ES Food and Agroforestry
DOI: https://dx.doi.org/10.30919/esfaf911

Isolation, Purification, and Identification of Antioxidant Peptides from Wastewater of Cheese Making Industry

Rushikesh Pol,[1] Alisha Rahaman,[1] Navanath Kumbhar,[3] Anupama Pable,[1] Gunderao Kathwate,[2] Vitthal T. Barvkar[4] and Umesh U. Jadhav[1,*]

Abstract

The present study describes the isolation and purification of antioxidant peptides. The cheese-making industry wastewater was collected to extract the proteins present in it. These proteins were used as substrates for the generation of antioxidant peptides. The *B. subtilis* National Collection of Industrial Microorganism (NCIM) 2724 was used for the digestion of these proteins. The digest was tested for 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity. It showed 56.24% free radical scavenging activity. The antioxidant peptides were purified from this digest using the 3 kDa membrane filter, Sephadex G-25, and reverse-phase high-pressure liquid chromatography. The liquid chromatography-mass spectrometry (LCMS) analysis of the purified peptide sample suggested the presence of 3-4 amino acid-containing peptide with a molecular mass of 410.21 Da. The LC-MS analysis provided 10 possible sequences for peptide. Further screening of these sequences using a peptide ranker tool resulted in two sequences Proline- Proline- Proline-Threonine (PPPT), Threonine-Proline- Proline- Proline (TPPP) having a good bioactivity score. The National Center for Biotechnology Information (NCBI) database search showed the presence of peptide sequence in signal transducer and activator of transcription 5A [*Bos taurus*]. Molecular docking studies of the peptides with the DPPH radical showed hydrogen and hydrophobic Pi-Alkyl bond formation. These results suggest that cheese-making industry wastewater can be useful resource for the generation of antioxidant peptides.

Keywords: Cheese-making Industry; Wastewater; Microbial digestion; Peptides; Antioxidant activity; Purification.
Received: 04 May 2023; Revised: 10 June 2023; Accepted: 16 June 2023.
Article type: Research article.

1. Introduction

Increased exposure to environmental pollutants, UV rays, as well as drug abuse, and stressful daily routines, can alter redox homeostasis, an important aspect of our health and well-being. The balance between free radicals such as reactive oxygen species, (ROS), reactive nitrogen species, (RNS) and antioxidants in the body plays an important role in maintaining the cell's functionality and integrity. On the other hand, free radical overproduction, along with a decrease in antioxidant levels, has recently been associated with oxidative stress, premature aging, and the occurrence of a variety of disorders.[1] Many of these disorders are categorized as non-communicable diseases (NCD). According to the world health organization (WHO), the NCD emerged as a major threat to global health. One of the prevention measures for NCD is the use of antioxidant compounds.[2] In this context, the antioxidants (natural or synthetic) received increasing attention. Synthetic antioxidants possess stronger antioxidant activities; however, their long-term use has potential health hazards.[3] Natural antioxidants have low molecular weight. They are safe, easy to absorb, and get cleared fast from the blood.[4] Hence, the search for alternative natural antioxidants became a hot research area and several researchers explored protein hydrolysates for the presence of antioxidant peptides.[5,6] The antioxidant peptides possess the ability to scavenge or neutralize free radicals and ROS in the body, thereby reducing oxidative stress. Several mechanisms are involved in this process. Antioxidant peptides have specific amino acid sequences that enable them to donate electrons or hydrogen

[1] *Department of Microbiology, Savitribai Phule Pune University, Pune 411007, Maharashtra India.*
[2] *Department of Biotechnology, Savitribai Phule Pune University, Pune 411007, Maharashtra India.*
[3] *Department of Biochemistry, Shivaji University, Kolhapur 416004, Maharashtra India.*
[4] *Department of Botany, Savitribai Phule Pune University, Pune 411007, Maharashtra, India.*
**Email:* ujadhav@unipune.ac.in, umeshjadhav02@gmail.com (U. T. Jadhav)

atoms to neutralize these free radicals, thereby preventing them from reacting with cellular components and causing oxidative stress.[2] Antioxidant peptides can modulate cellular signaling pathways involved in oxidative stress and inflammation. They can regulate the expression of genes associated with antioxidant defense systems, inflammatory responses, and cell survival pathways. Antioxidant peptides can inhibit the process of lipid peroxidation, which is the oxidative degradation of lipids leading to the production of lipid peroxides. Lipid peroxides can further propagate oxidative damage and contribute to various diseases. Antioxidant peptides can interrupt the lipid peroxidation chain reaction by donating electrons or hydrogen atoms to stabilize lipid radicals.[7] Recently antioxidant peptides are also found effective for the treatment of photoaging damage. The peptides reduced apoptosis of UV-irradiated cells. The peptides restored mitochondrial membrane potential (MMP), reduced the expression level of the apoptosis-executing protein, and up-regulated the expression levels of the nuclear factor erythroid 2–related factor 2 (Nrf2), NAD(P)H Quinone Dehydrogenase 1 (NQO1), and *Heme oxygenase-1 (*HO-1). This resulted in the reduction of intercellular ROS and malondialdehyde (MDA).[7,8] These benefits of antioxidant peptides highlighted their potential to be used in the areas of food, cosmetic products, and medicines. This also necessitates finding new sources of antioxidant peptides. The existence of antioxidant peptides in bovine casein, ovine casein, and buffalo casein has been reported.[9-11] The use of milk and milk products in India is prevailing. A large number of dairy and milk processing industries are established to fulfill the country's needs. The wastewater generated by these industries contains a high amount of milk proteins. Keeping this in view, we investigated the bioprospecting of this wastewater for the generation of antioxidant peptides (The manuscript is under consideration). In this work, the wastewater collected from cheese making industry was used as a protein source. The recovered proteins were hydrolyzed using *Bacillus* to obtain antioxidant peptides. Ultrafiltration, gel filtration chromatography, and reverse-phase high-performance liquid chromatography (RF-HPLC) were used to purify the antioxidant peptides. The LC-Q-TOF mass spectrometry was used for the identification of antioxidant peptides.

