuir monolayer was studied using compression-decompression cycles and constant compression at different

surface pressures. Spectroscopic studies such as absorbance and fluorescence of the tyrosinase Langmuir monolayer were carried out and compared to the measurements with the tyrosinase enzyme in solution. Enzyme conformation and orientation at air-aqueous interface was studied using IRRAS and CD spectroscopic was examined on LB films.

2. Experimental section

2.1. Materials

Mushroom tyrosinase was obtained from Sigma Aldrich with molecular weight 120 kDa as determined by electrophoresis. The water that was utilized in the experiments was obtained from Millipore® Direct-Q® 3 (Type1) water purifying system having resistivity of 18.2 M Ω cm, a surface tension of 72.1 mN m⁻¹, and a pH of 6.0 $\pm$ 0.5 at 20.0 $\pm$ 1.0 °C temperature. NaCl subphase was prepared using molecular biology grade NaCl purchased from MP Biomedicals, LLC.

2.2. Equipment

All the experiments were conducted in a clean room (class 1000) with temperature (20.0 $\pm$ 1.0 °C) and humidity (50% $\pm$ 1%) maintained constantly. A Kibron μ -trough (Kibron Inc., Helsinki, Finland) with area of 124.5 cm² (5.9 cm $\times$ 21.1 cm) was used for the study of surface pressure–area (π -A) isotherms, surface potential–area isotherms, stability, and compression–decompression cycles. A Kelvin probe that consists of a capacitor-like system was utilized to measure surface potential. The vibrating plate was adjusted to $\sim$ 1 mm above the surface of the Langmuir monolayer. To assess the surface pressure, the Wilhelmy plate method with a 0.51 mm diameter alloy wire probe having a sensitivity of $\pm$ 0.01 mN m⁻¹ was used. For the UV-vis and fluorescence experiments at air-subphase interface a KSV mini-trough (KSV

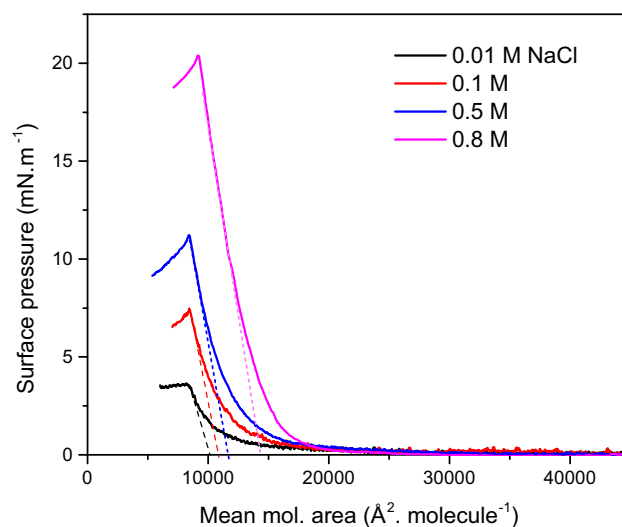


Fig. 1. Surface pressure–area isotherms of the tyrosinase Langmuir monolayer showing salting out effect due to varying concentration of NaCl in subphase (pH 6.0 $\pm$ 0.5; Temperature 20.0 $\pm$ 1.0 °C)

Table 1
Salting out effect of various concentration of NaCl in subphase.

Concentration of NaCl (M)	Collapse surface pressure	Limiting molecular area of Tyrosinase ($\text{\AA}^2 \cdot \text{molecule}^{-1}$)
0.01	3.5	10,269
0.1	7.37	10,871
0.5	11.23	11,721
0.8	20.3	14,336

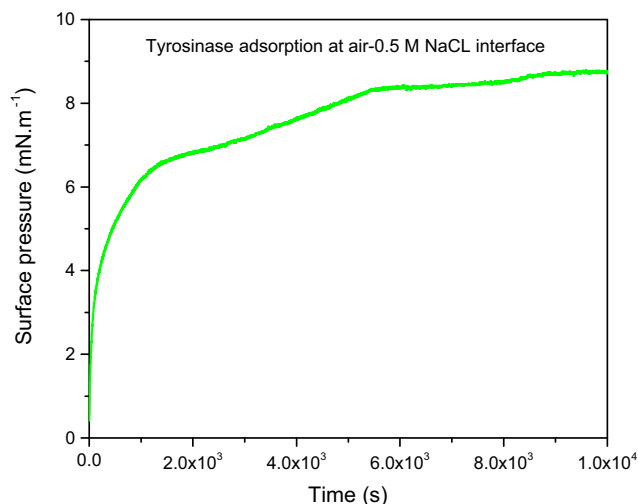


Fig. 2. Tyrosinase adsorption kinetics at air-subphase (NaCl 0.5 M; pH 6.0 $\pm$ 0.5) for a 3000 mm² surface area.

Instrument Ltd., Helsinki, Finland) having an area of 225 cm² (7.5 cm $\times$ 30 cm) was used.

2.3. Langmuir monolayer preparation

The tyrosinase solution was prepared in pure water (pH 6.0 $\pm$ 0.5) at the concentration of 0.1 mg.ml⁻¹. The tyrosinase was spread uniformly over the 0.5 M NaCl interface by using a 100 μ L syringe (Hamilton Co., Reno, Nevada). The spreading volume of the enzyme solution was 55 μ L for the surface chemistry and spectroscopic measurements. After spreading the solution, the Langmuir monolayer was allowed to attain the equilibrium state, i.e. 20 min. Then, Langmuir monolayer was compressed with the rate of 4800 $\text{\AA}^2 \text{molecule}^{-1} \text{min}^{-1}$. The experiments were repeated more than three times to assure reproducibility.

2.4. In situ UV-vis spectroscopy

The UV-vis absorption spectra of the Langmuir monolayer were taken with the help of a model 8452 A HP spectrometer fixed on a rail close to the KSV trough (KSV Instrument Ltd., Helsinki, Finland).

