Quality of Cold Plasma Treated Casein Peptide Targeting SARS-CoV2: An In-silico Approach

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Research Article

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Abstract

Raw cow milk being a highly nutritive but its contamination is one of the factors that has to be considered. Thermal treatment is generally adopted for decontamination of milk but at the same time it degrades the protein quality. Hence, there is a need for new treatment method with least processing technology to maintain the food quality. These challenges forced the scientist to introduce non-thermal technologies. The objective of the present study to elucidate the effect of optimized set up for plasma bubbling on casein protein and peptide with respect to raw cow milk. Structural characterization of casein was done using FTIR. The casein protein hydrophobicity was maintained well and Mascot result revealed a non-detrimental effect to α-s1 casein peptide upon the treatment. Specifically, casein peptides are good source of ACE inhibitory peptides. While, ACE2 receptor is responsible for binding of SARS-CoV2. Therefore, this study implemented on most effective ACE inhibitory peptide (RYLGY) which was observed in Mascot analysis, for both control and treated sample arising from peak at 1267m/z in MALDI-TOF of α-s1-casein. The peptide was considered for in-silico docking approach against SARS-CoV2. Interestingly, ACE2-RBD-peptide complex showed good binding score, suggesting that the peptide molecule disturbed the complex formation.

Introduction

Milk and milk products have been used for health benefits and widely consumed in various forms all over the world but at the same time, milk is perishable in nature (Rowlands 1952)(Council and others 1988). Consumer demands, that dairy product should be nutritious, decontaminated, minimally processed, and healthy (Mir, et al., 2016). Generally, the thermal treatment was implemented to decontaminate milk which ultimately denatures protein at high temperature (Rynne et al., 2004)(Deeth, 2019). Hence, the researchers introduced an emerging technology i.e., non-thermal technology to the food industry. Again, this technology is now being a powerful tool for modern medicine which is able to reduce microbial load without any significant negative effect on healthy tissue (Bran et al., 2020). Further, it was observed that in cold plasma treatment: the quality of food and its protein remains unchanged (Bagheri and Abbaszadeh, 2020) (Chaple et al., 2020). The cold plasma technology is an emerging decontamination technology in the recent era. The cold plasma technology which operates at ambient or moderate temperatures has gained attention as an alternative to chemical and thermal treatments for disinfection of foods products. The generating mode of cold plasma has both direct and indirect methods, the direct cold plasma generates a large variety of reactive species in a short life span (~ milliseconds) for decontamination. While the indirect method produces longer-span reactive species through the plasma which was generated in a separate chamber (Misra and Jo 2017) (Surowsky, et al.,2015). Cold plasma produces reactive species like Reactive Oxygen Species (ROS) and Reactive Nitrogen Species (RNS) (Graves, 2014). Specifically, it was investigated that the chemistry of reactive species mostly depends on the feed gases used for plasma generation which are basically the mixture of carbon dioxide, oxygen and nitrogen consist of O ,O₂⁻,O⁺,N⁺, NO⁺, N₂⁻, CO₂⁺ ions (Sharma and Singh 2022). To date, direct cold plasma has been well established in the food sector but indirect cold plasma setup is demanding (Misra
et al., 2011). So, the current study focused on the indirect Dielectric Barrier Discharge (DBD) generation of plasma (plasma bubbling). Further Chaple et al., (2020) reported that non-detrimental effect on the flour protein was observed in atmospheric pressure plasma: suggesting that cold plasma technology is successful technology introduced to the food industry. Therefore, it was hypothesized that plasma bubbling treatment may aid to restrain the quality of milk and it may not affect the milk proteins (caseins and whey). In fact, both casein and whey proteins are rich sources of ACE inhibitory peptides (Huth, et al., 2004). While, in humans, ACE2 is abundant in epithelia of respiratory tract and small intestine, which might provide possible entry points for SARS-CoV-2 (Donoghue et al., 2000 and Hamming, 2004). In case of humans, the SARS-CoV-2 is mainly transmitted by envelope fusion, which is the principal entry mechanism for corona viruses (Shang, Ye, et al., 2020) (Wędrowska et al., 2020). Initially, the virus attaches to host cell surface receptor, then enters the endosome and fuses with the lysosome membrane and spreads the infection (Shang, Ye, et al., 2020). The attachment of virus is mediated by spike glycoprotein, which binds to the ACE2 receptor of the host cell (Gallagher and Buchmeier, 2001) (Simmons et al., 2013). Therefore, the present study hypothesized that the plasma bubbling treated milk casein peptide might be helpful to block SARS-CoV-2 viral entry to human ACE cell receptor due to its successful microbial decontamination as well as its non-detrimental effect on the protein's quality. Specifically, the peptide “RYLGY” obtained from milk α-s1-casein protein showed anti-hypertensive effects by inhibiting ACE2 (Contreras et al., 2009). Hence, the present study has aimed the specific peptide (RYLGY) derived from milk α-s1-casein protein and to understand effect of peptide on ACE2-Spike complex, an in-silico observation was conducted.