2. Materials and methods

2.1 Microbial cultures, growth medium, and chemicals

Bacillus subtilis NCIM 2724, was procured from National Collection of Industrial Microorganisms (NCIM), India. *Bacillus subtilis* NCIM 2724 was selected due to its ability to produce protease. The stock cultures were stored in 70% Glycerol at -80 ℃. Skim Milk powder was procured from HiMedia. Skim milk medium (Skim Milk powder: 28 g/L, Dextrose: 1 g/L, pH 7) was used for the optimization of protease production and hydrolysis. Modified nutrient medium (Meat extract: 10 g/L, NaCl: 5 g/L, Peptone: 10 g/L, pH 7) was prepared according to the guidelines of NCIM. The stock culture of *Bacillus subtilis* NCIM 2724 was grown overnight and maintained in a modified nutrient medium (Meat extract: 10 g/L, NaCl: 5 g/L, Peptone: 10 g/L, pH 7) at 30 ℃. All chemicals and reagents used for further experiments were purchased from HiMedia lab, Sigma Chemicals, Merck, and Thermo Scientific.

2.2 Isolation of crude protein hydrolysate

The wastewater was generously provided by a local cheese-making industry at Yavat, Pune, Maharashtra, India. They provided the wastewater generated during the cheese-making process. The collected wastewater was stored at 4 ℃ for further use. The 1 L wastewater as received (without any pretreatment) was initially frozen at -20 ℃. Further water was removed by vacuum at a lower temperature using lyophilization or freeze drying. The lyophilization was performed at a lower temperature hence it does not alter the native structure of proteins. The lyophilized powder was used as a substrate for generating bioactive peptides. The medium was hence prepared by dissolving 2.8 g of lyophilized powder and 0.1 g of dextrose/glucose into 20 ml of sterile distilled water. *B. subtilis* NCIM 2724 was grown in a sterile modified nutrient medium for 12 hours to obtain log phase culture. The cells were then harvested by centrifugation at 7000 x g for 15 min and resuspended in 1 mL of sterile saline and OD600 of the culture was adjusted to 1. This suspension was used as inoculum (1%) for hydrolysis of proteins. The suspension was inoculated into 20 ml of lyophilized protein sample (obtained by lyophilization of wastewater) containing medium. This medium was prepared by adding 2.8 g of protein sample to 10 mL of sterile distilled water and stirring continuously until it mixed properly and was then autoclaved. This was followed by the addition of 0.1 g/mL dextrose (filter sterilized) and the volume was made up to 20 mL with sterile distilled water. A 1 ml culture suspension was inoculated in protein containing medium and incubated under optimized conditions. The cell-free supernatant was collected by harvesting cells at 5000 x g for 10 min at 4 ℃ then the collected supernatant was filter sterilized and used as crude protein hydrolysate.

2.3 Protein fractionation using ultra-centrifugal filters

In a previous study, the protocol was standardized for the fractionation of crude protein hydrolysate using Ultra-centrifugal filters Pierce™. Protein Concentrators PES 3K MWCO were used for the fractionation of crude protein hydrolysate (the manuscript is under consideration). The centrifugation filters were pre-washed with 10 mL of 10 mM PBS pH 7. A 20 mL of crude protein hydrolysate was transferred to the Protein Concentrators and was centrifuged (Eppendorf Centrifuge 5430 / 5430 R) at 3000 x g for 90 min at 4 ℃ to acquire a 3 kDa filtrate containing a mixture of peptides. The protein content of the supernatant and filtrate was determined by Bradford assay.

2.4 Determination of radical scavenging activity

The radical scavenging activity was determined using the 2,2-diphenyl-1-picryl-hydrazyl-hydrate (DPPH) method as described by Garrido *et al.*,[12] with slight modifications. The fixed amount of peptide samples was taken. A 0.5 mL of DPPH reagent was added to the peptide sample. DPPH solution (0.5 mL) and distilled water (1 mL) were used as a blank. The reaction mixtures were incubated in the dark for 30 minutes, and the absorbance was measured at 517 nm. The percent scavenging activity was calculated.

2.5 Isolation and purification of antioxidant peptides

A Sephadex G-25 gel filtration column (GE17-0851-01 disposable PD 10 desalting column, size: 1.45 × 5 cm) from Cytiva was used. It was equilibrated with distilled water as suggested by the supplier. The 3 kDa filtrate showing antioxidative activity was lyophilized, dissolved in water, and applied on a Sephadex G-25 column. The column was then eluted with distilled water at a 1 ml/min flow rate. Around 25 fractions were collected. The absorbance for each of the fractions was recorded at 280 nm to confirm the presence of proteins. Fractions were pooled and lyophilized, and then the antioxidative activity of each fraction was examined.

2.6 Fractionation of antioxidant peptides by RP-HPLC

The antioxidative fraction was separated by reversed-phase high-performance liquid chromatography (HPLC) on a Thermo C18 5U (150 × 4.6 mm) column using a linear gradient of acetonitrile (0–50%, v/v, 50 min) containing 0.1% formic acid at a flow rate of 1.0 ml/min.[3] The absorbance was recorded at 220 nm. Fractions showing antioxidative activity were identified.

2.7 Identification of antioxidant peptide using LC-Q-TOF-MS

The identification of potential antioxidant peptides was done using high-resolution mass spectrometry (HRMS) at the National Chemical Laboratory (Venture Center), Pune. Agilent Technologies Agilent 6550 UHD Accurate Mass QTOF MS with a Jet Stream ion source for electrospray ionization was used to conduct the ultra-high-performance LC-Q-TOF-MS analysis (Dual AJS ESI). The obtained LC/MS data was subsequently submitted to the Metlin database to identify the sequence of the peptides. The identified sequences were submitted to the peptide ranker (http://bioware.ucd.ie/~compass/biowareweb/) to find out the bioactivity score. The identified sequences of peptides were also analyzed for the Grand Average of Hydropathicity (GRAVY) using https://www.gravy-calculator.de/. This analysis helps to identify the hydrophilic or hydrophobic nature of peptides. NCBI database (https://www.ncbi.nlm.nih.gov/protein) was used to identify the distribution of the detected amino acid sequence in the parent proteins. The parent proteins considered for this analysis are casein, Lactoglobulin, Lactalbumin, Serum albumin, Lactoferrin, and Immunoglobulin. All the sequences were downloaded in FASTA format and analyzed for the presence of the detected amino acid sequence.