2.5. In situ fluorescence spectroscopy

The *in situ* fluorescence spectra of the tyrosinase Langmuir monolayer were measured with the help of an optical fiber detector on the top of the KSV trough, which was coupled to the Spex Fluorolog spectrofluorometer (Horiba, Jovin Yvon, Edison, NJ). The optical fiber used in the experiment has an area of 0.25 cm² and rests approximately 1 mm above the surface of the subphase and the slit width of 5 nm was used for excitation and emission. The instrument works in such a way that the excitation light gets

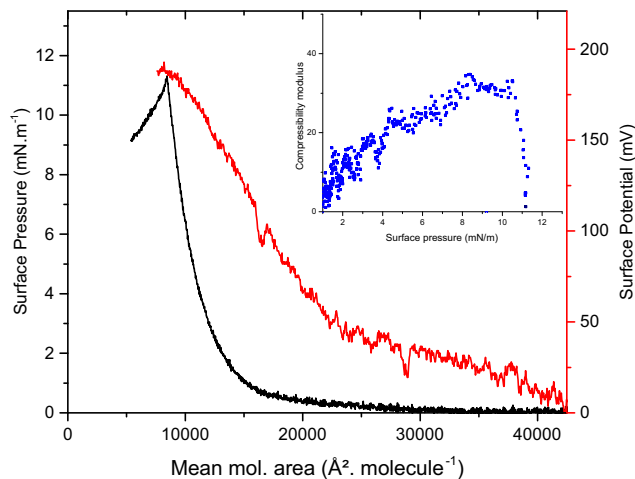


Fig. 3. Surface pressure and surface potential isotherms for the tyrosinase (0.1 mg.ml⁻¹) Langmuir monolayer at air-subphase (NaCl 0.5 M; pH 6.0 $\pm$ 0.5). Insert shows the compressibility modulus for the enzyme during compression.

transmitted through the optical fiber from the light source to the Langmuir monolayer and the emitted light from the Langmuir monolayer gets dispatched back to the detector through the optical fiber.

2.6. Infrared-reflection absorption spectroscopy

Bruker Equinox 55 FTIR (Billerica, MA) with XA-511 accessory was used to obtain the infrared spectra of the tyrosinase monolayer. P-polarized light and a mercury-cadmium-telluride (MCT) liquid nitrogen-cooled detector were used for the measurements. Each spectrum was acquired with 2048 scans with resolution of 8 cm⁻¹ at 60° incident angle. This angle has been chosen for its highest signal to noise ratio.

2.7. Langmuir-Blodgett films preparation

The transfer of preformed Tyrosinase monolayers to solid supports (quartz slide) was carried out by raising the slides up from the NaCl aqueous subphase to the air phase at a 2 mm.min⁻¹.

2.8. Circular dichroism

Circular Dichroism spectra were obtained using JASCO J-810 spectropolarimeter. Langmuir-Blodgett technique was used to transfer a monolayer of the tyrosinase enzyme on solid quartz support. At first the quartz support was dipped into the subphase; then the tyrosinase enzyme 55 μ L (0.1 mg.ml⁻¹) was spread on the subphase and waited for 20 min to reach equilibrium. After that, the Langmuir monolayer was compressed until the pressure reached 10 mN.m⁻¹ and this pressure was kept constant. Finally, the quartz support was slowly raised with the monolayer being transferred on it. The deposition ratio was about 1.0 $\pm$ 0.1.

2.9. AFM imaging

Images of the tyrosinase Langmuir-Blodgett films, were obtained using Agilent Technologies microscope model 5420 with tip thickness of 5.0 μ m, length of 225 μ m and a force constant of 20 N m⁻¹ in tapping mode. The parameters used for scanning includes a resonance frequency of 110 kHz, a scan rate of 1.0 Hz and 10 $\times$ 10 μ m scan area.

