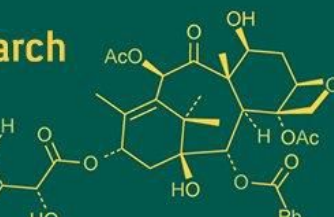
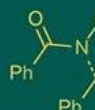


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Biofunctional properties of ultrafiltrate fraction of Kankrej milk casein hydrolysate

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Abstract

Bioactive peptides derived from milk proteins have attracted considerable interest due to their antioxidant and antihypertensive potential. This study focused on the production, isolation, and characterization of biofunctional peptides from Kankrej cow milk casein using trypsin-mediated enzymatic hydrolysis followed by ultrafiltration. Casein was isolated by acid precipitation and hydrolysed using trypsin at an enzyme-to-substrate ratio of 1:25 under optimized conditions (pH 8.0, 37 °C) for 8 h. The casein hydrolysates were then sequentially ultrafiltered using 10, 5 and 3 kDa molecular weight cut-off membranes. The permeate and retentate fractions were analysed for peptide content, antioxidant activity (ABTS, DPPH, and FRAP assays), and ACE inhibitory activity. Peptide content decreased with decreasing molecular weight cut-off, with the 3 kDa permeate showing the lowest peptide concentration (10.10 mg/ml). Despite this, the 3 kDa permeate exhibited the highest biofunctional activity, showing ABTS radical scavenging activity of 5524.79 $\mu\text{M TE/mg peptide}$, DPPH radical scavenging activity of 67.57%, FRAP value of 510.35 $\mu\text{M Fe}^{2+}/\text{mg peptide}$, and ACE inhibitory activity of 46.98%. Tricine SDS-PAGE confirmed the presence of low molecular weight peptides in the ultrafiltrate fractions. The results demonstrate that low molecular weight peptides (<3 kDa) derived from Kankrej cow milk casein possess superior antioxidant and ACE inhibitory activities, highlighting their potential application in functional foods and nutraceutical formulations aimed at managing oxidative stress and hypertension.

Keywords: Kankrej casein, ultrafiltrate, antioxidant activity, ace-inhibitory activity, SDS -page

Introduction

Casein, the major protein fraction in milk, is a well-established source of bioactive peptides that can be released through enzymatic hydrolysis. During hydrolysis by gastrointestinal enzymes or food-grade proteases such as trypsin, pepsin, chymotrypsin, or microbial enzymes, the inactive casein molecule is broken down to liberate specific peptide sequences with physiological functions. These casein-derived bioactive peptides exhibit a wide range of biological activities, including antihypertensive (ACE-inhibitory), antimicrobial, antioxidant, immunomodulatory, and opioid-like effects. Because these peptides are encrypted within the native protein structure and become active only after hydrolysis, casein is considered an important functional food ingredient for the development of nutraceuticals and health-promoting dairy products. The Kankrej is one of the heaviest dual purpose among 26 officially recognized indigenous cattle breeds in India. Characterized by large half-moon-shaped horns, drooping ears, and a silver-grey to dark coat, with high heat tolerance and disease resistance (Banerjee, 2005) [1]. Bioactive peptides or biopeptides are defined as food derived peptides with a distinct amino acid sequence of about 3-20 residues that endeavour a physiological effect in the body along with their nutritional value. The sequence of amino acids in the bioactive peptides can influence major organ systems, including the digestive, cardiovascular, nervous, and immune systems (Mohanty *et al.*, 2016) [22]. Bovine trypsin is a proteolytic enzyme that specifically hydrolyses peptide bonds on the carboxylic side of lysine and arginine, with an optimal pH range of 7 to 9 and a temperature range of 35° to 50 °C. Trypsin based hydrolysis typically generates protein hydrolysates with significant ACE-inhibitory, antioxidant and antidiabetic activity (Brunda, 2020) [4]. Kumar *et al.* (2016) [16] reported a higher radical reducing power in the casein hydrolyzed with trypsin. Short chain peptides (2-9 amino acid residues); containing hydrophobic amino acid residues in the

sequence (aromatic or branched side chains), and presence of proline, lysine or arginine residue at the C-terminal end and aliphatic amino acids (Leu, Ile and Val) at the N-terminal is a necessary structural features of ACE inhibitory peptides (Kitts and Weiler, 2003; Wu *et al.*, 2006)^[15, 38]. Ile-Pro-Pro (β-casein f 74-76; κ-casein f 108-110) and Val-Pro-Pro (β-casein f84-86) were among the first dietary peptides found to have a hypotensive effect in spontaneously hypertensive rats (Nakamura *et al.*, 1995; Bougle and Bouhallab, 2017)^[26, 39].