2.8 Docking studies of predicted peptides and DPPH

The interaction process between discovered peptides and free radicals such as DPPH was simulated using the AutoDock Tools software, according to Sun *et al.*.[13] BIOVIA Discovery Studio Visualizer was used to predict the structures of the peptides PPPT and TPPP. An annotation of atom types, charges, and hydrogen atoms has been employed to pre-process the peptides and ligand molecules. The AutoDock 4.2 program and the Lamarckian genetic algorithm were used to determine the binding sites between the peptides and ligands. A hundred conformers were produced by a docking simulation and selected based on the lowest binding energy between peptides and ligands. The results were exported in pdbqt format and conformations were analyzed in BIOVIA Discovery Studio Visualizer.

2.9 Statistical analysis

The data analysis was performed utilizing OriginPro 2020 SR19.7.0.188 statistical analysis software. The statistical significance of the differences between means was assessed using a one-way analysis of variance (ANOVA) followed by Tukey's test. All experiments were conducted three times, and the presented values represent the means ± standard deviation (SD). Symbols with different letters indicate significant differences ($p < 0.05$) between the groups being compared.

3. Results and discussion

3.1 Gel filtration chromatography of 3 kDa membrane filtrate

The proteins present in wastewater of cheese making industry were recovered using lyophilization. These proteins were digested using *B. subtilis* NCIM 2724. The digest was tested for DPPH radical scavenging activity. It showed 56.24% free radical scavenging activity. It was further passed through a 3 kDa membrane cut-off filter. The filtrate collected from this process also showed DPPH radical scavenging activity (56.45%). This filtrate was then employed for the purification and identification of antioxidant peptides. The 3 kDa membrane filtrate was initially loaded on a Sephadex G-25 column to fractionate the peptides according to their molecular size. Several researchers have suggested that gel filtration chromatography is an effective method for separating bioactive peptides.[14,15] Around 25 fractions were collected using Sephadex G-25 column chromatography. Results showed that 10 fractions have prominent protein content (Fig. 1a). All the fractions were tested for antioxidant activity. Fraction 10 showed maximum DPPH radical scavenging activity (20.56%) (Fig. 1b).

3.2 Isolation of peptides from Fraction-10 by RP-HPLC

The content of fraction 10 was lyophilized and purified with RP-HPLC. Seven peaks at various retention times were

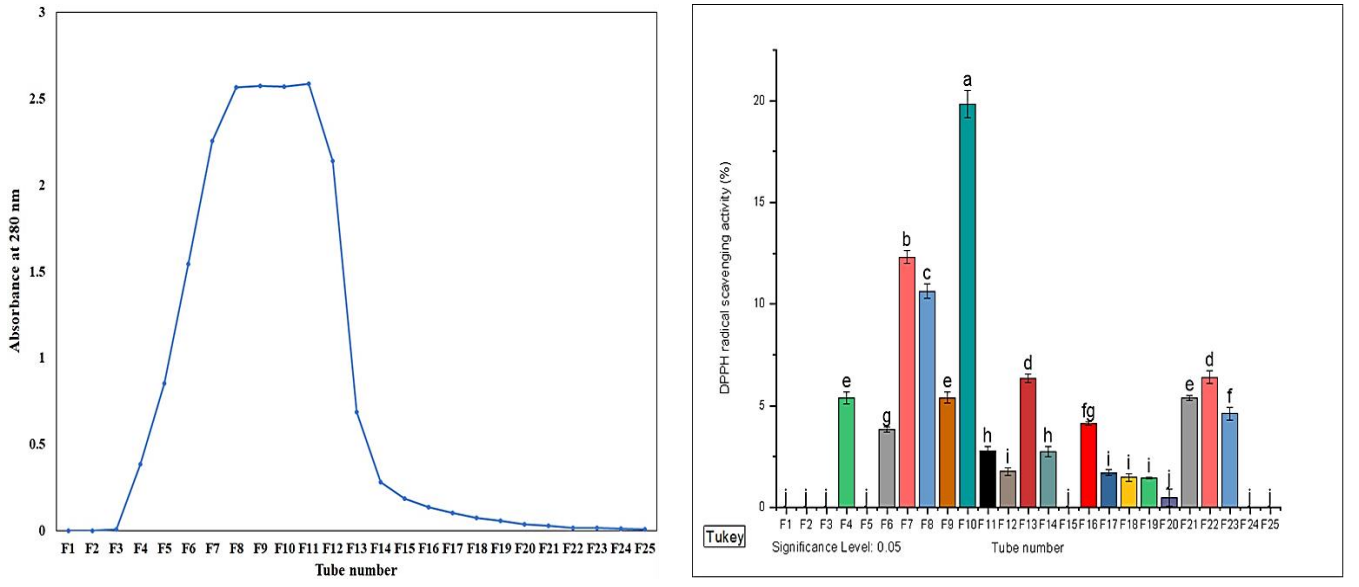

Fig. 1 (a) Elution profile on the Sephadex G-25 column, (b) DPPH radical scavenging activity of collected fractions. The data were subjected to one-way ANOVA analysis using Tukey's test. Fig. 1b displays the mean ± standard deviation (SD) of each fraction. The experiments were performed in triplicate (n=3). Symbols with different letters indicate significant differences ($p < 0.05$) between the groups being compared.

obtained during purification (Fig. 2 and Table 1). The peak at retention time 8.040 minutes showed a 48.03% area. The sample from this fraction showed 10.3% DPPH radical scavenging activity.