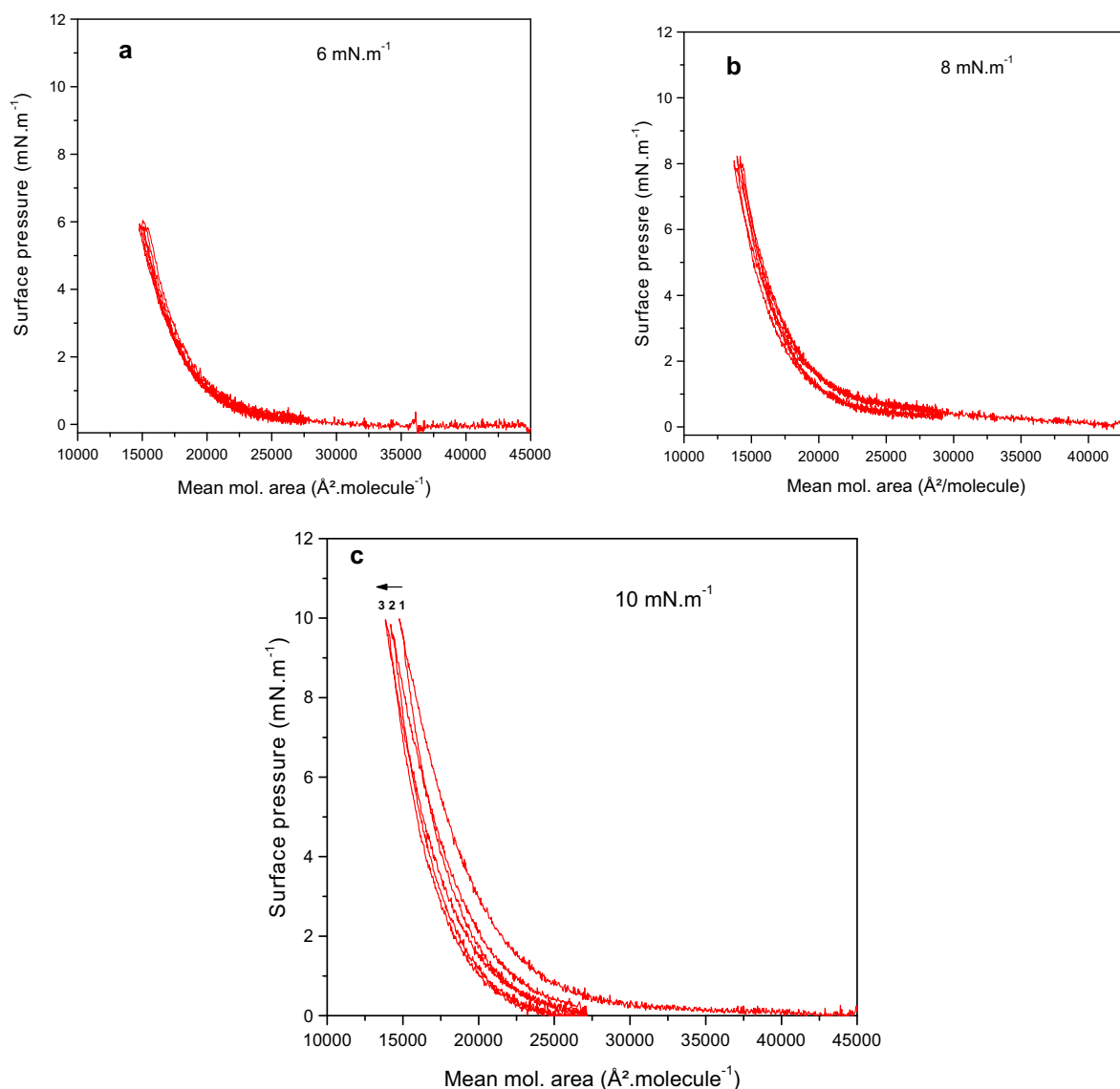


Fig. 4. Compression–decompression cycles of the tyrosinase Langmuir monolayer at air-subphase (NaCl 0.5 M; pH 6.0 ± 0.5), maintained at the surface pressure of a) 6, b) 8 and c) 10 mN.m⁻¹.

3. Results and discussion

3.1. Surface pressure - area isotherms and adsorption kinetics

The Langmuir monolayer behavior and interfacial properties of the tyrosinase on the surface of saline solution were investigated by spreading 55 μL of the enzyme (0.1 mg.ml⁻¹) on different concentrations of NaCl subphase solution. In order to check the salting out effect on the number of tyrosinase molecules adsorbed at the air-aqueous interface, different concentrations of NaCl (0.01, 0.1, 0.5 and 0.8 M) were tested. In the absence of NaCl solution, the monolayer was unable to form, however, using different concentrations of NaCl, various Langmuir monolayer isotherm could be obtained as shown in Fig. 1.

When the Langmuir monolayer was compressed, at the beginning the surface pressure remained zero in the range of 45,000 to 20,000 $\text{\AA}^2 \cdot \text{molecule}^{-1}$ which can be correlated to the gaseous phase. Further compression resulted an increase in the surface pressure with the decrease in area per molecule. In 0.5 M NaCl subphase, the surface pressure started increasing when the mean molecular area reached 17,000 $\text{\AA}^2 \cdot \text{molecule}^{-1}$. This phase is

considered as the beginning of the liquid expanded phase which is observed until the area reaches 10,000 $\text{\AA}^2 \cdot \text{molecule}^{-1}$. Upon further compression, there was sharp increase in the surface pressure from 10,000 to 8500 $\text{\AA}^2 \cdot \text{molecule}^{-1}$; this can be correlated to liquid condensed phase. The limiting molecular area and subphase surface pressure kept on increasing with increase in the NaCl concentration in the subphase as shown in Table 1. Based on the salting out effect on the surface-pressure area isotherm, 0.5 M NaCl subphase was selected for all further experiments.

Tyrosinase enzyme is water-soluble, therefore, to induce its adsorption onto the surface, sub phase of a NaCl was used. A volume of 55 μL from a 0.1 mg.ml⁻¹ tyrosinase solution (dispersed in ultrapure water) was spread onto the surface of four different NaCl concentrations (0.01, 0.1, 0.5 and 0.8 M), as shown in Fig. 1. As expected, the enzyme molecules were forced out of the aqueous solution proportionally to the subphase salt concentration indicated by the shift in the surface pressure-area isotherms towards higher mean molecular areas. Moreover, an overall increase in the maximum surface pressure attained by the interfacial layer is also observed as presented in Table 1.