Ultrafiltration employing molecular cut-off membranes is specifically used for fractionating peptides, removal of proteins and also remaining macromolecules based on their molecular cut off. UF has successfully been used to concentrate ACE inhibitory peptides (Holder *et al.*, 2013; Bhandari *et al.*, 2020)^[10, 3] and antioxidative peptides (Roblet *et al.*, 2012)^[34]. Studies of Mendis *et al.* (2005)^[21] showed that the low molecular weight peptides have better antioxidant activities and the observation was similar to that reported by various workers (Fang *et al.*, 2012)^[6].

Numerous methods are available for characterization of bioactive peptides. Tricine based SDS-PAGE is generally used to characterize the proteins smaller than 30 kDa as it gives better resolution than glycine SDS PAGE (Schagger and Von, 1987)^[35].

This research work has been planned to characterize and separate the casein derived hydrolysates by Ultrafiltration from Kankrej cow milk and to evaluate its biofunctional properties.

Material and Methods

Materials

Kankrej cow was collected from Livestock Research Centre (LRC) of Kamdhenu University, Anand. Fresh pooled raw milk was cooled to 4 °C after collection. The trypsin enzyme was procured from Sigma-Aldrich Chemical Pvt Ltd, India (coded as T1426). The enzyme was from bovine pancreas source which was in the lyophilized form. The optimal pH of trypsin enzyme selected for this study was 8.0 (as suggested by the Sigma-Aldrich catalogue).

and also Brunda (2020)^[4] reported that the pH of 8 for Deoni cow milk casein hydrolysates by trypsin obtained from the same source) and optimum temperature maintained for hydrolysis was 37 °C. After hydrolysis the enzyme was inactivated by heat treatment at 90°C for 10 min. The glass distilled water (double distilled water)/ HPLC water was used for preparation of all reagents. 2, 2-diphenyl-1-picrylhydrazyl (DPPH), 2, 2'-Azino bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), 2, 4, 6-tri-(2-pyridyl)-5-triazine (TPTZ), o-phthalaldehyde, sodium dodecyl sulphate (SDS), molecular markers for SDS-PAGE were purchased from Sigma-Aldrich. Inc., India.

Preparation of Casein

Freshly drawn Kankrej cow milk samples maintained at 4°C were preheated at 33 °C and subjected for cream separation. The skim milk was warmed to 37 °C and the pH was adjusted to 4.6 at 37 °C slowly by adding 1.0 N HCl and with a constant stirring with spatula. After clear precipitation of casein, the suspension was then filtered through muslin cloth. The casein curd was washed four times, first two washings in cold distilled water (5±2 °C) followed by warm distilled water (37 °C) and finally with cold distilled water. The wet casein curd was dried in

vacuum drier at 45 °C under 600 mmHg. The casein was powdered and stored in an air tight container at -20 °C till further use.

Analysis of Casein

The moisture content of casein was estimated by gravimetric method (FSSAI, 2022)^[8], Fat content of casein was determined by acid digestion method as described in (FSSAI, 2022)^[8], Protein content in casein was estimated by Kjeldahl method (FSSAI, 2022)^[8] using block digester and automatic distillation unit. The ash content of casein sample was determined by following standard method (IS: SP-18, 1981)^[12] using 3 g of sample. The total acidity of casein sample was determined by following standard method (IS: SP-18, 1981)^[12]. The acid Insoluble ash content of casein sample was determined by following standard method (IS: SP-18, 1981)^[12].