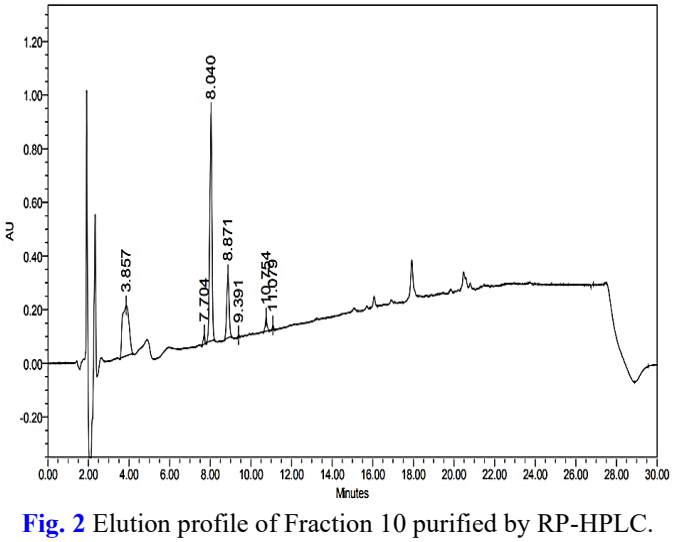

Fig. 2 Elution profile of Fraction 10 purified by RP-HPLC.

Table 1. Retention time, %area of the peaks observed during HPLC analysis.

Sr. No.	Retention time (minutes)	Area	% Area
1	3.857	4253492	32.25
2	7.704	228493	1.73
3	8.040	6333648	48.03
4	8.871	1909999	14.48
5	9.391	40489	0.31
6	10.754	342743	2.60
7	11.079	78594	0.60

3.3 Characterization of purified antioxidant peptides

The fraction with the highest antioxidant activity was further analyzed by LC-MS/MS. LCMS chromatogram showed two peaks. The information on the prominent peak at 11.816 minutes was searched in the database (Fig. 3). It gave ten sequences. These peptides have 3–4 amino acids. These sequences were having a molecular mass of 410.21 Da. These sequences were submitted to the peptide ranker to find out the bioactivity score. Out of ten peptide sequences only two sequences Proline-Proline-Proline-Threonine, and Threonine-Proline-Proline- Proline showed a good score (above 0.70) (Table 2). It is reported that proline plays an important role in the antioxidant activity of peptides due to its hydrophobic nature.[3,16] This is further confirmed by the Grand Average of Hydropathicity (GRAVY) analysis. The GRAVY score can be calculated as the sum of the hydropathy values for all the amino acids in a protein divided by the total number of residues in it. A negative GRAVY value indicates that the protein is non-polar and a positive value indicates that the protein is polar. The GRAVY score obtained for TPPP and PPPT was -1.375. Several researchers also reported that the low-molecular-weight peptides showed relatively high antioxidant activities. It is related to the steric hindrance and the enhanced interaction between peptides and free radicals.[17,11,13] NCBI database was used to identify the distribution of the detected amino acid sequence in the parent proteins such as casein, Lactoglobulin, Lactalbumin, Serum albumin, Lactoferrin, and Immunoglobulin. It provided 97, 56, 48, 1, 30, and 17 hits respectively. Only two peptide sequences Proline-Proline- Proline-Threonine, Threonine-Proline- Proline-Proline were present in the signal transducer and activator of transcription 5A [*Bos taurus*]. Further, it was observed that the peptide amino acid sequence was concentrated at positions 780-783 in the signal transducer and activator of transcription

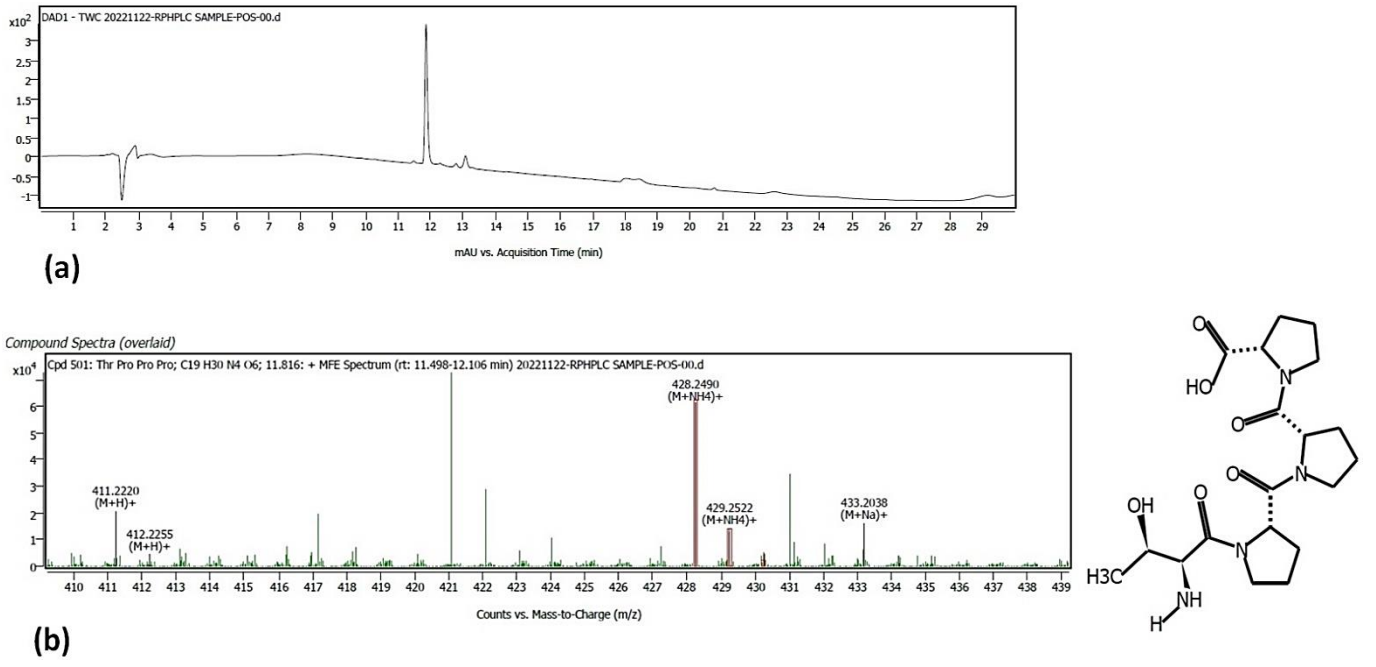

Fig. 3 LCMS profile of purified fraction (a), the mass spectrogram of the purified peptide obtained on a Q-TOF system (b).