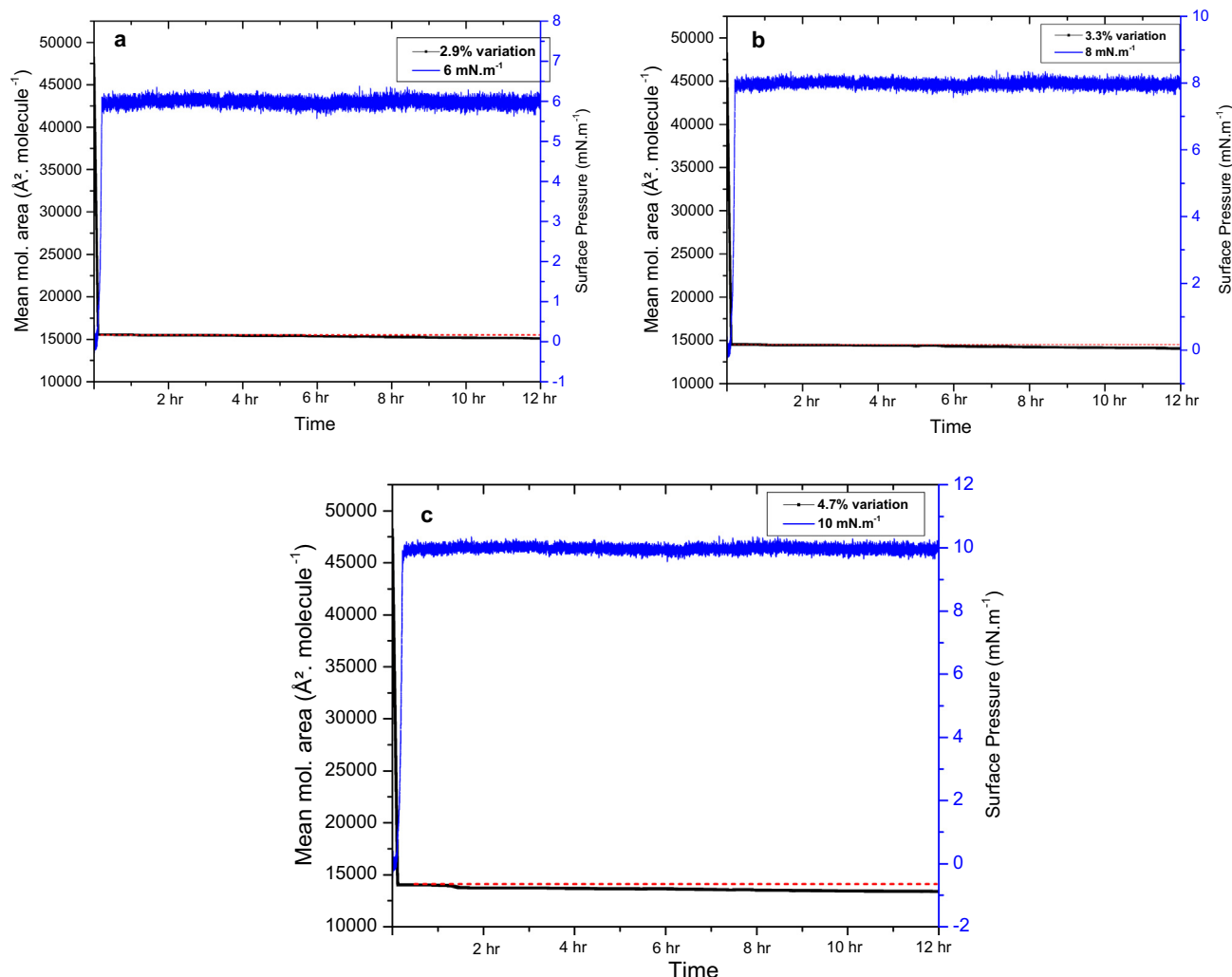


Fig. 5. Stability measurements of the tyrosinase Langmuir monolayer at three different surface pressures a) 6 mN.m^{-1} , b) 8 mN.m^{-1} and c) 10 mN.m^{-1} at air-subphase (NaCl 0.5 M ; pH 6.0 ± 0.5 ; Temperature 20.0 ± 1).

Subphase concentrations higher than 0.8 M were also tested, leading to monolayers with greater limiting molecular areas and higher maximum surface pressures (not shown). It is important to mention that elevated ionic strengths may lead to enzyme denaturation, which makes the intermediate concentration of 0.5 M appropriate for further analysis.

The adsorption kinetics in Fig. 2 shows that most of the enzyme adsorption at the interface started between 1.2×10^3 and $1.5 \times 10^3 \text{ s}$ with a maximum surface pressure of 8.5 mN.m^{-1} . Using the adsorption profile surface chemistry experiments were performed after 20 min lag time after spreading the tyrosinase enzyme to attain the equilibrium. This shows that the tyrosinase enzyme is surface active.

3.2. Surface pressure - surface potential isotherm

Surface potential evaluates the potential difference, which is the difference in dipole moment above and below the Langmuir monolayer film. The interaction of molecules at longer distances is regarded as dipole-dipole interaction and surface pressure quantifies the interactions between molecules in close contact (van der Waals interaction). The surface potential-area isotherms exhibit the molecular interactions that occur before and during phase change of the monolayer as seen during the compression [20]. When compression of the Langmuir monolayer is started, the

surface potential-area isotherm exhibits an increase in the surface potential which is due to the charges that are present on tyrosinase. When the Langmuir monolayer begins to move from a liquid expanded phase to liquid condensed phase a kink appears on the surface potential curve at about $23000 \text{ Å}^2 \text{ molecule}^{-1}$ and a change in surface potential is observed. The other small bumps seen in the surface potential curve at very low surface pressure are due to tyrosinase molecules rapidly moving on the subphase surface under the vibrating electrode. The maximum voltage of 185 mV relates to the compact packing structure of the enzyme molecules. At this value, due to short distance between molecules some of the dipole-dipole interactions get canceled out which ultimately results in a steady value in surface potential.

The inserted graph in Fig. 3 shows the compressibility modulus ($C_s^{-1} = -A(\frac{\partial \pi}{\partial A})_T$) of the tyrosinase monolayer extracted from its surface pressure-area isotherm during its compression. Compressibility modulus is a rheological parameter which gives physical information about interfacial elasticity of Langmuir monolayer during compaction. Based on rheological published data, it has been summarized that C_s^{-1} between 0 and 12.5 mN.m^{-1} refers to the gas phase, from 12.5 to 50 mN.m^{-1} the liquid - expanded phase and from 100 to 250 mN.m^{-1} the liquid - condensed phase. The values above 250 mN.m^{-1} are features of the solid phase [21]. C_s^{-1} values from Fig. 3 showed that the C_s^{-1} was never above 40 mN.m^{-1} which tells us that the tyrosinase Langmuir monolayer stayed

