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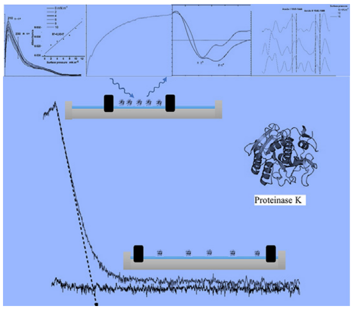
Interfacial behavior of Proteinase K enzyme at air-saline subphase

Suraj Paudyal[a], Ganesh Sigdel[a], Sujit K Shah[a,e], Shiv K. Sharma[a], John D. Grubb[b], Miodrag Micic[b,c], Luciano Caseli[d], Roger M. Leblanc[a,*]

[a] Department of Chemistry, University of Miami, 1301 Memorial Drive, Coral Gables, FL 33146, USA
[b] Gojira Fine Chemicals LLC, 5386 Majestic Pkwy , Suite #7, Bedford Heights, OH 44146, USA
[c] Department of Engineering Design Technology, Cerritos College, 11110, Alondra Blvd, Norwalk, CA 90650, USA
[d] Department of Chemistry, Federal University of São Paulo, Diadema, Sao Paulo, Brazil
[e] Department of Chemistry, Mahendra Morang Adarsh Multiple Campus, Tribhuvan University, Biratnagar 56613, Nepal

GRAPHICAL ABSTRACT



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ABSTRACT

This study investigates the interfacial behavior of the proteinase K enzyme at air–water interface. Adsorption of enzyme on the surface was induced using saline subphase. The surface packing and stability of the enzyme was investigated using of surface pressure-area (π-A) and surface potential-area (ΔV-A) isotherms. Proteinase K enzyme forms film at air-aqueous interface and demonstrates good stability as shown through compression-decompression cycle experiments. To characterize the surface assembly morphology of the interfacial enzymes UV–vis and fluorescence spectroscopic techniques were used. The data revealed that the enzyme Langmuir monolayer has good homogeneity with no evidence of aggregates during compression. The secondary structure of the enzyme at interface was determined to be α-helix using p-polarized infrared-reflection absorption spectroscopy. This was confirmed through Circular dichroism spectra of the enzyme Langmuir-Blodgett (LB) film which showed that the major conformation present were α-helices.

1. Introduction

In this report we present the results of the investigations of the interfacial behavior of the proteinase K enzyme at air–water interface. Proteinase K is a highly potent proteolytic enzyme in nature found in fungus *Tritirachium Album Limber*[1,2] *(*also known as *Engyodontium album*) and its recombinant variants are the most used enzyme for general digestion of tissues and proteins. Proteinase K got its name from its unique ability to digest keratin, such as hair or nails. Proteinase K is a broad-spectrum serine protease lytic enzyme. It's a member of the S8 (subcilisin) family of pro-

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* Corresponding author.
E-mail address: rml@miami.edu (R.M. Leblanc).

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teases. Proteinase K is also known under the names of protease K, endopeptidase K, and Tritirachium alkaline proteinase. Proteinase K digests native proteins by clearing peptide bonds adjacent to the carboxyl group of aromatic and aliphatic amino acids with blocked alpha amino groups. It is activated by Ca++ ions, and it is stable in the presence of chelating agents. It is active over a wide pH range (4–12) and remains active in presence of detergents as well. It is extensively used in a sample preparation workflow for decontamination of DNase, RNase from nuclei acid preparation for molecular diagnostics/PCR assays and for sequencing applications[3]. Proteinase K has also been used for sample preparation and digestions of prions and amyloids[4] as well as in cell culture applications for releasing mammalian cells from the tissue extracellular matrix. Recent major application of proteinase K is for sample preparation workflows of molecular diagnostics assays for coronavirus PCR test, where it is used for lysis/digestion of samples from blood or from nasopharyngeal swab[5].

Proteinase K enzyme is the main "work-horse" of sample preparation for molecular biology and molecular diagnostics applications. Proteinase K is considered the essential lab reagent for molecular and cell biology, and is one of the most important *in vitro* diagnostics enzyme used in molecular diagnostics assays sample prep stage and is commonly used for its high activity and broad specificity in tissues and cellular sample digestion/lysis In molecular diagnostics assays workflow, proteinase K main application is in isolation of high quality genomic DNA and RNA out of microbial and mammalian samples and in clearing out any lysate from proteins. During the process of tissue lysis or homogenization, proteinase K digest cellular proteins and furthermore protects nucleic acids by rapidly degrading RNAses and DNAses enzymes, and as such is essential component of many molecular diagnostics, forensics and life sciences protocols and kits.