Preparation of Casein hydrolysate

Casein was dissolved in double distilled water and small quantities of 0.1N NaOH solution was added to get 5% casein solution. The casein solution was preheated to 90 °C/10 min in order to inactivate the native proteases present. Trypsin enzyme was added at 1:25 Enzyme:Substrate ratio and incubated at pH 8.0 and temperature 37 °C for a period of 8 h (BRENDA, 2025).

Aliquot of the sample after 8h of hydrolysis, were inactivated by heating to 90 °C for 10 min. Samples were then centrifuged at 14000 rpm for 20 min at 5°C to remove the unhydrolyzed casein.

Ultrafiltration of Casein Hydrolysates

The casein hydrolysates thus obtained was ultrafiltered sequentially using different molecular weight cut-off membranes (MWCO) viz., 10, 5 and 3 kDa. The permeate and retentate fractions were collected, lyophilized and stored at -20 °C till further analysis.

Peptide Quantification by Lowry's Method of Casein Ultrafiltrates

The concentration of peptide in the ultrafiltrate fractions was estimated by the method of Lowry *et al.* (1951)^[19].

Determination of biofunctional property

The permeates and retentates obtained after ultrafiltration using different molecular weight cut-off membranes were analysed for selected biofunctional properties (*in-vitro*) namely antioxidant activity and ACE inhibitory assay (*in-vitro*).

Antioxidant Activity

ABTS method

The antioxidant activity was determined using 2, 2'-azinobis-3-ethyl benzothiazoline-6-sulfonic acid (ABTS) radical cation decolourization assay developed by Re *et al.* (1999)^[33] with some modifications as described by Tachakittirungrod *et al.* (2007)^[37].

$$\% \text{ ABTS radical scavenging activity} = \frac{(A_0 - A_1)}{A_0} \times 100$$

Standard curve was prepared using trolox. Exactly 0.2 ml of

the different concentrations of Trolox or sample solution was added to 1.8 ml of ABTS solution. The contents were mixed for 5 s, incubated for 10 min away from direct light and then the absorbance was measured at 734 nm against solvent blank (HPLC grade water). For control HPLC grade water was used in the place of sample or standard. Based on the per cent inhibition of sample, trolox equivalent was determined from standard curve. The results were expressed as trolox equivalent antioxidant capacity (TEAC) i.e. μM Trolox equivalence per mg of the protein.

Where,

A_0 is the absorbance of control, A_1 is the absorbance of sample

FRAP Method

Ferric reducing antioxidant power (FRAP) activity in samples was carried out according to the method of Benzie and Strain (1996)^[2]. Ninety microlitres of sample or ferrous sulphate standard solutions was added to 2.7 ml FRAP reagent. The content was mixed for 5 s and incubated at 37 °C for 30 min. The absorbance was measured at 595 nm using spectrophotometer. Based on the result obtained from the standard curve, antioxidant activity was expressed as μM Fe^{2+} per mg of protein.

DPPH Method

Antioxidant activity based on 2, 2 diphenyl-1-picryl hydrazyl (DPPH) radical scavenging was analysed as per the method given by Mao *et al.* (2011)^[20]. Exactly 750 μl of sample was added to 750 μl of DPPH solution (0.2 mM). The contents were mixed for 5 s, incubated at 37 °C for 30 min and absorbance was measured at 517 nm against methanol. For control, methanol was used in the place of sample. The results were expressed as per cent inhibition.

$$\% \text{ DPPH radical scavenging activity} = \frac{(A_0 - A_1)}{A_0} \times 100$$

Where,

A_0 is the absorbance of control, A_1 is the absorbance of sample

Angiotensin converting enzyme (ACE) inhibition assay

Angiotensin Converting Enzyme (ACE) Inhibitory activity of the samples were measured using the modified method given by Hernández-Ledesma *et al.* (2004)^[9] Exactly 20 μl of sample was transferred into an eppendorf of 2 ml capacity. To this 110 μl of buffer substrate was added and incubated at 37 °C for 5 min followed by the addition of 20 μl of ACE (1:5 diluted) was added and incubated at 37 °C for 80 min. Then the reaction was arrested by adding 40 μl of chilled and microfiltered 1.0 N HCl. Hippuric acid formed was extracted in 1.0 ml ethyl acetate by centrifugation at 3000 rpm for 10 min at 4 °C. The solvent layer of extract (about 750 μl) was dried at 95 °C for 20 min in a fresh 2.0 ml eppendorf. Dried hippuric acid was dissolved in 1.0 ml of HPLC water and absorbance was read at 228 nm using 1 ml cuvette, spectrophotometrically. The above procedure was repeated for negative control by replacing the sample by 20 μl of distilled water and the blank is prepared by adding 40 μl of distilled water instead of sample and ACE.