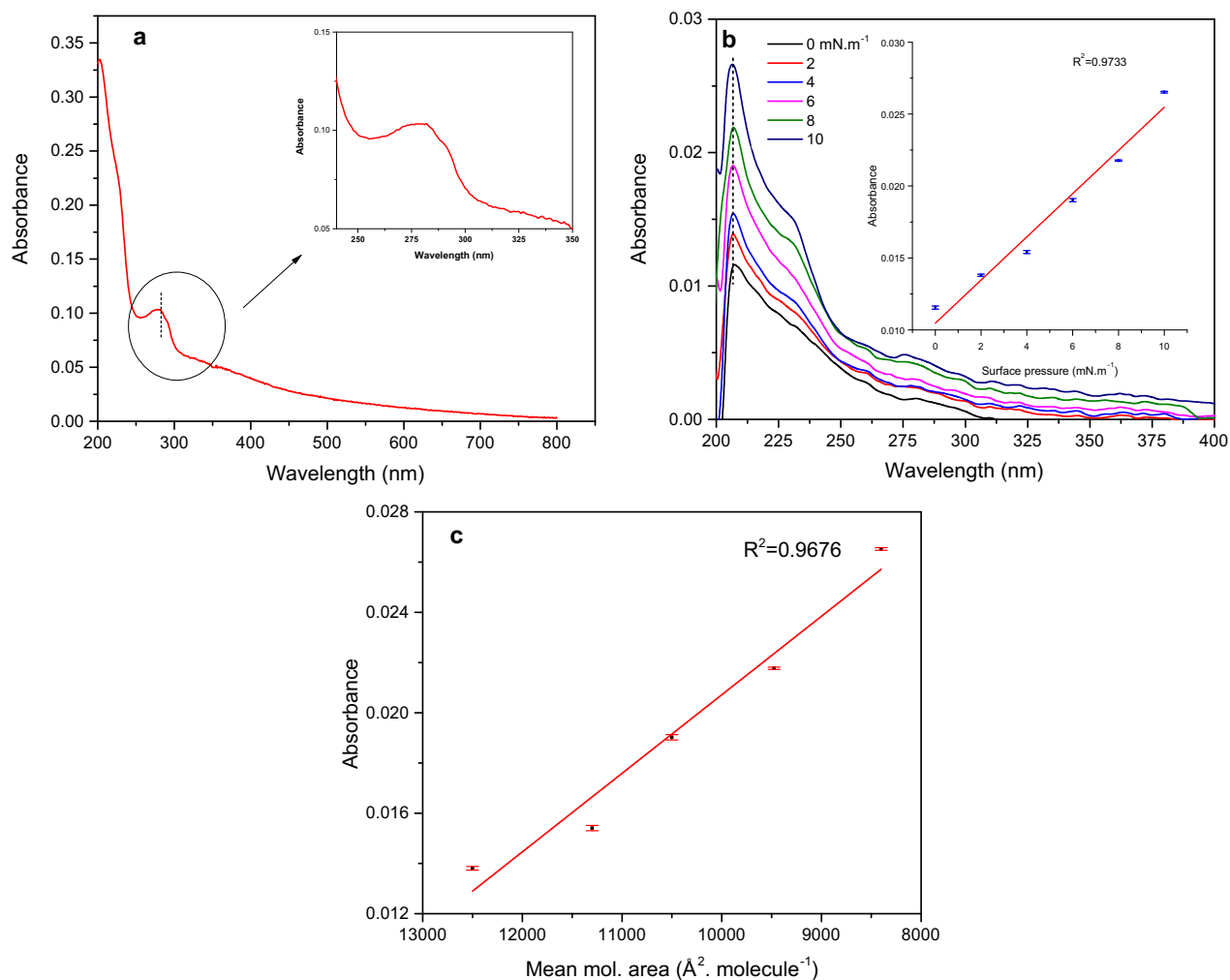


Fig. 6. UV-vis absorption spectra of the tyrosinase a) in solution (0.04 mg.ml⁻¹ concentration with optical path length of 1 cm of quart cell); b) Langmuir monolayer at air-subphase (NaCl 0.5 M pH 6.0 ± 0.5). The inserted graph consists a plot of absorbance at different surface pressures; c) Plot of absorbance versus mean molecular area.

in the liquid-expanded range for majority of compression process. Lower values of C_s^{-1} generally refer to the low rigidity and compaction of monolayer, which shows that tyrosinase forms a fluid interfacial layer with low viscoelasticity.

3.3. Compression-decompression cycles and stability studies

The compression-decompression cycles show the extent of loss of analyte to desorption from the interface into the subphase, which is known as hysteresis. Moreover, the compression-decompression cycle gives information relating to the stability of the film [22,23]. The surface pressure for compression and decompression cycles were chosen 6, 8, and 10 mN.m⁻¹. For the successive compression-decompression cycles (3 cycles) that followed, a hysteresis behavior of the isotherm was observed. From Fig. 4, it was found that the hysteresis was 2.7, 3.8 and 11% with 6, 8 and 10 mN.m⁻¹ surface pressure, respectively. At lower surface pressure (6 and 8 mN.m⁻¹) the loss of material or change of conformation of the enzyme at the subphase was minimal while increasing the pressure to 10 mN.m⁻¹ caused a small increase of hysteresis. As compression-decompression takes appreciable time to complete the cycles, it can also be used as one of the parameters to check the stability of the Langmuir monolayer.

To further analyze the stability, the tyrosinase Langmuir monolayer experiments were performed at different surface pressures, 6,

8 and 10 mN.m⁻¹ [Fig. 5]. For this, the monolayer was kept at a constant pressure and the stability was observed over the period of 12 hr. At all three surface pressures, the collapse of the monolayers was not observed. This gives evidence that the tyrosinase enzyme has good stability at the air-subphase interface over a period. The variations in the mean molecular area were calculated after 12 h at 6, 8 and 10 mN.m⁻¹ which were 2.9, 3.3 and 4.7% respectively. This shows that, there is slight loss of enzyme into subphase over the period which increases with higher surface pressure.