**Materials And Methods**

**Plasma bubbling of milk**

The optimized plasma bubbling parameter setup was configured at 200 volts (V), 10 liters per hour (L/h), time interval of 15 minutes (min), and 100 mL sample volume. The atmospheric gas was supplied as feed gas to carry out the plasma bubbling and it bubbled through the milk sample. This parameter set up successfully decontaminated E. coli, coliform, yeast, and mould. The treated and control (raw cow milk) samples were tested for E.coli, coliform, yeast, and mould counts using MacConkey agar (Parseelan et al., 2018), violet red bile agar (Ray & Speck 1978) and chloramphenicol yeast glucose agar (Torkar and Teger 2006) respectively. The current study was observed both control and the optimized set-up parameters for plasma bubbling to better understand the protein quality with respect to control sample.

**Extraction Of Casein Protein**

Casein protein was extracted from control, optimized plasma bubble treated sample and then compared with standard casein protein purchased commercially (Sigma-Aldrich, USA). For extraction of casein, the milk was purchased from a local vendor in Thanjavur. An extensive comparison was made for the commercial, control and treated casein protein sample. The extraction procedure of casein protein was
followed according to Pavia et al., (1998). Briefly, 100 mL volume of milk was centrifugated at 4000 rpm for 20 minutes at ambient temperature for removing the fats or lipids from the mixture. After centrifugation, the lipids containing supernatant were removed. Further, the milk was transferred to beakers and pH (Laqua pH1100, Horiba Scientific, Singapore) was adjusted using 0.2N HCL until it reaches the isoelectric point at pH 4.6 which resulted in precipitation of casein. The curdy precipitate was separated from the supernatant using filter paper (Whatman no 1) of size 25*50 mm and allowed for sedimentation of casein protein. Then, the sedimented precipitate was washed using distilled water to remove the salts, followed by washing with diethyl ether and ethanol in the ratio of 1:1 i.e., 50% of diethyl ether: 50% ethanol to remove excess fat. The precipitated casein protein was lyophilized (Lyophilizer LYO 10 T, Borg Scientific, Chennai) with vacuum at temperature of -55°C to -60°C for 18 hours following Deshwal et al., (2020).

**Ftir- Characterization**

The FTIR analysis was performed for standard, control and treated casein protein sample to observe the secondary structures of the casein upon the plasma bubbling treatment. The spectrum using 50 Nicolet FTIR spectrophotometer (Thermoscientific, USA) was analyzed with a scan range from 400-4000cm-1 and a resolution of 4 cm-1 (Rutten et al., 2011).