Due to sudden increase in demand of molecular assays for COVID-19, proteinase K enzyme is in a short supply and has become a de-facto bottleneck for further proliferation of PCR tests. Thereby, it will be beneficial to device a way to reduce use of this enzyme per test, for example by immobilizing it on a substrate Recently published study immobilized proteinase K on Whatman paper no. 1[6] for the sample prep of urine for tuberculosis molecular assay. In this study, we will investigate the Langmuir and Langmuir Blodgett monolayer properties of proteinase K, to determine if it is possible to create a stable monolayer at air–water interface and then transfer it to solid support using Langmuir-Blodgett technique, which can lead to a novel way of immobilization of proteinase K on substrates and dramatically reducing amount of enzyme needed per test, if such heterogeneous catalyst is used instead of the enzyme solution. This report represents first step in this direction, as we investigate the formation and stability of the enzyme monolayers under different surface pressure and examine stability and uniformity of the solid-state deposited Langmuir Blodgett film of the enzyme.

The Langmuir monolayer and Langmuir-Blodgett (LB) films have been successfully used as models for biological interfaces since its introduction. These techniques provide vital information about the interfacial properties of molecules which varies from bulk phase[7–9]. Information, specific to Langmuir monolayer of molecules, at air–water interface are obtained from coupling of surface pressure-area (π-A) and surface potential-area (ΔV-A) isotherms, UV–vis, fluorescence, and Fourier transform infrared spectroscopies *in situ* [10,11]. Furthermore, LB technique is used for controlled preparation of thin films which are transferred to a solid support from a pre-formed stable Langmuir monolayer at air-water interface[12]. In recent years, this technique has particularly gain importance for immobilizing biomolecules such as enzymes on solid support for the purpose of biosensing specific analytes [12]. Enzyme conformation plays an important role for maintaining its activity, therefore, conformational changes of enzyme before and after immobilization can be studied using LB technique through Circular dichroism (CD) spectroscopy[13]. Information about the secondary structure and orientation of enzyme Langmuir monolayer can be studied using Infrared reflection absorption spectroscopy (IRRAS).

Most of the enzymes are soluble in water, therefore when spreading on air–water interface they partition between interface and aqueous subphase. To increase the portion of enzymes on interface, usually salt is incorporated in subphase which helps adsorption of enzymes on the surface. Herein, interfacial properties of proteinase K enzyme has been studied at air-aqueous/NaCl subphase. In this study, adsorption of proteinase K on air-subphase interface has been investigated. The stability of proteinase K Langmuir monolayer is studied using compression-decompression cycles and constant compression at different surface pressures. Spectroscopic properties of the enzyme are emphasized by comparing UV–vis, fluorescence spectra between the Langmuir monolayer and bulk solution. Moreover, the enzyme conformation and orientation at air-subphase interface has been studied using IRRAS *in situ* and CD of LB film. Immobilization of an enzyme is always the crucial phase for industrial application, as immobilization increases the endurance of enzyme and reduces its quantity. Therefore, this study model can be very useful as it provides interfacial properties of proteinase K required for many potential applications. In comparison to the bulk aqueous solutions, solid–liquid interfaces constitute different microenvironments which can affect the structure and activity of enzymes. The enzyme close to the interface possess different electronic interaction than inside the bulk. Surface chemistry studies of Proteinase K enzyme coupled with its immobilization on solid support allows this biocatalyst to be reused which has point of interest for future applications. Furthermore, various surface sensitive spectroscopic technique has been applied on this study which allowed the structure and orientation of Proteinase K enzyme to be probed at air-subphase interface.

2. Experimental section

2.1. Materials

The recombinant proteinase K enzyme with molecular weight 28.93 kDa (Activity of > 30 U/mg of protein) was obtained from Gojira Fine Chemicals LLC, Bedford Heights, OH. (Cat. No. PK1001). Ultrapure water used in this experiment was obtained from Millipore® Direct-Q® 3 (Type1) water purifying system having resistivity of 18.2 MΩ cm, a surface tension of 72.1 mN m^{-1}, and a pH of 6.0 ± 0.5 at 20.0 ± 1.0C temperature. ACS/USP/FCC grade NaCl from Gojira Fine Chemicals LLC (Cat. No. SC1015) was utilized to prepare the saline subphase. Dialysis tube with molecular weight cutoff 3500 was purchased from Thermo Scientific.

2.2. Experiments

A clean room (class 1000) with temperature (20.0 ± 1.0 °C) and humidity (50% ± 1%) was used for every experiments. The experiments for surface pressure–area (π–A) isotherms, surface potential–area isotherms, stability, and compression–decompression cycles, a Kibron μ-trough S (Kibron Inc., Helsinki, Finland) with area of 124.5 cm^2 (5.9 cm × 21.1 cm) was utilized. For measurement of surface potential, a Kelvin probe (MicroSpot surface potentiometer, product code: 6270, Kibron Inc., Helsinki, Finland) that consists of a capacitor-like system was used and the vibrating plate was adjusted 1 mm above the surface of Langmuir monolayer. The surface pressure was measured using Wilhelmy plate technique which utilizes alloy wire probe, with a 0.51 mm diameter having a sensitivity of ± 0.01 mN m^{-1}. The surface pressure and surface potential data were acquired every 0.25 s which measures the minuscule difference. The UV–vis and fluorescence measurements at air-surface interface was conducted on KSV mini-trough (KSV Instrument Ltd., Helsinki, Finland) having an area of 225 cm^2 (7. 5 cm × 30 cm).