3.4. UV-vis absorption and fluorescence spectroscopies:

To further study the homogeneity of the enzyme at the air-subphase interface, UV-vis spectra of tyrosinase Langmuir monolayer at different surface pressures were recorded. Fig. 6a shows that the absorbance spectra of 0.04 mg.ml⁻¹ of tyrosinase enzyme solubilize in water. The absorbance peak at 280 nm (Fig. 6a) can be attributed to the tryptophan molecules present in the enzyme as shown by the amino acid sequencing. The absorbance spectra at the monolayer of the enzyme (Fig. 6b) shows an intense peak at 207 nm and a broad peak around 230 nm. These bands can be attributed to $\pi - \pi^*$ transition at 207 nm and $n - \pi^*$ transitions at 230 nm of the peptide bonds present in the enzyme. When plotting the absorbances values vs surface pressures, a linear relationship was established at wavelength 207 nm. The linearity of absorption

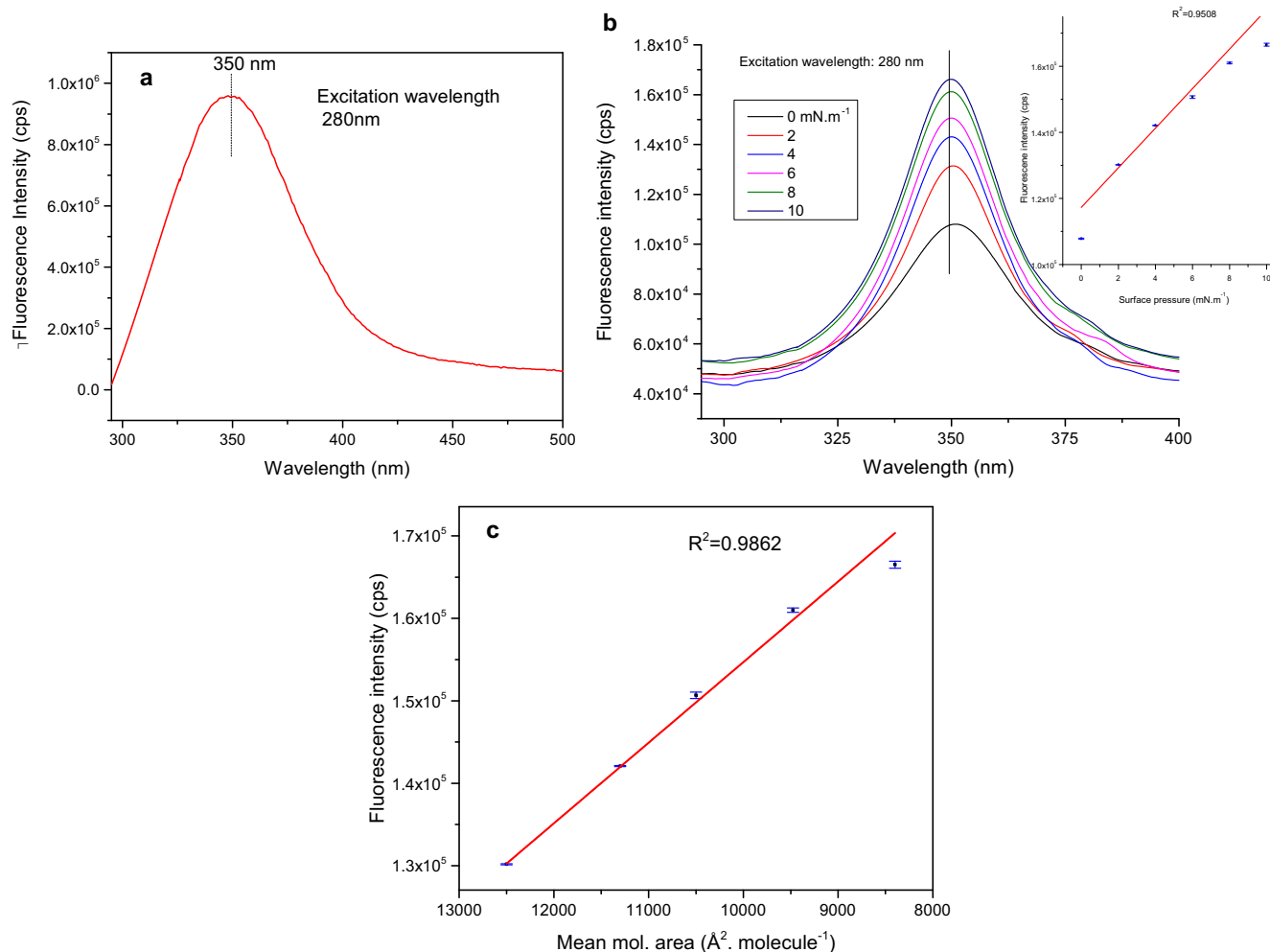


Fig. 7. Fluorescence spectra of the tyrosinase a) in solution (0.04 mg.ml^{-1} concentration with optical path length of 1 cm of quart cell and slit width of 5 nm) b) Langmuir monolayer at air-subphase (NaCl 0.5 M; pH 6.0 ± 0.5). The inserted graph consists a plot of fluorescence intensity at 350 nm at different surface pressure; c) Plot of fluorescence intensity at 350 nm versus mean molecular area.

suggests that during compression the number of molecules per unit area was increasing linearly. To confirm that there is not any aggregation, absorbance versus mean molecular area was plotted which also shows linear relationship (Fig. 6c). This observation reinforces the interpretation that the Langmuir monolayer was retained at the interface without aggregation during compression.

The fluorescence spectra of tyrosinase showed the emission maxima at 350 nm when excited at 280 nm both in solution and in the Langmuir monolayer as presented in Fig. 7a and 7b. The fluorescence emission at 350 nm can be attributed to the emission from tryptophan. The fluorescence intensity at different surface pressure shows linearity corresponding to the observation from absorbance data at similar surface pressures. The lack of quenching of the fluorescence when there is an increase in the molecules per unit area rules out any aggregation effect in the Langmuir monolayer. Moreover, linear relationship between fluorescence intensity and mean molecular area as shown in Fig. 6c further confirms that aggregates were not formed during compression of tyrosinase Langmuir monolayer.