5A [*Bos taurus*] of the parent proteins (https://www.ncbi.nlm.nih.gov/protein/NP_001012691.1) (Fig. 4).

Table 2. Bioactivity prediction using peptide ranker.

Sr. no.	Peptide sequence	Activity score
1	PPPT	0.763
2	TPPP	0.72
3	PPTP	0.693
4	PTPP	0.661
5	KYT	0.087
6	YKT	0.087
7	TYK	0.080

3.4 Mechanism of DPPH radical scavenging

The ability of compounds to function as free-radical scavengers and hydrogen suppliers is determined using the DPPH which possesses a nitrogen-free radical.[18,19] It does not dimerize due to the delocalization of the spare electron. This delocalization imparts violet color when DPPH solution is prepared in ethanol. When a substance having the ability to donate a hydrogen atom is mixed with a DPPH solution then it is changed to colorless or light yellow due to the reduction and transformation of DPPH into DPPHH.[19,20] In the DPPH assay, an odd electron displays a strong absorption band at a wavelength of 519 nm, which loses absorption once the odd electron is paired off by a hydrogen or electron-donating antioxidant (Fig. 5).

Further investigation of the antioxidant mechanism of peptides was carried out by studying the binding mode between DPPH and peptides using molecular docking. Several types of research showed that the interaction of peptides with

signal transducer and activator of transcription 5A [Bos taurus]

NCBI Reference Sequence: NP_001012691.1

GenPept Identical Proteins Graphics

>NP_001012691.1 signal transducer and activator of transcription 5A [Bos taurus]
MAGWIQAQQLQGDALRQMQVLYGQDFPIEVRHYLAQWIESQPWDAIDLDNPQDRAQATQLLEGLVQELQK
KAEHQVGEDGFLLKIKLGHYATQLQNTYDRCPMELVRCIRHILYNEQRLVREANNGSSSAGILVDAMSQK
HLQINQTFEELRLVTQDTENELKKLQQTQEYFIIQYQESLRIQAQFAQLAQLNPQERLSRETALQQKQVS
LEAWLQREAQTLQQYRVELAEKHQKTLQLLRKQQTIILDDELIQWKRRQQLAGNGGPPEGSLDVLQSWCE
KLAEIIWQNRQQIRRAEHLCQQLPIPGPVEEMLAEVNATITDIISALVTSTFIIEKQPPQVLKTQTKFAA
TVRLLVGGKLNVHMNPPQVKATIISEQQAKSLLKNENTRNECSGEILNNCCVMEYHQATGTLSAHFRNMS
LKRIKRADRRGAESVTEEKFTVLFESQFSVGSNELVFQVKTLSLPVVVIVHGSQDHNATATVLWDNAFAE
PGRVPFAVPDKVLWPQLCEALNMKFKAEVQSNRGLTKENLVFLAQKLFNSSSSHLEDYNGMSVSWSQFNR
ENLPGWNYTFWQWFDGVMEVLKKHHKPHWNDGAILGFVNKQLAHDLLINKPDGTFLMRFSDSEIGGITIA
WKFDSPDRNLWNLKPFTTRDFSIRSLADRLGDLNYLIYVFPDRPKDEVFSKYYTPVLAKAVDGYVKPQIK
QVVPEFVSASADSAGSNATYMDQAPSPAVCPQPHYNMYPQNPDPVLDQDGEFDLDETMDVARHVEELLRR
PMDSLEPSLPPPTGLFTPGRGSLS

Fig. 4 The sequences of the identified peptides in the parent protein signal transducer and activator of transcription 5A [*Bos taurus*].

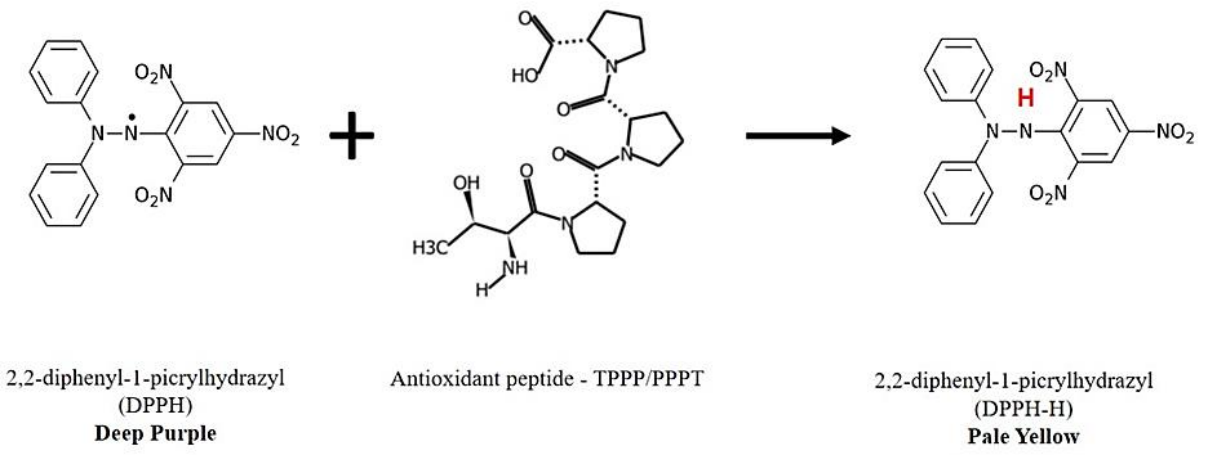

Fig. 5 Possible reaction mechanism of DPPH with antioxidant peptides.