2.3. Purification of Proteinase K enzyme

1 g proteinase K enzyme was dissolved in 25 ml of water. The solution was transferred to a molecular weight cutoff (MWCO) 3500 dialysis bag and was dialyzed with 5 L deionized water for 5 days. The water was changed every 12 h. The solution was frozen and lyophilized to obtain dry powder.

2.4. In situ UV–vis spectroscopy

8452 A HP spectrometer fixed on a rail close to the KSV trough (KSV Instrument Ltd., Helsinki, Finland) was used to measure the UV–vis spectrum of the Langmuir monolayer.

2.5. In situ fluorescence spectroscopy

An optical fiber detector on the top of the KSV trough, which was coupled to the Spex Fluorolog spectrofluorometer (Horiba, Jovin Yvon, Edison, NJ) was used for the fluorescence measurement of the Proteinase K Langmuir monolayer. The optical fiber was rested approximately 1 mm above the subphase and 5 nm slit width was used for both excitation and emission. The instrument uses the optical fiber to transmit the excitation light from the source to Langmuir monolayer and dispatches back the emitted light from Langmuir monolayer to detector.

2.6. In situ Infrared-Reflection absorption spectroscopy

PM-IRRAS (PMI-550 KSV Instruments) was used to obtain the infrared spectra of Proteinase K monolayer, which was compressed up to the desired surface pressure. Each spectrum was acquired with 6000 scans at 80° incident angle.

2.7. Langmuir-Blodgett films preparation

The preformed Langmuir monolayer of proteinase K was transferred to a solid support (quartz slides) by rising the slides up from NaCl aqueous subphase to air at the rate of 2 mm min^{-1} at constant pressure.

2.8. Circular dichroism

JASCO J-810 was used to obtain circular dichroism spectra for proteinase K in solution and Langmuir-Blodgett film. A single layer of proteinase K was transferred to a quartz solid support before measurement.

2.9. Langmuir monolayer preparation

Proteinase K enzyme solution was prepared in pure water (pH 6.0 ± 0.5) at the concentration of 0.5 $mg.ml^{-1}$. 65 μL of the enzyme solution was spread uniformly over 0.5 M NaCl subphase by using a 100 μL syringe (Hamilton Co., Reno, Nevada). The Langmuir monolayer was allowed to attain the equilibrium state for 30 min before every experiment. Then, Langmuir monolayer was compressed with the rate of 1800 $Å^2$ $molecule^{-1}$ min^{-1}. The experiments were repeated more than three times to assure reproducibility.

3. Results and discussion

3.1. Surface pressure - area isotherms and adsorption kinetics

The interfacial properties of Proteinase K enzyme were investigated on surface of saline solution by spreading 65 μL of the enzyme with 0.5 $mg.ml^{-1}$ concentration. It does not present relevant surface activity when spread on pure water as subphase since the surface pressure increase is not significant at end of the compression (Fig. 1). This low surface activity is related to its high solubility in water, which provides a low surface excess. With 0.5 M of NaCl as subphase, the isotherm shows a higher surface activity for the enzyme since the compression at the air–water interface lead the surface pressure to reach values as high as 15 $mN.m^{-1}$. This increase of the surface activity of proteins with high salt concentration is a strategy to boost the adsorption of proteins at the air–water interface owing to the salting out effect[14,15]. In 0.5 M NaCl subphase, the surface pressure started increasing when the mean molecular area reached 600 $Å^2$.$molecule^{-1}$. This phase can be considered as the beginning of the liquid expanded phase. Upon further compression, there was sharp increase in the surface pressure from 450 to 250 $Å^2$.$molecule^{-1}$; this can be correlated to liquid condensed phase. The limiting molecular area was determined to be 480 $Å^2$.$molecule^{-1}$ which was determined by extrapolating a tangential line near the collapse surface pressure towards the x-axis which means the limiting molecular area as shown in Fig. 1.

The adsorption kinetics in Fig. 2 shows that most of the enzyme adsorption at the interface started between 30 min and 1 h with a maximum surface pressure of 9 $mN.m^{-1}$. Measuring the adsorption profile, surface chemistry experiments were performed after 30 min lag time after spreading the Proteinase K enzyme to attain the equilibrium.