3.5. Infrared reflectance-absorbance spectroscopy (IRRAS)

IRRAS has been one of the important techniques for *in situ* characterization of the secondary structures of proteins at the air-aqueous interface [24,25]. For orientational changes of the protein at Langmuir monolayer p-polarized light is usually used, as it

probes the dipole moments both parallel and perpendicular to the interface compared with the s-polarized light which only probes parallel to the interface [26]. The incident angle and orientation of transition dipole moment determines, whether the measured signal have positive or negative bands [27]. Those dipole moments which are parallel to the air-aqueous interface have negative bands below the Brewster angle (54.2° at 2850 cm^{-1} for IR light) and positive bands above it [26,28].

In this technique the p-polarized IR beam at a 60° angle of incidence (best signal-noise ratio in the spectrum) was used to investigate tyrosinase Langmuir monolayer on 0.5 M NaCl subphase for information about orientation and conformation. For biomolecules like proteins and enzymes, spectroscopic analysis of amide bands (I and II) helps to study conformational changes at the air-aqueous interface during compression of the Langmuir monolayer. For α -helix structures, absorption spectrum for amide I (C=O stretching from peptide bond) ranges from 1650 to 1660 cm^{-1} and amide II (N-H bending and C-N stretching) ranges from 1540 to 1550 cm^{-1} . Similarly, for β -sheets structures absorption for amide I and amide II are at 1620 – 1640 and 1520 – 1535 cm^{-1} , respectively [17,29].

The IRRAS spectra showed the orientation of the enzyme changes with respect to the aqueous surface during compression. At 0 mN.m^{-1} both α -helix and β -sheets coexist as Fig. 8 shows amide I and amide II bands at 1660 and 1540 cm^{-1} for α -helix

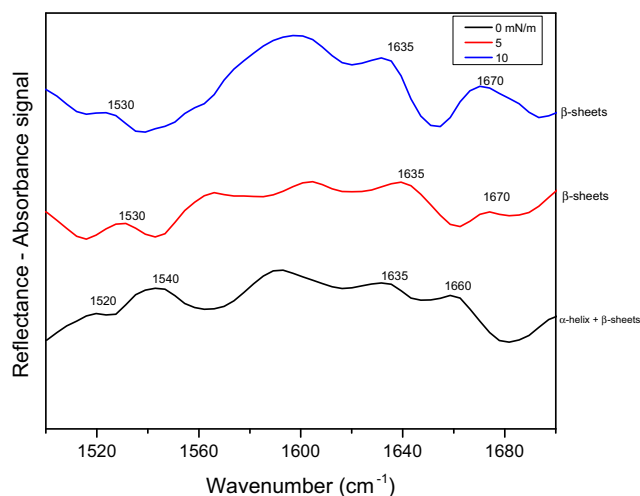


Fig. 8. p-polarized IRRAS spectra of the tyrosinase Langmuir monolayer at air-subphase (0.5 M NaCl, pH 6.0 $\pm$ 0.5) at 60° incident angle and at surface pressure 0, 5 and 10 mN.m⁻¹.

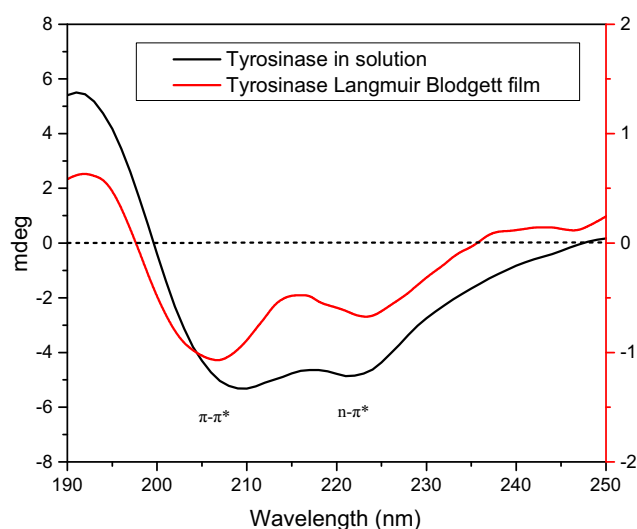


Fig. 9. Circular dichroism spectra of the tyrosinase LB film on quartz substrate deposited at 10 mN.m⁻¹ and the tyrosinase enzyme in solution (0.02 mg.ml⁻¹).

and 1635 and 1520 cm⁻¹ for β -sheets, respectively. However, while increasing the surface pressure to 5 and 10 mN.m⁻¹ the α -helix bands disappear and bands at 1635 and 1530 cm⁻¹ becomes dominant which can be assign to amid I and amide II bands of β -sheet structures. These bands are accompanied by appearance of a new band at 1670 cm⁻¹ representing β turns. This phenomenon shows that initially, at low surface pressure, the transition dipole moment of the helical axis of enzyme was parallel to the interface so the peaks were observed and upon compression the helical axis became more perpendicular therefore there was no signal. Simultaneously, for β -sheets conformation, the orientation of the dipole moments appeared more parallel with compression which caused appearance of peaks for β -sheets along with β turns.

3.6. Circular dichroism and AFM imaging

Circular dichroism (CD) is one of the spectroscopic techniques which measures the differential absorption of left and right circularly polarized light by compounds having chirality [30]. CD spectra ranging from 190 to 250 nm has been extensively used to analyze conformations of proteins. Information about secondary structure of proteins having different conformations have been characterized by CD spectroscopy. Proteins with α -helical conformation have negative bands at 208 and 222 nm and positive band at 193 nm. However, proteins with β -pleated sheets have negative band around 218 nm and positive band at 195 nm [31,32]. In Fig. 9, the black curve of the tyrosinase in solution clearly shows two bands at 208 and 222 nm which can be attributed to π - π^* and n - π^* transitions for peptide bonds in the α -helix conformation of enzyme. Langmuir-Blodgett (LB) film technique was used to transfer the tyrosinase enzyme on a solid quartz surface. The red curve in Fig. 9 was obtained for the tyrosinase Langmuir monolayer transferred at 10 mN.m⁻¹ surface pressure. Bands at similar positions, compared with the tyrosinase in solution were observed for the LB film, with slight shift in the peak position. This suggests that the tyrosinase enzyme, both in solution and on the LB film has α -helical conformation.