free radicals occurs through hydrogen bonds, hydrophobic, van der Waals, and electrostatic interactions.[21,22,13] The results of the present study show that hydrogen bonds were formed between the antioxidative peptides and free radicals. To understand the possible interactions involved in that might be the antioxidant mechanism, molecular docking was performed. There was a strong interaction shown by both the peptides with DPPH free radicals. Bond interactions like Hydrogen and hydrophobic Pi-Alkyl bonds are formed as a result of molecular docking studies of the peptides PPPT, TPPP, and the DPPH free radical. Peptide PPPT and DPPH form a conventional H-bond with proline (PRO1) amino acid with a 1.90 Å bond length. Peptide PPPT has also formed two hydrophobic Pi-Alkyl bonds DPPH-PRO1 with 4.65 Å and 5.49 Å bond length and one Pi-Anion DPPH-THR4 with 3.38Å (Fig. 6). Similarly, interaction between TPPP and DPPH shows two conventional H-bonds threonine (THR1)-DPPH and proline (PRO4)-DPPH with bond lengths of 2.08 Å and 2.5 Å and hydrophobic Pi-Alkyl bond DPPH -proline (PRO2) with a bond length of 5.25 Å (Fig. 6). The calculated binding energy of PPPT and DPPH is -3.64 Kcal/mol and for TPPP and DPPH is -4.50 Kcal/mol, with an inhibition constant of 2.15 mM and 0.5 mM respectively. When compared with PPPT-DPPH interaction which shows the involvement of a single amino acid (proline-PRO1), TPPP shows the interaction of three different amino acids with DPPH. Additionally, the TPPP-DPPH interaction has lower binding energy than PPPT-DPPH. These results indicate that TPPP has stronger and more stable interaction with DPPH than PPPT. It is possible to conclude that amino acid residues in peptides can successfully

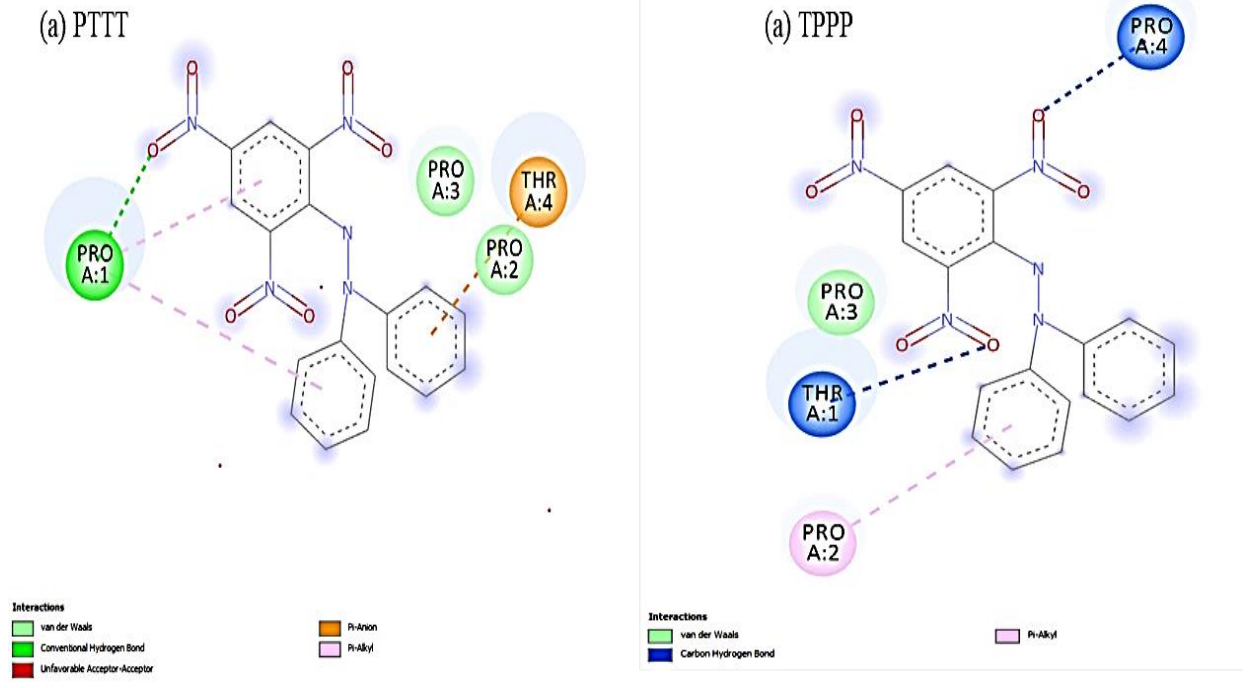

Fig. 6 Interaction of PPPT with DPPH radical in two-dimensional space. amino acids of the peptide are shown in green and brown color while DPPH is in grey. Bond interaction as Hydrogen in green, pi alkyl in pink, pi-Anion in brown colors (a) Interaction of TPPP with DPPH radical in two-dimensional space. amino acids of the peptide are shown in green, blue, and pink color while DPPH is in gray. Bond interaction as van der Waals in green, pi alkyl in pink color, carbon-hydrogen bond in blue color (b).