**Sample Preparation For Maldi-tof**

The sample preparation for MALDI-TOF was followed by Sodium Dodecyl Sulphate–Poly Acrylamide Gel Electrophoresis (SDS-PAGE) using 10% (w/v) gels according to Laemmli, (1970). Known quantities (2–3 µg) of casein protein along with standard molecular weight protein markers were loaded in appropriate wells for running SDS-PAGE. The silver staining was used to stain the gel (Morrissey, 1981). The gel slice was diced to small pieces, de-stained using destaining solution (ammonium bicarbonate: acetonitrile = 1:1) for 10-minute intervals (3–4 times) until the gel pieces become translucent white. The gels were dehydrated completely using acetonitrile in SpeedVac instrument, rehydrated with Dithiothreitol (DTT) by incubation for 1hour. After incubation, the DTT solution was removed and the gel pieces were treated with Iodoacetamide (IDA) for 45min. The supernatant was removed and the gel was incubated with ammonium bicarbonate solution for 10 mins and then dehydrated with acetonitrile for 10 mins in SpeedVac till complete dryness. A concentration of 15µL trypsin solution was added and incubated overnight at 37°C. The digested solution was transferred to fresh Eppendorf tubes. The gel pieces were extracted thrice with extraction buffer Acetonitrile and Trifluoracetic acid (ACN and 0.1% TFA = 1:1 ratio) and the supernatant was collected each time into the Eppendorf and then SpeedVac was used for complete dryness.

**MALDI-TOF**
The purified casein (control and treated) proteins bands obtained in SDS-PAGE were excised and further subjected to trypsin digestion and were analyzed using Matrix Assisted Laser Desorption/Ionization-Time of Flight Mass Spectrometry (MALDI-TOF/TOF MS Bruker Daltonics Ultraflex III). Briefly, dried pepmix was suspended in TA buffer (ACN and 0.1% TFA = 1:2 ratio). The peptides obtained were mixed with α-Cyano-4-Hydroxycinnamic Acid (HCCA) matrix in 1:1 ratio and the resulting 2µl was spotted onto the MALDI plate. After air drying the sample was analyzed on the MALDI TOF/TOF ULTRAFLEX III instrument and further analysis was done with flex analysis software for obtaining the peptide mass fingerprint. The obtained masses were submitted for Mascot search in “concerned” database for protein identification. These peptide mass values were searched against the databases such as National Center for Biotechnology Information Non-Redundant Proteins (NCBI nr), SWISS-PROT to obtain information about the identity of protein.

**Peptide Toxicity Prediction**

Generally, peptide is being studied as a potential target for therapeutic candidate. However, some peptides showed toxicity towards the eukaryotic cell. Therefore, it is important to estimate the toxicity level before use for *in vitro* toxicity studies. We have aimed to check the toxicity level of identified peptide molecule using online servers. We have examined toxicity of the peptide using three servers which has shown non-toxic and non-allergen to the human. The predicted peptide (RYLGY) was analyzed for the toxicity using ToxinPred ([http://crdd.osdd.net/raghava/toxinpred/](http://crdd.osdd.net/raghava/toxinpred/)) (Gupta et al., 2013), VaxiJen ([http://www.ddg-pharmfac.net/vaxijen/vaxijen/vaxijen.html](http://www.ddg-pharmfac.net/vaxijen/vaxijen/vaxijen.html)) (Chukwudozie et al., 2021) and AllerTOP v.2.0 ([https://www.ddg-pharmfac.net/allertop/method.html](https://www.ddg-pharmfac.net/allertop/method.html)) (Dimitrov et al., 2014) servers.

**Preparation And Docking Studies**

The crystal structure of the ACE2-RBD complex (PDB ID: 6M0J) was obtained from protein data bank (PDB) database ([http://www.rcsb.org/pdb](http://www.rcsb.org/pdb)). The structure was prepared using protein preparation wizard implemented in the Schrodinger software suite 2021 ([http://www.schrodinger.com/](http://www.schrodinger.com/)) (Mariadasse et al., 2015). The OPLS-2015 (Optimized Potential for Liquid Simulations) (Choubey et al., 2017) all-atom force field incorporated in the Schrodinger software was used for the energy minimization to obtain the improved quality of the ACE2-RBD complex. H-bonds, ionization, missing side chains, and tautomerization of the residues were incorporated besides removal of steric clashes. According to previous report by Kong et al., (2019), the ACE2-RBD interface region was considered as peptide-binding region confirmed through the CoDockPP server ([http://codockpp.schanglab.org.cn/](http://codockpp.schanglab.org.cn/)) and sitemap module. The grid was generated in the ACE2-RBD interface region of the complex. Further, the peptide (RYLGY) molecule was built using Avogadro software and minimized through the protein preparation wizard of Schrodinger software suite 2021. The minimized structure of ACE2-RBD and peptide molecule was considered for the docking studies using Schrodinger software.
Molecular Dynamics Simulation Studies