Per cent ACE inhibitory activity was calculated using the following formula.

$$\% \text{ ACE Inhibition} = \left[1 - \frac{(C-D)}{(A-B)} \right] \times 100$$

Where,

A-Control, OD with enzyme, HHL and no sample; B-Blank, OD with HHL, no sample and enzyme; C- Sample, OD with enzyme, HHL and sample; D- Sample blank, OD with HHL and sample, no enzymes.

Characterization of casein ultrafiltrate fractions by Tricine SDS - PAGE

The permeates of casein ultrafiltrate fractions obtained using 10, 5 and 3 kDa cutoff membranes were characterized.

Tricine Sodium Dodecyl Sulphate - Polyacrylamide Gel Electrophoresis was carried out to characterize the peptides in the casein hydrolysates obtained from different hours of hydrolysis per the method of Schagger and von Jagow (1987)^[35] as modified by Pardo and Natalucci (2002)^[28].

Tricine SDS-PAGE was performed using a 16.5% separating gel and a 4% stacking gel in an OmniPAGE Mini Vertical Electrophoresis unit. Gel solutions were poured into cassettes, overlaid with butanol to prevent air interference, and allowed to polymerize; after removal of butanol, the stacking gel was added, combs were inserted to form wells, and wells were rinsed with cathode buffer after polymerization. Casein hydrolysate samples were mixed with 2 $\times$ sample buffer (1:1), heated at 100 °C for 5 min, vortexed, and stored at 4 °C prior to loading, while ultra-low molecular weight markers (26.6-1.06 kDa) were used as standards. Electrophoresis was conducted at room temperature, initially at 50 V until samples migrated through the stacking gel, followed by 90-100 V until the tracking dye reached the bottom of the gel. After electrophoresis, gels were fixed in acetic acid:methanol:water (10:40:50), stained overnight with 0.1% Coomassie Brilliant Blue R-250, and destained until clear background and visible protein bands were obtained.

Statistical Analysis

The data related to biofunctional properties of ultrafiltrates (casein hydrolysate fraction) were analysed using Completely Randomized Design (Steel and Torrie, 1980)^[36].

Results

Proximate Composition of Casein

The casein prepared by acid precipitation of Kankrej cow milk was analysed for its proximate composition, and the moisture, fat, protein, and ash contents were found to be 4.54%, 1.97%, 83.29%, and 2.33%, respectively.

Analysis of Permeates and Retentates

The casein hydrolysate obtained using trypsin enzyme from Kankrej cow milk casein exhibited antioxidant activity and ACE inhibitory activities. The casein hydrolysates which were separated from the unhydrolyzed casein by centrifugation are made up of peptides and polypeptides of varying molecular weight. Since, bioactive peptides typically contains 3-20 amino acid residues per molecule,

the hydrolysates obtained from Kankrej cow milk casein using trypsin at a 1:25 E:S ratio after 8 h of hydrolysis were subjected to sequential ultrafiltration using 10, 5 and 3 kDa molecular weight cut off membranes.

Permeates and retentates obtained after ultrafiltration were subsequently analysed for selected biofunctional properties and peptide content. The permeate fractions were also subjected to electrophoresis.

Peptide Content of Casein Ultrafiltrates

The peptide content of casein ultrafiltrate fractions was estimated by Lowry's technique. The result of analysis is presented in the Table 1.