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 quantify the interactions between molecules in close contact (van der

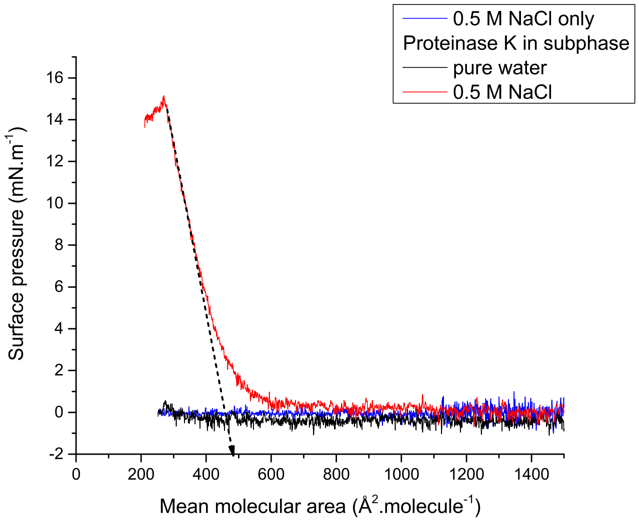

Fig. 1. Surface pressure- area isotherms of 0.5 M NaCl only- blue line; Proteinase K Langmuir monolayer at air-subphase (water- black line and 0.5 M NaCl- red line; pH 6.0 ± 0.5) by spreading 0.5 $mg.ml^{-1}$ enzyme aqueous solution (65 μL) above subphase.

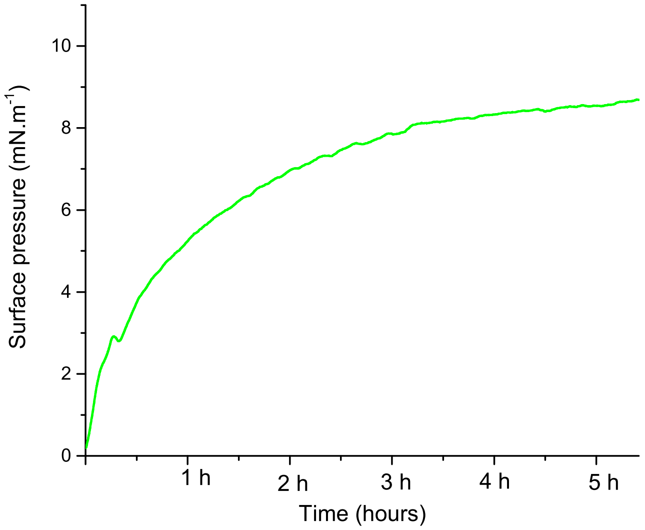

Fig. 2. Proteinase K adsorption kinetics at air-subphase (NaCl 0.5 M; pH 6.0 ± 0.5) for a 3000 mm^2 surface area.

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[16]. When the Langmuir monolayer is slowly compressed the dipole moments associated to Proteinase K enzyme starts to reorient at the interface (Fig. 3, blue curve), which can be seen through gradual increase in the surface potential. The surface potential start to increases steadily from 1200 to 800 mV showing a liquid expanded phase and a change of phase is detected from 800 to 600 mV. From 600 to 400 mV a solid expanded film is formed up to the collapse at about 200 mV. The surface potential curve (Fig. 3), initially at large areas surface potential is close to zero and remains constant until a critical area is reached on compression, after which an increase is observed. At first, the surface potential close to zero in large areas, suggests that the Proteinase K enzyme was sufficiently distributed with no aggregation. The appearance of gradual rise of the surface potential indicates the structuring of the monolayer during compression. With compression, the orientation of electric moments gives the monolayer a positive surface potential with a maximum of 200 mV.

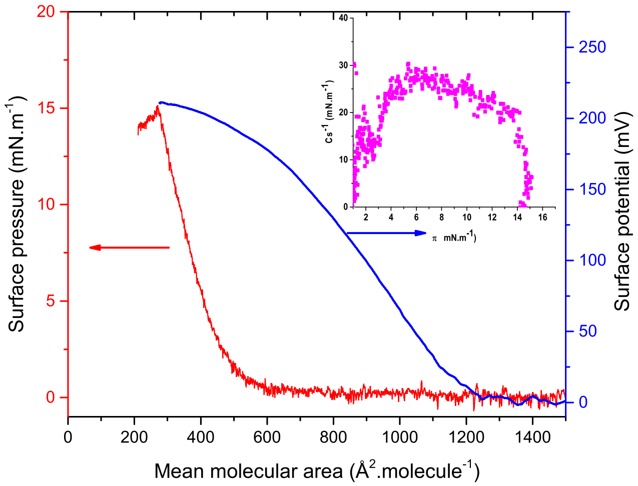

Fig. 3. Surface pressure and surface potential isotherms for proteinase K enzyme (0.5 mg.ml^{-1}) Langmuir monolayer at air-subphase (NaCl 0.5 M; pH 6.0 ± 0.5 temperature 20 ± 1.0 ℃). Inset shows the compressibility modulus for the enzyme during compression.

The inserted graph in Fig. 3 shows the surface compressibility modulus (C_s^{-1}= - A $(\frac{\partial \pi}{\partial A})_T$) of Proteinase K 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 [17]. 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. 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 Langmuir monolayer stayed 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 Proteinase K forms a fluid interfacial layer with low viscoelasticity.