The energy minimized ACE2-RBD and ACE2-RBD-peptide complex were used for the molecular dynamics simulation studies by GROMACS (GROningen Machine for Chemical Simulations) version molecular dynamics package 5.1 (Choubey et al., 2017). The GROMACS 56a force field was employed for ACE2-RBD and ACE2-RBD-peptide complex to obtain the energy minimized conformation. Both, the complex systems were fixed with cubic boxes with the dimension of 1 nm and solvated with SPC216 water models. Periodic boundary conditions (PBC) were applied in all directions for the approximation of infinite systems. Further, the net charges of both the systems were neutralized by adding appropriate ions (Na+) counterions. Steepest Descent Algorithm (SDA) was used for energy minimization of the ACE2-RBD and ACE2-RBD-peptide complex through the 50000 steps with a maximum tolerance of 1000 KJ mol\(^{-1}\) nm\(^{-1}\). The complexes were equilibrated with two steps; the first step includes the NVT ensemble (constant number of particles, volume, and temperature) for 100 ps to stabilize the system at 310 K, and the second step, the NPT (constant number of particles, pressure, and temperature) equilibrium using coupling reference pressure of 1 bar (atm) for 100 ps. All bond lengths were constrained with the LINCS (Linear Constraint Solver) algorithm. The PME (Particles Mesh Ewald) electrostatic and periodic boundary conditions were applied in all directions. A cut-off value of 9 Å for coulomb interactions and 10 Å for Van der Waals interactions were assigned. This pre-equilibrated system of the complexes were subjected to 100 ns molecular dynamic simulations (Richard, et al.,2019).

Mmpbsa Analysis

The Molecular Mechanics Poisson-Boltzmann Surface Area (MMPBSA) method was used to calculate the binding free energies of ACE2-RBD and ACE2-RBD-peptide complex (Eq-1). Here, the last 2 ns frames of the trajectories were obtained to calculate the binding free energy analysis as a method described previously (Richard, et al., 2019).

\[
\Delta G_{\text{bind}} = G_{\text{complex}} - (G_{\text{protein}} + G_{\text{ligand}})
\]

Where, the \(\Delta G_{\text{complex}}\) is the total free energy of the complex, whereas, \(\Delta G_{\text{protein}}\) and \(\Delta G_{\text{ligand}}\) are the total free energies of ACE2-RDB and ACE2-RDB-peptide complex.

Results And Discussion

FTIR Analysis

FTIR plot (Fig. 1) shows that FTIR spectrum of the commercial casein (std), casein obtained from raw cow milk (control) and plasma bubbling treated casein at 200V, 10L/h, 100mL, 15 min represented as “CP”. Casein is highly flexible in nature with a disappearance of crystalline structure. Generally, hydrophilic and hydrophobic domains are created in casein protein by clustering the amino acids (Siroć et al., 2016). In the present study, there was no observation on the spectra of casein protein for α-helix, β-
turn and random coil in treated casein protein sample, the control sample and standard casein protein sample. Whereas an increase in % of β-sheet was observed for CP i.e., 77.62% at 1623.39 cm\(^{-1}\), while in control it was 69.17% at 1633.41 cm\(^{-1}\): revealing the formation of hydrogen-bonded β-sheet which is responsible for the aggregate formation (Zhao et al., 2013) (Ellepola, Siu, & Ma 2005). The findings were discussed based on Bandekar, 1992: Pelton & Mclean, 2000; Schmidt, et al., (2005). A similar finding was observed for increase in % of β-sheet of whey protein when subjected to spark discharge cold plasma. Generally, hydrophobicity of protein is defined by the β-sheet (Schmidt, et al., 2005). Due to increase % of β-sheet in the treated sample the finding proved that the hydrophobicity of casein protein increases which is an important factor for the folding of protein that helps to keep the protein stable and biologically active as it decreases the surface area of protein as well as reduce unwanted interaction with water molecule (Tanford, 1962; Strub et al., 2004) (Gembloux and Biophysique, 2018). Mostly, it was observed that generation of cold plasma helps to increase in β-sheets which is associated with improvement of protein gelation behaviour suggesting that it gives desirable texture to the food products (Sharma and Singh 2022) (Nicolai 2019). Similar observation of increase in β-sheets of zein powder and peanut protein upon cold plasma treatment was revealed (Dong et al., 2017) (Ji et al., 2018) respectively. Moreover, this study revealed that the plasma bubbling treated casein protein is biologically stable and active than the control sample without microbial contamination which is important information to proceed for the discovery of peptide derived drug.