Table 1: Peptide content of casein ultrafiltrates

Hydrolysate fraction	Peptide content (mg/ml)
10 kDa retentate	24.56±0.19
10 kDa permeate	22.83±0.09
5 kDa retentate	21.43±0.09
5 kDa permeate	21.16±0.09
3 kDa retentate	20.36±0.09
3 kDa permeate	10.10±0.09

Values are mean ± SD; n=4

The peptide content of 10, 5 and 3 kDa permeates was 22.83, 21.16 and 10.1 mg/ml, and that of corresponding retentates was 24.56, 21.43 and 20.36 mg/ml, respectively.

Biofunctional Properties of Permeates and Retentates

The permeates and retentates obtained after ultrafiltration were analysed for their antioxidant activity by ABTS, DPPH and FRAP assay as well as for ACE inhibitory activity.

ABTS radical scavenging activity of casein ultrafiltrates

The permeates and retentates obtained after ultrafiltration were analysed for ABTS radical scavenging activity and the results of analysis is presented in Table 2.

Table 2: ABTS radical scavenging activity of casein ultrafiltrates

Hydrolysate fraction	ABTS (μM TE/mg peptide)
10 kDa permeate	2421.2±34.24 ^c
10 kDa retentate	1956.62 ±31.79 ^a
5 kDa permeate	3416.94±38.95 ^c
5 kDa retentate	2335.88±24 ^b
3 kDa permeate	5524.79±51.0 ^f
3 kDa retentate	2549.87±38.95 ^d

Values are mean ± SD; n=4 Values with different superscripts within column differ significantly ($p<0.05$)

The ABTS radical scavenging activity of the 10, 5 and 3 kDa permeates was 2421, 3416, and 5524 μM TE/mg peptide, respectively while that of their corresponding retentates was 1956, 2335, and 2549 μM TE/mg peptide, respectively. It may be observed that the 3 kDa permeate of casein ultrafiltrates showed the highest ABTS radical scavenging activity of 5524 μM TE/mg peptide among all permeates and retentates. Also it may be noted that among all the fractions ABTS radical scavenging activity of permeates were higher than their respective retentates and the difference among them was statistically significant ($p<0.05$). This study revealed that the low molecular weight peptides have superior antioxidant properties which is in consistent with the findings of Mendis *et al.* (2005) [21]. Jeon

(1999) [13], also quoted that the peptide's molecular weight had a significant impact on their antioxidant properties.

DPPH radical scavenging activity of casein ultrafiltrates

The permeates and retentates obtained after ultrafiltration of casein hydrolysates were analysed for DPPH radical scavenging activity and the results of analysis is presented in Table 3. The 10, 5 and 3 kDa permeates of casein hydrolysate ultrafiltrate fractions gave DPPH radical scavenging activity of 59.70, 65.95, and 67.57 per cent inhibition, respectively while those of corresponding retentates gave 58.26, 61.84, and 62.93 per cent of inhibition, respectively. It may be observed that the 3 kDa permeate of casein ultrafiltrates showed the highest DPPH radical scavenging activity of 67.57 per cent of inhibition among all permeates and retentates. Also it may be noted that among all fractions, DPPH radical scavenging activity of permeates were higher than their respective retentates and difference among them was statistically significant ($p<0.05$).

Table 3: DPPH radical scavenging activity of casein ultrafiltrates

Hydrolysate fraction	DPPH (% inhibition)
10 kDa permeate	59.70±0.46 ^b
10 kDa retentate	58.26 ±0.28 ^a
5 kDa permeate	65.95±0.61 ^e
5 kDa retentate	61.84±0.28 ^c
3 kDa permeate	67.57±0.45 ^f
3 kDa retentate	62.93±0.36 ^d

Values are mean ± SD; n=4 Values with different superscripts within column differ significantly ($p<0.05$)

Ferric Reducing Antioxidant Power (FRAP) of casein ultrafiltrates

The antioxidant potential of permeates and retentates of casein ultrafiltrates obtained from Kankrej cow milk casein were analysed by FRAP assay and the results of analysis is presented in Table 4. The FRAP assay values of the 10, 5 and 3 kDa permeates were 207, 235, and 510 μM Fe²⁺/mg peptide, respectively while that of their corresponding retentates were 186, 223, and 242 μM Fe²⁺/mg peptide, respectively. It may be observed that the 3 kDa permeate of casein ultrafiltrates gave the highest value of 510 μM Fe²⁺/mg peptide on analysis by FRAP assay inhibition among all permeates and retentates. Also it may be noted that among all samples, FRAP values of permeates were higher than their respective retentates and difference among them was statistically significant ($p<0.05$).