Atomic force microscopy has been a useful technique to study properties of protein biomolecules when combined with other spectroscopic methods [33,34]. Images of the adsorbed proteins on the solid support helps to know the morphology of LB films [35]. Fig. 10a shows the image of the tyrosinase enzyme LB film, deposited on a quartz substrate at 10 mN.m⁻¹ surface pressure, using tapping mode. The bright yellow bumpy regions throughout

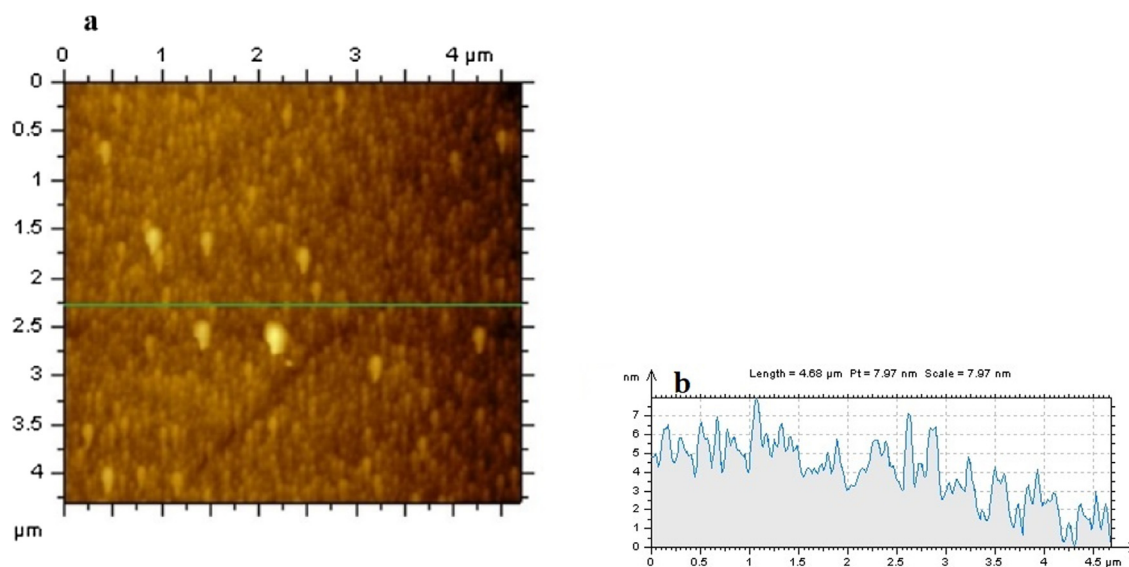


Fig. 10. AFM image of the tyrosinase enzyme (a) LB film on a quartz substrate and (b) extracted height profile.

the image indicates that the deposition was regular and homogeneous. The extracted height profile from Fig. 10b shows that the mean thickness of the tyrosinase film is in the range of 3–4 nm which is usual for biomolecules like proteins [35].

4. Conclusions

The present work reports the use of Langmuir monolayer technique to study the behavior of tyrosinase enzyme at air-aqueous NaCl subphase and its transferred films on solid support. It was found that *salting out* effect has a major role in producing stable monolayer as limiting molecular area and the Langmuir monolayer collapse surface pressure kept on increasing with increasing concentration of NaCl in subphase. The adsorbed tyrosinase enzyme forms a fluid interfacial film as revealed by the compressibility modulus calculation. The compression-decompression cycles and stability at constant pressure showed that tyrosinase forms stable monolayers at air-water interface in presence of salt. The UV-vis and fluorescence measurements throughout the compression shows linearity suggesting that the Langmuir monolayer presented good homogeneity. Data obtained from IRRAS indicated that at lower surface pressures both α -helix and β - sheets structures were conserved whereas at higher surface pressure there is a predominance of β - sheets structures. Spectra from circular dichroism showed that the immobilized layer of tyrosinase on quartz glass has an α -helix conformation. Images from AFM showed that the immobilized LB film presents a homogenous and regular deposition with mean thickness ranging from 3 to 4 nm. Overall, we believe that the present work enhances the fundamental understanding of formation of the Langmuir monolayer and Langmuir-Blodgett films especially for enzymes. In addition, this work can be particularly important in the design of the Tyrosinase covered electrodes for the detection of polyphenols as biofilm sensors.

CRedit authorship contribution statement

Suraj Paudyal: Conceptualization, Data curation, Investigation, Methodology, Project administration, Software, Validation, Writing - original draft. **Shiv K. Sharma:** Data curation, Formal analysis, Investigation, Methodology, Project administration, Software, Validation, Writing - original draft. **Rafael L.C.G. da Silva:** Investigation, Project administration, Validation. **Keenan J. Mintz:** Investigation, Software, Validation, Writing - review & editing. **Piumi Yasodha Liyanage:** Methodology, Validation. **Abdulrahman O. Al-Youbi:** Funding acquisition, Resources, Visualization. **Abdulaziz S. Bashammakh:** Funding acquisition, Resources, Visualization. **Mohammad S. El-Shahawi:** Funding acquisition, Resources, Visualization. **Roger M. Leblanc:** Formal analysis, Funding acquisition, Project administration, Resources, Supervision, Supervision, Writing - review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgment

The authors greatly appreciate the generous funding support provided by National Science Foundation (NSF), United States, grant # GR-011298.

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