Mass Spectrometry Analysis (Maldi-tof)

In this study, the comparison of casein protein from the control and the plasma bubbled milk were considered to evaluate the α-s1casein protein quality. The result of MALDI-TOF analysis of α-s1casein showed the spectra with a highest peak at 1267 m/z for both control and treated sample, suggesting that a non-detrimental effect on the protein quality (Fig-2). Specifically, α-s1casein contains most active ACE inhibitory peptides, among all the peptides present in α-s1 casein, RYLGY shows higher activity towards hypersensitivity inhibition (in vivo) (Contreras et al., 2013). The peptide RYLGY arising from (m/z 1267) in control as well as from treated sample (Fig − 3). Therefore, the analysis proved that the casein peptide is irrespective to the plasma bubbling treatment and the peptide was maintained well along with microbial decontamination.

Peptide Toxicity Prediction

The result of Toxin Pred, Vaxi Jen, Aller Pro software observation proved that the peptide (RYLGY) is a non-toxin peptide derived obtained from the α-s1-casein protein (Table-1). The peptide (RYLGY) further considered as a therapeutic candidate and used for in silico docking and dynamics studies.
Table 1
Toxicity Analysis for Casein ACE Inhibitory Peptide “RYLGY”.

<table>
<thead>
<tr>
<th>Server</th>
<th>Peptide</th>
<th>Result</th>
</tr>
</thead>
<tbody>
<tr>
<td>Toxin Pred</td>
<td>RYLGY</td>
<td>Non-toxin</td>
</tr>
<tr>
<td>Vaxi Jen</td>
<td>RYLGY</td>
<td>Threshold for the model :0.4</td>
</tr>
<tr>
<td>Aller Pro</td>
<td>RYLGY</td>
<td>Probably non-allergen</td>
</tr>
</tbody>
</table>

**Molecular Docking Studies Of Peptide With Ace2-rbd Complex**

Angiotensin-Converting Enzyme 2 (ACE 2) is a human receptor located in the cell membrane which is responsible for the binding of receptor binding domain (RBD) in the spike protein of SARS-CoV-2 (Fig. 4). Binding of ACE2 with RBD of spike protein mediates the viral entry to the host cell and is involved in the viral invasion. The crystal structure of ACE2 protein complex with RBD was solved and targeted for the drug designing to inhibit the viral entry to the host cell. The crystal structure of ACE2-RDB complex showed potential interactions (13 hydrogen bond interactions and 2 salt bridges) including N487, K417, Q493, Y505, Y449, T500, N501, G446, Y449, Y489, N487, G502, and Y505 from RBD of spike SARS-COV-2 and interaction with Q24, D30, E35, E37, D38, Y41, Q42, Y83, Q325, E329, N330, K353 and R393 of ACE2 receptor. This complex formation region was considered for docking with peptide in order to deform its interactions. Docking of peptide with ACE2-RDB complex showed affinity of -8.65 kcal/mol and the peptide showed two potential H-bond interactions namely Tyr2-Glu484 and Tyr5-Gly485 with the complex (Fig. 5). In addition, the electrostatic interactions were found between the peptide and ACE2 receptor complex interface.