Table 4: Ferric reducing antioxidant power of casein ultrafiltrates

Hydrolysate fraction	FRAP (μM Fe ²⁺ /mg peptide)
10 kDa permeate	207.39±1.76 ^b
10 kDa retentate	186.68 ±2.34 ^a
5 kDa permeate	235.47±1.14 ^d
5 kDa retentate	223.90±1.49 ^c
3 kDa permeate	510.35±3.26 ^f
3 kDa retentate	242.41±1.20 ^e

Values are mean ± SD; n=4 Values with different superscripts within column differ significantly ($p<0.05$)

FRAP values of permeates obtained from Kankrej cow milk casein on hydrolysis with trypsin increased with decreasing molecular weight.

ACE inhibitory Activity of Casein ultrafiltrates

The permeates and retentates obtained after ultrafiltration of casein hydrolysates were analysed for ACE inhibitory activity and the results of analysis is presented in Table 5.

Table 6: ACE inhibitory activity of casein ultrafiltrates

Hydrolysate fraction	ACE inhibitory activity (%)
10 kDa permeate	34.28±0.96 ^c
10 kDa retentate	26.58±0.36 ^a
5 kDa permeate	39.12±0.27 ^c
5 kDa retentate	31.74±0.55 ^b
3 kDa permeate	46.98±0.60 ^f
3 kDa retentate	34.44±0.55 ^d

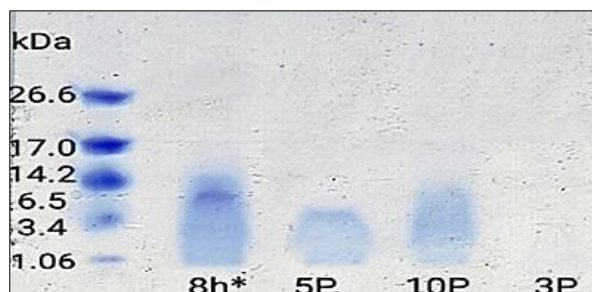
Values are mean ± SD; n=4 Values with different superscripts within column differ significantly ($p<0.05$)

The ACE inhibitory activity of the 10, 5 and 3 kDa permeates was 34.28, 39.12, and 46.98 per cent, respectively, while that of their corresponding retentates was 26.58, 31.74, and 34.44 per cent, respectively.

It may be observed that the 3 kDa permeate of casein ultrafiltrates showed the highest ACE inhibitory activity of 46.98 per cent among all permeates and retentates. Also it may be noted that among all samples, ACE inhibitory activity of permeates were higher than their respective retentates and difference among them was statistically significant ($p<0.05$).

Characterization of Casein Ultrafiltrates by Electrophoresis

In this study casein hydrolysate obtained from the Kankrej cow milk casein by trypsin enzyme (E:S of 1:25) hydrolysis for 8 h at 37°C were subjected to sequential ultrafiltration using 10, 5 and 3 kDa membranes. The casein hydrolysate (8h) along with its different ultrafiltrate permeate fractions were subjected to electrophoresis to access their molecular weight. Figure 4.3 clearly shows that the molecular weight of each permeates fractions was within the range. The casein hydrolysate obtained after 8 h (8h*) hydrolysis showed protein bands in the range of 14.2 to 1.06 kDa. In contrast, its 10 (10P) and 5 kDa (5P) permeate fractions exhibited protein bands below the 10 kDa and 5 kDa, molecular weight respectively in a permeate (3P) could not be detected by Coomassie blue stain. reference to molecular marker (ladder from 26.6 kDa to 1.06 kDa). The bands of 3 kD