3.3. Compression – decompression cycles and stability studies

The compression – decompression cycle gives information relating to the stability of the monolayer film. It shows the extent of loss of analyte to desorption from the interface into the subphase, which is known as hysteresis[18]. The surface pressure for compression and decompression cycles were chosen at 10, 12.5, and 15 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 2.5, 3.5 and 6% with 10, 12.5 and 15 mN.m^{-1} surface pressure, respectively. At lower surface pressure (10 and 12 mN.m^{-1}) the loss of material or change of conformation of the enzyme at the subphase was minimal while increasing the surface pressure to 15 mN.m^{-1} which 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[19].

To further analyze the stability, Proteinase K Langmuir monolayer was kept at a constant pressure 15 mN.m^{-1} and the stability was observed over the period of 4 hrs (Fig. 5). At 15 mN.m^{-1} surface pressure, the collapse of the monolayers was not observed. This gives evidence that Proteinase K has good stability at the air-subphase interface over this time period. The variations in the mean molecular area were calculated after 4 hrs at 15 mN.m^{-1} which was less than 10%. The optical properties of the Langmuir monolayer were performed within one hour which means a stable Langmuir monolayer during the spectroscopic measurements.

3.4. UV – vis absorption and fluorescence spectroscopies:

Fig. 6a shows the UV–vis spectra in aqueous solution (0.06 mg. ml^{-1}). The absorbance peak at 275 nm is the characteristic band of the tryptophan amino acid present in the enzyme[20]. Further experiments were performed to study the *in situ* UV–vis spectra of the Langmuir monolayer compressed at different surface pressure as shown in Fig. 6b. The absorbance spectra of the monolayer of the enzyme shows two bands at 210 and 230 nm which is attributed to $\pi \rightarrow \pi^*$ and $n \rightarrow \pi^*$ transitions, respectively. The peak of the tryptophan absorption can be seen at 27 5 nm, but quite small to be measured within the experimental error. The inset of the Fig. 6b shows the absorbance values at wavelength, λ = 210 nm versus surface pressures. A linear relationship suggests that during the compression the number of molecules per unit area increased as the surface pressure was increased. This observation gives strong evidence that the Langmuir monolayer of Proteinase K