**Molecular Dynamics Simulation Studies Of Ace2-rbd And Ace2-rbd-peptide Complex**

The structural stability as well as potential interactions of ACE2-RBD and ACE2-RBD-peptide complex were analyzed through 100 ns MD simulation studies. The Root Mean Square Deviation (RMSD) and Root Mean Square Fluctuation (RMSF) profiles are reliable parameters to understand the structural stability of the complexes. The overall RMSD profile of ACE2-RBD complex showed that the complex was stable during the dynamics in the average deviation values of 0.1 to 0.6 nm. The structural deviation was observed in the ACE2-RBD complex during the dynamics and reached the stabilized complex at the end of simulation. Also, the structural deviation was analyzed separately to the ACE2 and RBD complex. The RMSD profile of ACE2 and RBD receptor showed the structural deviation present in the average value between 0.15 to 0.4 nm. The RMSF profile of ACE2 receptor showed the residual fluctuation between 0.1 to 0.5 nm while the RBD of spike protein showed residual fluctuation ranging from 0.1 to 0.6 nm suggesting the both the proteins found to be stable complex formation during the dynamics. Also, the
initial residues (50 residues) in the RBD domain fluctuate high in the range between 0.1 to 0.6 nm and remaining residues were found below the 0.4 nm except N- and C-terminal residues. The solvent accessible of ACE2-RBD complex for the conformational changes were measured through the Solvent Accessible Surface Area (SASA) analysis. The SASA values of ACE2-RBD complex showed the values sharply fall from 355 nm$^2$ to 315 nm$^2$. The effect of peptide molecule on ACE2-RBD complex was analyzed in term of RMSD, RMSF, SASA and MMPBSA analysis. The overall RMSD profile of ACE2-RBD-peptide complex showed structural stability up to 78 ns in the average range of 0.4 nm, followed by the sudden deviation was observed in the range of 0.4 to 0.7 nm exhibiting conformational changes due to the peptide disturbance (Fig. 6A). Also, the RMSD profile of ACE2 and RBD receptor showed the deviation values in between 0.15 to 0.5 nm and 0.15 to 0.4 nm respectively (Fig. 6B and C). The ACE2 in peptide bound complex had structural deviation higher than the native ACE2 receptor due to the interactions of peptide molecule. The trajectory analysis showed that, peptide molecule has undergone selective conformational changes in the interface region and disturbs the complex formation. Also, RMSF plot of ACE2 and RBD receptor of peptide complex showed that the higher residues fluctuation comparatively to the ACE2-RBD complex in the range between 0.1 to 0.6 nm and 0.1 to 0.45 nm respectively (Fig. 6D and E). The peptide driven interaction on ACE2-RBD disturb the complex formation by increasing the higher residual fluctuations. In terms of SASA, the value decreased in 78 nm$^2$ till the end of simulation in the range between 355 to 305 nm$^2$ suggesting the conformational changes of two molecules and weakening of the complex formation (Fig. 6F). The conformational changes and disturbance of ACE2-RBD complex rely upon the interactions and driven force of peptide molecule in the interface of the complex. The RMSD of peptide molecule has shown deviation in 0.4 nm till 80 ns and it showed structural deviation till the end of simulation in range of 0.5 nm (Fig. 6G). The conformational changes of peptide molecule affect the complex formation of ACE2-RBD complex leading to weaken its interactions. The potential H-bond interactions of ACE2-RBD and ACE2-RBD-peptide complex. Further the study was extended to understand the role of peptide molecule on ACE2-RBD complex for its destabilizing interactions.