Discussion

Ultrafiltration of Kankrej cow milk casein hydrolysates produced permeate and retentate fractions with decreasing peptide content as molecular weight cut-off decreased, with the 3 kDa permeate showing the lowest peptide concentration, similar to earlier reports on bovine and Deoni cow milk casein hydrolysates (Brunda, 2020; Mor *et al.*,

2021) [4, 24]. Despite lower peptide content, the antioxidant activities measured by ABTS, DPPH, and FRAP assays were significantly higher in low-molecular-weight permeates, particularly the 3 kDa fraction, indicating that bioactivity is primarily governed by peptide size and composition rather than concentration. Comparable trends have been reported for casein hydrolysates obtained from buffalo, bovine, camel, and goat milk using different proteolytic enzymes (Parmar *et al.*, 2015; Nichal, 2015; Mor *et al.*, 2020; Qiao *et al.*, 2022) [29, 27, 23, 30].

ABTS radical scavenging activity of casein ultrafiltrates

This study revealed that the low molecular weight peptides have superior antioxidant properties which is in consistent with the findings of Mendis *et al.* (2005) [21]. Jeon (1999) [13], also quoted that the peptide's molecular weight had a significant impact on their antioxidant properties.

Rao *et al.* (2020) [31] reported that the 10 kDa and 3 kDa permeates of casein hydrolysate obtained by flavorzyme enzyme showed radical scavenging activity of 1258.40 and 1271.38 Trolox eq./mg peptide respectively.

Brunda (2020) [4] reported that the 3 kDa permeate of casein ultrafiltrates obtained by combination of protease K and flavorzyme showed 3380.74 μ M TE/mg peptide and was highest among all the permeates and retentates (10, 5 and 3 kDa ultrafiltrate fractions).

The findings of this study are in agreement with the studies of Nichal (2015) [27], Rao *et al.* (2016) [32] and Brunda (2020) [4] who reported that the antioxidant activity of peptides are influenced by their molecular weight with lower molecular weight peptides giving higher ABTS radical scavenging activity.

DPPH radical scavenging activity of casein ultrafiltrates

The results of this analysis showed that 3 kDa permeates with the lowest molecular weight peptides gave highest DPPH radical scavenging activity among all ultrafiltrate fractions. Irshad *et al.* (2015) [11] observed that the 10 kDa and 1 kDa permeates of casein hydrolysate obtained by trypsin enzyme hydrolysis showed 17.3 and 24.4 per cent of DPPH radical scavenging activity after 4 h of hydrolysis period.

Nichal (2015) [27] observed that the 10 kDa and 3 kDa permeates of casein hydrolysate obtained by alcalase enzyme exhibited DPPH radical scavenging activity of 3.78 and 5.50 μ M TE/mg protein while those obtained by flavorzyme gave activity of 4.03 and 5.50 μ M TEAC/mg protein, respectively. Mor *et al.* (2020) [23] reported that the 10, 5 and 3 kDa permeates of casein hydrolysate obtained by flavorzyme enzyme displayed DPPH radical scavenging activity of 2.8, 3.29, and 4.02 μ M TE/mg protein, respectively while that of their respective retentates were 2.32, 2.54, and 3.28 μ M TE/mg protein, respectively.

The results of this analysis is in agreement with the reports of Nichal (2015) [27] and Mor *et al.* (2020) [23] who made similar findings.

Ferric Reducing Antioxidant Power (FRAP) of casein ultrafiltrates

FRAP values of permeates obtained from Kankrej cow milk casein on hydrolysis with trypsin increased with decreasing molecular weight. Parmar *et al.* (2015) [29] reported that the 3 kDa ultrafiltrate fractions obtained by hydrolysing the buffalo milk casein with alcalase enzyme (2.0 nkat/mg

protein at pH 7.5 at 50 °C/3 h) showed 78 µM TE/mg protein of radical scavenging activity when assayed by FRAP method. Nichal (2015) [27] observed that the 10 kDa and 3 kDa permeates of casein hydrolysate obtained by alcalase enzyme showed antioxidant potential of 77 and 75 µM TEAC/ mg peptide for FRAP assay while those obtained by flavorzyme gave FRAP values of 81 and 90.55 µM TEAC/mg peptide, respectively. The antioxidant activity of permeates and retentates of ultrafiltrate fractions of Kankrej cow milk casein hydrolysates obtained using 10, 5, and 3 kDa molecular cut off membranes differed significantly ($p < 0.05$) when analysed by ABTS, DPPH and FRAP assay methods. Irrespective of the antioxidant assay method, the 3 kDa permeate of casein hydrolysate showed the higher antioxidant activity. These findings are in agreement with the studies of various workers who reported that the antioxidant activities of peptides were highly influenced by their molecular weight and the low molecular weight peptides had better antioxidant activity.