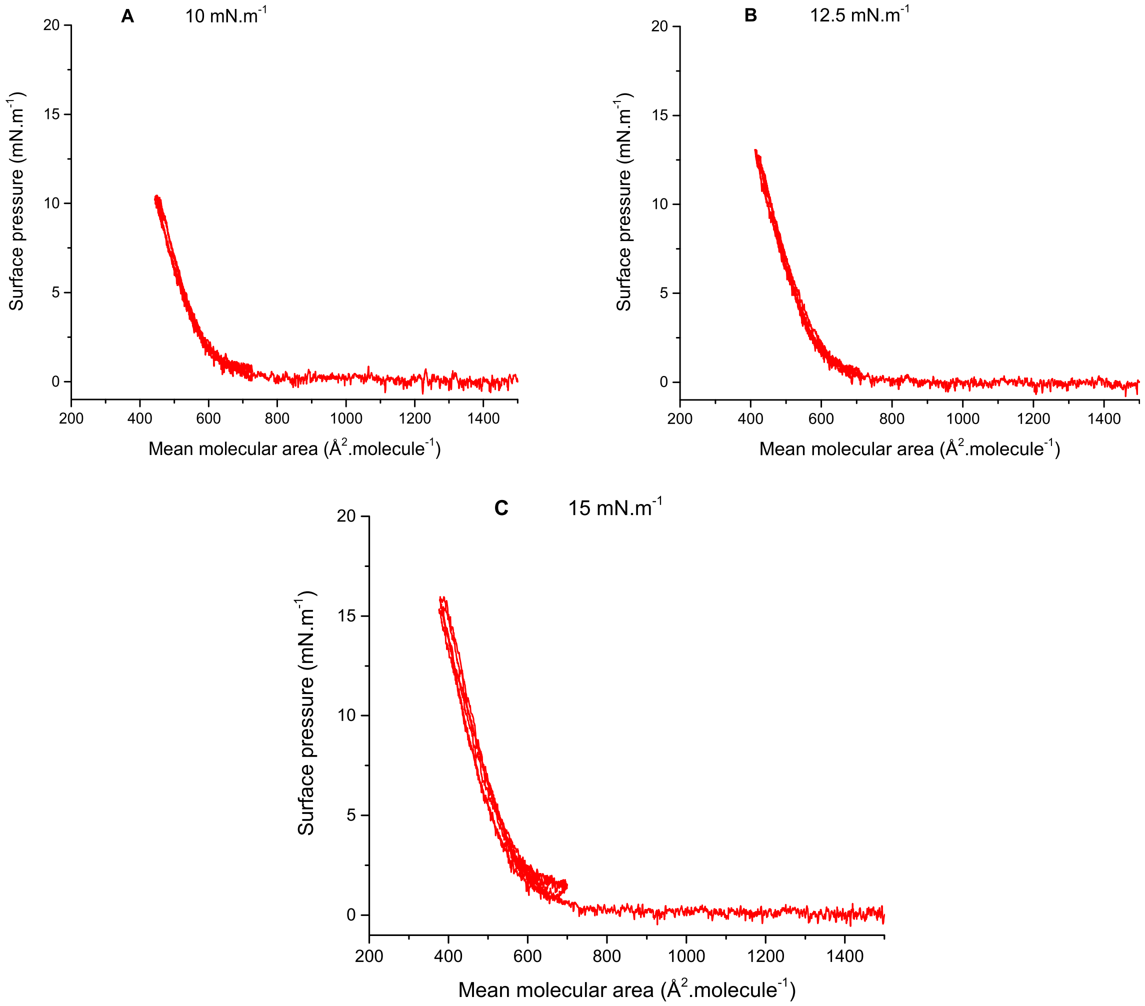

Fig. 4. Compression – decompression cycles of Proteinase K Langmuir monolayer at air-subphase (NaCl 0.5 M; pH 6.0 ± 0.5), maintained at the surface pressure of a) 10, b) 12.5 and c) 15 mN.m^{-1}.

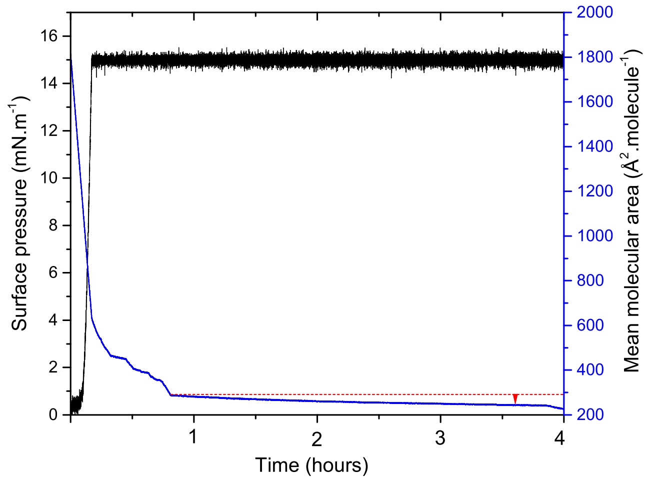

Fig. 5. Stability measurement of Proteinase K Langmuir monolayer at 15 mN.m^{-1} surface pressure at air-subphase (NaCl 0.5 M; pH 6.0 ± 0.5; temperature 20.0 ± 1.0 °C).

enzyme presents a good homogeneity at the air-subphase interface. To confirm this observation, we performed *in situ* fluorescence spectroscopy of the Langmuir monolayer as shown in Fig. 7b. As compared to the UV–vis observation in solution, we obtained a linear relationship between fluorescence intensity at 340 nm versus surface pressure. This observation signifies a homogeneous increase in the surface concentration of tryptophan moiety upon compression. Although there was weak absorption band for tryptophan at 275 nm in *in situ* UV–vis spectroscopy, the *in situ* fluorescence of the interfacial film presented emission bands for the amino acid at 340 nm when excited at 275 nm. This is due to the high fluorescence quantum yield of the tryptophan residue present in the enzyme.

3.5. Circular dichroism and IRRAS

To study the changes in the secondary structure of Proteinase K enzyme at air-subphase interface, circular dichroism (CD) experiment was done. CD has been extensively used in past few decades for both qualitative and quantitative assessment of the secondary structures of enzymes[21,22]. Various surface immobilization techniques for enzymes have been used, as immobilization can further prevent enzymes conformational changes and enzymatic inactivation[23]. In our experiment, we have used Langmuir-Blodgett (LB) technique to immobilize the enzyme from air-subphase interface to a solid quartz support. CD spectra of enzymes with α-helical conformation have negative bands around 208 and 222 nm and positive band around 193 nm. However, proteins with β-pleated