**Mmpbsa Analysis**

MMPBSA is a powerful method to estimate the binding affinity of the complexes during the dynamics. MMPBSA calculation showed the energy components in term of Van der Waals, electrostatic, polar solvation, solvent accessible surface area and total energies of the complexes. In ACE2-RBD complex, the Van der Waals (-31291.2 kJ/mol), electrostatic (-1543.4 kJ/mol), polar solvation (-47784.3 kJ/mol), solvent accessible surface area (0.018.6 kJ/mol) and total energies (-56.5 kJ/mol) were found for the complex formation. However, the ACE2- RBD-peptide complex showed relatively lesser complex formation energy in the Van der Waals (14768 kJ/mol), electrostatic (-405.28 kJ/mol), polar solvation (-1563.5 kJ/mol), solvent accessible surface area (-0.15 kJ/mol) and total energies (-245.3 kJ/mol) of the complex suggesting that, the identified peptide molecule hindered the complex formation of ACE2-RBD receptor (Table 2). The identified peptide may have potential features to disturb the invasion of SARS-CoV-2 viral entry.
<table>
<thead>
<tr>
<th>Energy Components</th>
<th>ACE2-Spike complex (kJ/mol)</th>
<th>ACE2-Spike-peptide (kJ/mol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vander Waal energy</td>
<td>-31291</td>
<td>14768</td>
</tr>
<tr>
<td>Electrostatic energy</td>
<td>-1543</td>
<td>-405.289</td>
</tr>
<tr>
<td>Polar solvation energy</td>
<td>-47784</td>
<td>1563.587</td>
</tr>
<tr>
<td>SASA energy</td>
<td>0.018</td>
<td>-15.293</td>
</tr>
<tr>
<td>Binding energy</td>
<td>-56.53</td>
<td>245.31</td>
</tr>
</tbody>
</table>

**Conclusion**

The milk without contamination of microbes along with maintenance of its nutritional quality is challenging. Interestingly, the optimized plasma bubbling set up was able to decontaminate microbes from milk with a non-detrimental effect to the milk casein protein and peptide. While, FTIR and UHPLC study revealed that the hydrophobicity of the casein protein in post plasma bubbling treatment was maintained well, suggesting that treated casein protein was biologically active as well as stable in nature. In the mascot result search analysis, the peptide RYLGY was observed in both control and treated sample which was being arisen at highest peak of MALDI-TOF i.e., 1267 m/z. The RYLGY is a therapeutic peptide which could be able to successfully inhibit the interaction of SARS-CoV-2 spike protein with ACE2, thus blocking the cellular entry of virus. *In silico* docking studies showed a better binding affinity (-8.65 kcal/mol) of peptide to the ACE2-RBD complex. The MD study demonstrated that native ACE2-RBD complex showed high stability and binding affinity for the complex formation. However, the MMPBSA analysis confirmed a successful interruption in the native ACE2-RBD complex formation by the peptide. Therefore, the identified peptide can be a potential molecule to hinder the ACE2-RBD complex formation and inhibit the viral entry to human. This study tried to illuminate the route that introduce the plasma bubbling system for the decontamination of milk which may helpful to food industry due to its non-detrimental effect to milk quality and can be an effective approach for the treatment of Covid-19. Further, in-vivo study is required to better understand the effect of plasma bubbling treated peptide for an effective target of Covid-19.

**Declarations**

**Acknowledgement**

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Competing Interest

The author declares there is no conflict of interest.

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Figures
Figure 1

FTIR spectra of standard, control, treated casein proteins. Where, (std) denotes commercial casein, (Control) denotes raw cow milk casein and (CP) denotes the plasma bubbling treated casein.

Figure 2
MALDI Spectra of control and plasma bubbled α-s1 casein with a peak value of 1267 m/z. Where, A and B is represented as control and treated samples respectively.

Figure 3

MALDI-TOF analysis obtained MS/MS spectra of α-S1 casein peptides a) Mass spectra showing high monoisotopic peak at m/z 1267, b) Mascot search engine at spectra 1267 m/z showing 106-115 amino acid indicating “RYLGY” peptide obtained from casein protein.
Figure 4

The schematic diagram represents A) Binding of RBD domain of SARS-CoV-2 with ACE2, B) Docked complex of RBD-ACE2 with peptide

Figure 5
Representation of potential H-Bond interaction of peptide “RYLGY” on RBD-ACE2 complex visualized through the Ligplot. The peptide residues Tyr2 and Tyr5 formed H-bond interactions between Glu484 and Gly485 residues of RBD domain.

Figure 6

Molecular dynamics stimulation of ACE2-RBD-peptide complex. Where, (A) over all RMSD of ACE2-RBD-peptide complex (B) RMSD of ACE2-peptide bound complex (C) RMSD of RBD-Peptide (D) RMS fluctuation analysis of ACE2 receptor-Peptide (E) RMSF of RBD – Peptide (F) SASA of ACE2-RBD-peptide (G) RMSD analysis of Peptide (RYLGY)

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