ACE inhibitory activity of casein ultrafiltrates

In this study 3 kDa permeates exhibited highest ACE inhibitory activity. De Oliveira *et al.* (2020) [5] reported that the 1 kDa ultrafiltrate fractions of bovine milk casein hydrolysate obtained using trypsin enzyme (E:S ratio of 1:150 for 2 h of hydrolysis period) showed 80.7 per cent of ACE inhibitory activity. Brunda (2020) [4] found that the 3 kDa permeate of casein ultrafiltrates obtained by hydrolysing Deoni cow milk casein with trypsin (E:S ratio of 1:25 for 8 h of hydrolysis period) showed 38.86 per cent inhibition and was highest among all the permeates and retentates (retentates of 10, 5 and 3 kDa ultrafiltrate fractions).

Qiao *et al.* (2022) [30] stated that 1 kDa permeate of casein ultrafiltrates obtained by hydrolysing goat milk casein with trypsin (0.4 per cent E: S (w/w) for 2.5 h) showed 71.19 per cent inhibition and was the highest among all the ultrafiltrate fractions. Lower molecular weight peptides derived from casein consistently demonstrate enhanced ACE inhibitory activity. This occurs because peptides with lower molecular weight (typically under 3 kDa) binds more effectively to the catalytic sites of ACE due to their compact size, which facilitates optimal interaction with the enzyme's active pockets. Hydrophobic residues like valine, proline, or phenylalanine at terminal positions further strengthen this binding, significantly boosting inhibitory potency. Studies confirmed that enzymatic hydrolysis of casein to produce low molecular weight fragments is a key strategy for maximizing antihypertensive effects (FitzGerald *et al.*, 2004) [17].

Characterization of Casein Ultrafiltrates by Electrophoresis

In the present study, the ultrafiltrate permeates fraction when subjected to Tricine-SDS PAGE gave protein bands only for 10 and 5 kDa permeates.

Kundu (2013) [17] reported that the ultrafiltrates of buffalo casein phosphate hydrolysate obtained by trypsin hydrolysis for 12 h did not show any visible protein bands when subjected to Tricine-SDS PAGE. The researcher reasoned that it may be possible that hydrolysis resulted in the production of low molecular weight peptides (< 3 kDa) that cannot be depicted in electrophoresis plate or may be the concentration of high molecular peptide were too low to be

detected by Coomassie blue stain. Brunda (2020) [4] reported 10, 5, and 3 kDa permeates fractions of the casein ultrafiltrates of Deoni milk cow milk obtained by trypsin hydrolysis after 8 h gave protein bands less than their respective molecular weight when characterized by Tricine-SDS PAGE. Jodhani (2021) [14] also made similar findings that the 10 and 3 kDa permeates fraction ultrafiltrates of sheep milk fermented with *Lactobacillus plantarum* KGL3A, did not produced any protein bands less than 10 kDa when subjected to electrophoresis. The SDS-PAGE bands obtained in the study also similar observation is in agreement with the observations made by Kundu (2013) [17] and Jodhani (2021) [14].

Conclusion

The 10, 5 and 3 kDa ultrafiltrate fractions of the casein hydrolysate obtained by using trypsin enzyme at a E: S ratio of 1:25, at pH 8.0 after 8 h of hydrolysis period at 37°C exhibited biofunctional properties, namely antioxidant activity and ACE inhibitory activity. Among all fractions, the 3 kDa permeate showed the highest biofunctional activity. This study provides the first evidence of biofunctional peptide generation from Kankrej cow milk casein, highlighting its potential application in functional food and nutraceutical development.

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