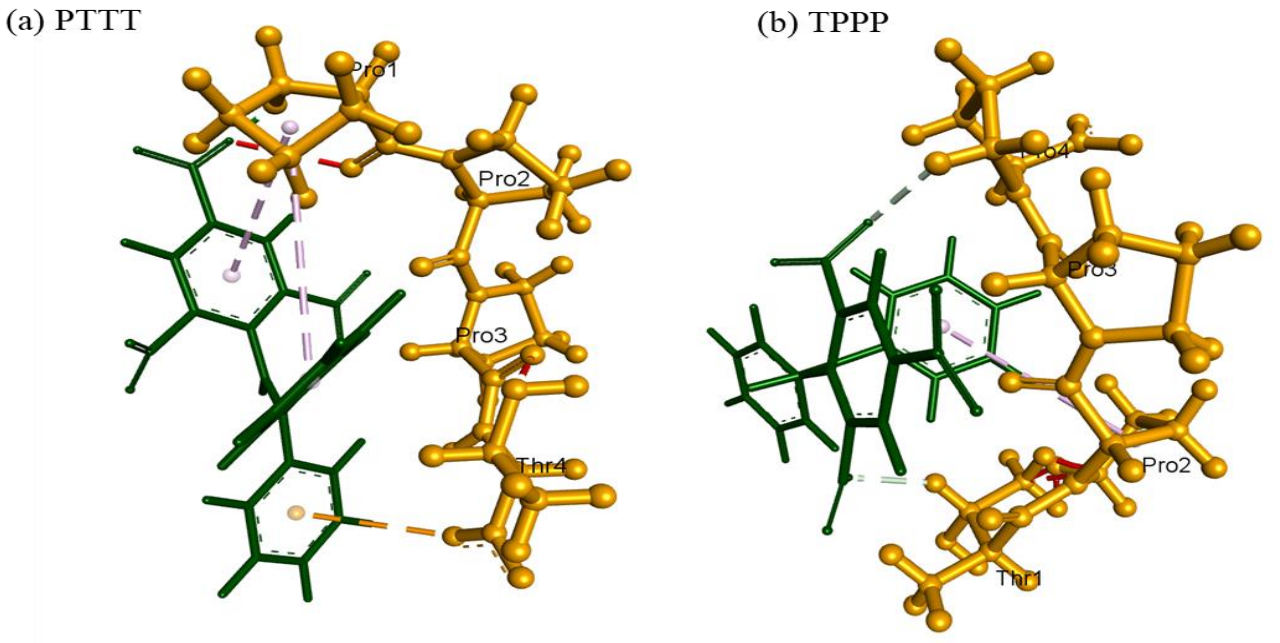

Fig. 7 Molecular docking of the antioxidant peptides with DPPH. (a) Interaction of PPPT with DPPH in three-dimensional space. DPPH radical is dark green in color interacting with PPPT peptide in brown color forming various bonds like green color hydrogen, red color pi bond, pi-Anion in brown and pink color alkyl bond. b) Interaction of TPPP with DPPH in three-dimensional space. DPPH radical is dark green in color interacting with TPPP peptide in brown color forming various bonds like green color hydrogen, red color pi bond, and pink color alkyl bond.

Table 3. Molecular docking of the peptide with DPPH.

Name of compound	Interactions (Bond distance and angles)	Binding Energy	Inhibition Constant
PPPT	Conventional H-Bond PRO1- DPPH (1.90/152.885) Hydrophobic Pi-Alkyl DPPH-PRO1 (4.65485) DPPH-PRO1 (5.49532) Pi-Anion DPPH-THR4 (3.38)	-3.64 Kcal/mol	2.15 mM
TPPP	Hydrogen Bond THR1-DPPH (2.08014/141.975) PRO4-DPPH (2.49703/117.687) Hydrophobic Pi-Alkyl DPPH -PRO2 (5.25)	-4.50 Kcal/mol	0.5 mM

form various bonds with free radicals of DPPH, resulting in powerful free radical scavenging potential (Fig. 7, Table 3). It is possible to conclude that antioxidant scavenging residues in peptides can successfully form hydrogen bonds with free radicals and reduction leads to forming an equivalent number of hydrazine. This results in free radical scavenging and decolorization of DPPH.

4. Conclusion

The cheese-making industry wastewater is a good source of proteins. The microbial digestion of these proteins using *B. subtilis* NCIM 2724 successfully generated antioxidant peptides. The 3 kDa membrane filtration and several chromatographic techniques helped the purification of antioxidant peptides. The generated antioxidant peptides contained 3-4 amino acids. The peptides were observed to be containing hydrophobic amino acids. The molecular docking results showed hydrogen bonds between the peptides and free radicals formed. This resulted in free radical scavenging and decolorization of DPPH. The results of the present study encourage to use of wastewater generated during cheese making process as a source of protein. The real field application of this research will help in two ways. Being an agriculture-based economy, India hosts several small and large manufacturing plants for dairy products. These units generate huge amounts of wastewater which is a threat to the

environment. The recycling of this wastewater for the generation of antioxidant peptides will protect the environment. It will also provide the manufacturer with an alternate source of revenue generation and they can share this increased profit with the farmers to strengthen the economy.

Acknowledgment
Mr. Rushikesh Pol acknowledges the Indian Council of Medical Research for the fellowship and Department of Microbiology, Savitribai Phule Pune University (SPPU) (Formerly Pune University), Pune for providing the required facilities.

Conflict of Interest
There is no conflict of interest.

Supporting Information
Not applicable.