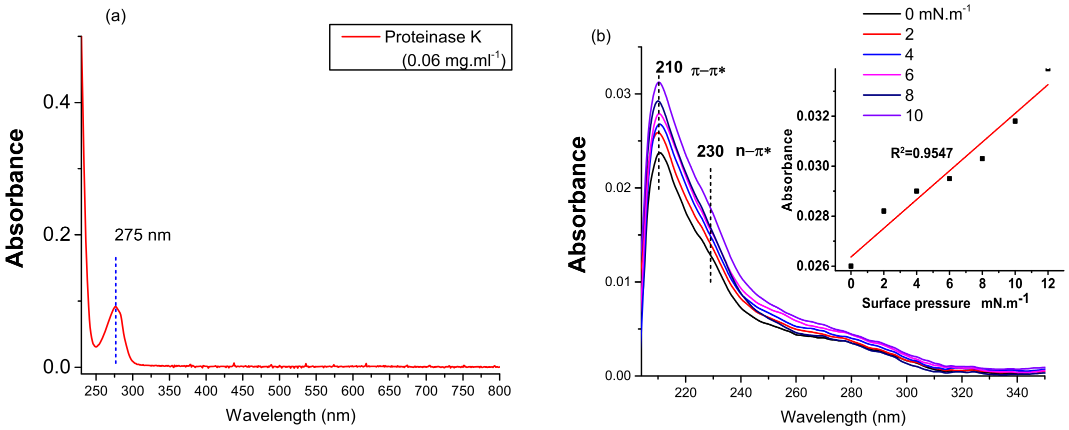

Fig. 6. UV – vis absorption spectra of the Proteinase K a) in solution (0.06 mg.ml^{-1} concentration with optical path length of 1 cm quart cell); b) Langmuir monolayer at air-subphase (NaCl 0.5 M, pH 6.0 ± 0.5 and temperature 20.0 ± 1.0 ℃) at different surface pressure. Inset in the graph shows plot of absorbance versus surface pressure.

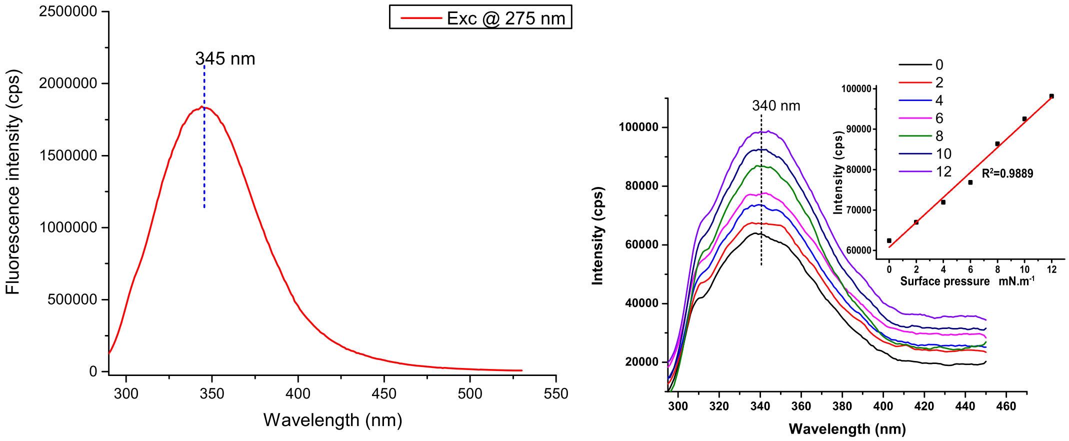

Fig. 7. Fluorescence spectra of Proteinase k a) in solution (0.06 mg.ml^{-1} concentration with optical path length of 1 cm of quart cell and slit width of 5 nm) b) Langmuir monolayer at air-subphase (NaCl 0.5 M; pH 6.0 ± 0.5) at different surface pressure. Inset in the graph shows plot of fluorescence intensity versus surface pressure.

sheets have negative band around 218 nm and positive band around 195 nm[24,25]. Fig. 8 shows the CD spectra of Proteinase solution (blue curve) and LB film (red curve) transferred at 15 mN.m^{-1} surface pressure. The two bands in Fig. 8, blue and red can be attributed to π → π* and n → π* transitions for α-helix structure of the enzyme. The small shift in the wavelength between solution spectra and the one for the transferred LB film can be related to the light scattering due to the refractive index of the slides vs water as solution.

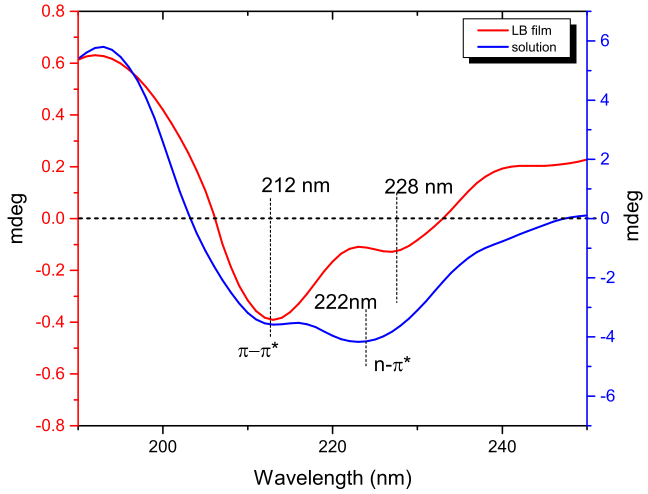

Fig. 8. Circular dichroism spectra of the Proteinase K LB film on quartz substrate deposited at 15 mN.m^{-1} surface pressure at air subphase (NaCl 0.5 M; pH 6.0 ± 0.5 temperature 20 ± 1.0 ℃) and the proteinase K in solution (0.06 mg.ml^{-1}).