References
[1] C.-Z. Zhu, W.-G. Zhang, G.-H. Zhou, X.-L. Xu, Z.-L. Kang, Y. Yin, Isolation and identification of antioxidant peptides from Jinhua ham, *Journal of Agricultural and Food Chemistry*, 2013, **61**, 1265-1271, doi: 10.1021/jf3044764.
[2] Y. Sheng, W.-Y. Wang, M.-F. Wu, Y.-M. Wang, W.-Y. Zhu, C.-F. Chi, B. Wang, Eighteen novel bioactive peptides from monkfish (lophius litulon) swim bladders: production, identification, antioxidant activity, and stability, *Marine Drugs*, 2023, **21**, 169, doi: 10.3390/md21030169.
[3] H.-G. Byun, J. K. Lee, H. G. Park, J.-K. Jeon, S.-K. Kim, Antioxidant peptides isolated from the marine rotifer, Brachionus rotundiformis, *Process Biochemistry*, 2009, **44**, 842-846, doi: 10.1016/j.procbio.2009.04.003.
[4] J. Yang, L. Hu, T. Cai, Q. Chen, Q. Ma, J. Yang, C. Meng, J. Hong, Purification and identification of two novel antioxidant peptides from perilla (Perilla frutescens L. Britton) seed protein hydrolysates, *PLoS One*, 2018, **13**, e0200021, doi: 10.1371/journal.pone.0200021.
[5] A. Bougatef, N. Nedjar-Arroume, L. Manni, R. Ravallec, A. Barkia, D. Guillochon, M. Nasri, Purification and identification of novel antioxidant peptides from enzymatic hydrolysates of sardinelle (Sardinella aurita) by-products proteins, *Food Chemistry*, 2010, **118**, 559-565, doi: 10.1016/j.foodchem.2009.05.021.
[6] Chang-Feng, Chi, Isolation and characterization of three antioxidant peptides from protein hydrolysate of bluefin leatherjacket (Navodon septentrionalis) heads, *Journal of Functional Foods*, 2015, **12**, 1-10, doi: 10.1016/j.jff.2014.10.027.
[7] J. Kong, X.-M. Hu, W.-W. Cai, Y.-M. Wang, C.-F. Chi, B. Wang, Bioactive peptides from skipjack tuna cardiac arterial bulbs (II): protective function on UVB-irradiated HaCaT cells through antioxidant and anti-apoptotic mechanisms, *Marine Drugs*, 2023, **21**, 105, doi: 10.3390/md21020105.
[8] Shuang-Yi, Zhang, Gelatins and antioxidant peptides from Skipjack tuna (Katsuwonus pelamis) skins: purification, characterization, and cytoprotection on ultraviolet-a injured human skin fibroblasts, *Food Bioscience*, 2022, **50**, 102138, doi: 10.1016/j.fbio.2022.102138.
[9] S. G. Rival, C. G. Boeriu, H. J. Wichers, Caseins and casein hydrolysates. 2. antioxidative properties and relevance to lipoxygenase inhibition, *Journal of Agricultural and Food Chemistry*, 2001, **49**, 295-302, doi: 10.1021/jf0003911.
[10] J. Á. Gómez-Ruiz, I. López-Expósito, A. Pihlanto, M. Ramos, I. Recio, Antioxidant activity of ovine casein hydrolysates: identification of active peptides by HPLC-MS/MS, *European Food Research and Technology*, 2008, **227**, 1061-1067, doi: 10.1007/s00217-008-0820-3.
[11] V. P. Shanmugam, S. Kapila, T. K. Sonfack, R. Kapila, Antioxidative peptide derived from enzymatic digestion of buffalo casein, *International Dairy Journal*, 2015, **42**, 1-5, doi: 10.1016/j.idairyj.2014.11.001.
[12] T. Garrido, M. Gizdavic-Nikolaidis, I. Leceta, M. Urdanpilleta, P. Guerrero, K. de la Caba, P. A. Kilmartin, Optimizing the extraction process of natural antioxidants from chardonnay grape marc using microwave-assisted extraction, *Waste Management*, 2019, **88**, 110-117, doi: 10.1016/j.wasman.2019.03.031.
[13] J. Sun, C. Zhou, J. Cao, J. He, Y. Sun, Y. Dang, D. Pan, Q. Xia, Purification and characterization of novel antioxidative peptides from duck liver protein hydrolysate as well as their cytoprotection against oxidative stress in $HepG_2$ cells, *Frontiers in Nutrition*, 2022, **9**, 848289, doi: 10.3389/fnut.2022.848289.
[14] L. Sun, Y. Zhang, Y. Zhuang, Antiphotoaging effect and purification of an antioxidant peptide from tilapia (Oreochromis niloticus) gelatin peptides, *Journal of Functional Foods*, 2013, **5**, 154-162, doi: 10.1016/j.jff.2012.09.006.
[15] M. Zhang, T. H. Mu, M. J. Sun, Purification and identification of antioxidant peptides from sweet potato protein hydrolysates by Alcalase, *Journal of Functional Foods*, 2014, **7**, 191-200, doi: 10.1016/j.jff.2014.02.012.
[16] Y. A. A. H. Neto, J. C. Rosa, H. Cabral, Peptides with antioxidant properties identified from casein, whey, and egg albumin hydrolysates generated by two novel fungal proteases, *Preparative Biochemistry & Biotechnology*, 2019, **49**, 639-648, doi: 10.1080/10826068.2019.1566147.
[17] S. Ranathunga, N. Rajapakse, S.-K. Kim, Purification and characterization of antioxidative peptide derived from muscle of conger eel (Conger myriaster), *European Food Research and Technology*, 2006, **222**, 310-315, doi: 10.1007/s00217-005-0079-x.
[18] M. Yangthong, N. Hutadilok-Towatana, W. Phromkunthong, Antioxidant activities of four edible seaweeds from the southern coast of Thailand, *Plant Foods for Human Nutrition*, 2009, **64**, 218-223, doi: 10.1007/s11130-009-0127-y.
[19] S. Baliyan, R. Mukherjee, A. Priyadarshini, A. Vibhuti, A. Gupta, R. P. Pandey, C.-M. Chang, Determination of antioxidants by DPPH radical scavenging activity and quantitative phytochemical analysis of ficus religiosa, *Molecules*, 2022, **27**, 1326, doi: 10.3390/molecules27041326.

[20] S. B. Kedare, R. P. Singh, Genesis and development of DPPH method of antioxidant assay, *Journal of Food Science and Technology*, 2011, **48**, 412-422, doi: 10.1007/s13197-011-0251-1.
[21] A. T. Girgih, R. He, R. E. Aluko, Kinetics and molecular docking studies of the inhibitions of angiotensin converting enzyme and renin activities by hemp seed (Cannabis sativa L.) peptides, *Journal of Agricultural and Food Chemistry*, 2014, **62**, 4135-4144, doi: 10.1021/jf5002606.
[22] M. Mirzaei, S. Mirdamadi, M. R. Ehsani, M. Aminlari, Production of antioxidant and ACE-inhibitory peptides from Kluyveromyces marxianus protein hydrolysates: purification and molecular docking, *Journal of Food and Drug Analysis*, 2018, **26**, 696-705, doi: 10.1016/j.jfda.2017.07.008.