IRRAS techniques have been successfully applied *in situ* for studies of orientation and conformation of different molecular films including enzymes[26]. For the PM-IRRAS technique, p and s-polarized infrared beams, perpendicular and parallel to the plane of incidence, respectively, are oriented at a defined angle of incidence which interacts with oriented dipoles of analyte present on the surface. The reflected light is converted to a reflection–absorption signal by adjusting the detector at an angle equal to the angle of incidence[27]. It is to be noted that good S/N ratio is difficult to obtain for p-polarized radiation at incident angles closer to the Brewster angle for air–water interface (≈ 54°). Around this angle, the intensity of the reflected light is weak, therefore angle 80°which is further away from the Brewster angle was chosen so that the spectra are obtained. The incident angle and orientation of transition dipole moment determines whether the measured signal have positive or negative bands to the baseline[28]. 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[27,29]. Herein, the IRRAS spectrum (Fig. 9) we are looking for the positive bands

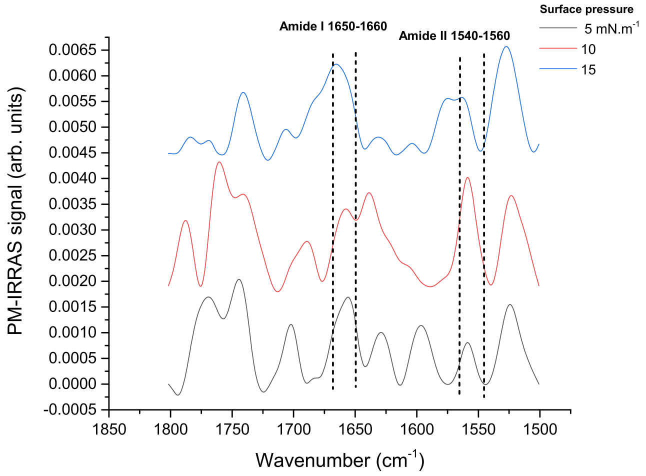

Fig. 9. PM-IRRAS spectra of the Proteinase K Langmuir monolayer at air-subphase (0.5 M NaCl, pH 6.0 ± 0.5; Temperature 20.0 ± 1.0 ℃) at 80° incident angle and at surface pressure 5, 10 and 15 mN.m$^{-1.}$

for α-helix. 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. In general, α-helix structures shows positive bands from 1650 to 1660 cm^{-1} for amide I (C═O stretching from peptide bond) and 1540 to 1560 cm^{-1} for amide II (N—H bending and C—N stretching). However, for β-sheets structures amide I and amide II are in the regions at 1620–1640 and 1520–1535 cm^{-1}, respectively[30,31]. Fig. 9 shows PM-IRRAS spectra for Proteinase K enzyme at three different surface pressures 5, 10 and 15 mN.m^{-1} with increasing intensity of absorbance at higher surface pressure. At lower surface pressure, 5 mN.m^{-1} (black curve) two major absorbance bands at 1655 cm^{-1} for amide I and 1560 cm^{-1} for amide II can be seen, which indicates majority of the enzymes present are in α-helix conformation. Small intensity band at 1630 cm^{-1} can also be seen which shows presence of β-turns in the enzyme. As the enzyme is further compressed to the higher surface pressure of 10 and 15 mN m^{-1} (red and blue curves, respectively), bands relating to α-helix structure can still be seen indicating the maintenance of confirmation of enzyme at higher surface pressure.

4. Conclusion

The present work reports the significance aspects about the enzyme proteinase K Langmuir monolayer at air/aqueous NaCl subphase. It is important to emphasize, that a stable and reproducible Langmuir monolayer was achieved due to the presence of NaCl at the subphase owing to the *salting out* effect. The stability studies including compression-decompression cycles indicate Proteinase K can form a stable Langmuir monolayer at air-subphase interface. Throughout the compression, Proteinase K presented good homogeneity which can be confirmed from the linearity in the increase of UV–vis absorption and fluorescence intensity. The enzyme maintained its secondary structure during immobilization through LB technique which could be confirmed using CD spectra. The enzyme monolayer along its compression showed predominance of α-helix structures which was indicted through PM-IRRAS data. We believe that this present work can contribute to understand characteristics of enzymes Langmuir monolayer. The demonstrated stability of the Langmuir Blodgett film of proteinase K open doors for the further studies of catalytic activity of such films, and open a door for the possible application of proteinase K Langmuir Blodgett film as a heterogeneous catalyst for molecular biology assays proteins sample preparation applications, which will be a subject of the future studies.

CRediT authorship contribution statement

Suraj Paudyal: Writing – original draft, Investigation, Methodology. **Ganesh Sigdel:** Data curation. **Sujit K Shah:** Investigation. **Shiv K. Sharma:** Methodology, Software. **John D. Grubb:** . **Miodrag Micic:** Writing – review & editing. **Luciano Caseli:** Data curation. **Roger M. Leblanc:** Supervision.

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.

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