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Nivedita PG

M.Tech Scholar, Department of Dairy Chemistry, ICAR–National Dairy Research Institute, Southern Regional Station, Bengaluru, Karnataka, India

Lakshman Naik

Principal Scientist, Department of Dairy Chemistry, ICAR–National Dairy Research Institute, Southern Regional Station, Bengaluru, Karnataka, India

Balisana Kamyabali

M.Tech Scholar, Department of Dairy Chemistry, ICAR–National Dairy Research Institute, Southern Regional Station, Bengaluru, Karnataka, India

Antioxidant activity of trypsin-derived β -casein hydrolysates from Deoni and Malnad *Gidda* cattle milk

Nivedita PG¹, Lakshman Naik², Balisana Kamyabali³

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ABSTRACT

Milk-derived bioactive peptides have gained increasing attention because of their antioxidant potential and their applications in functional foods. β -Casein hydrolysates exhibited high antioxidant activity in both breeds. This study evaluated the antioxidant activity of trypsin-derived β -casein hydrolysates prepared from the milk of Deoni and Malnad *Gidda* indigenous cattle breeds. β -Casein was isolated from whole casein obtained from pooled milk samples and hydrolysed with trypsin under optimized conditions (enzyme-to-substrate ratio 1:25, pH 8.0, 37 °C) for 0, 8, and 12 h. The antioxidant activity of the resulting hydrolysates was assessed using ABTS and DPPH radical scavenging assays and expressed as Trolox equivalent antioxidant capacity (TEAC). Among the casein fractions, β -casein exhibited the highest antioxidant activity in both breeds. Enzymatic hydrolysis markedly enhanced antioxidant activity, reaching its maximum after 8 h of hydrolysis. The ABTS radical scavenging activity increased from 1302.2 to 2972.5 $\mu\text{M TE mg}^{-1}$ protein in Deoni hydrolysates and from 1534.2 to 3202.4 $\mu\text{M TE mg}^{-1}$ protein in Malnad *Gidda* hydrolysates. Likewise, DPPH radical scavenging activity increased from 10.59 to 19.7 $\mu\text{M TEAC mg}^{-1}$ protein in Deoni and from 10.66 to 21.1 $\mu\text{M TEAC mg}^{-1}$ protein in Malnad *Gidda* hydrolysates. A slight decline in antioxidant activity was observed after 12 h of hydrolysis in both breeds. Overall, β -casein hydrolysates from Malnad *Gidda* cattle showed higher antioxidant activity than those from Deoni cattle. These findings demonstrate that controlled trypsin hydrolysis effectively enhances the antioxidant potential of β -casein and highlight the potential of milk from indigenous cattle breeds as a valuable source of natural antioxidant peptides for the development of functional dairy ingredients.

Keywords: Antioxidant activity, B-casein, ABTS, DPPH, Deoni, Malnad *Gidda*

Introduction

Milk proteins are widely recognized as a valuable source of bioactive peptides that can be released during gastrointestinal digestion, fermentation, or controlled enzymatic hydrolysis. These peptides exhibit a wide range of biological activities, including antioxidant, antihypertensive, antimicrobial, immunomodulatory, and mineral-binding properties, making them attractive ingredients for functional foods and nutraceuticals (Clare and Swaisgood, 2000; Haque *et al.*, 2008; El-Sayed and Awad, 2019).

Caseins account for nearly 80% of the total protein in bovine milk and consist of four major fractions: αs_1 -, αs_2 -, β -, and κ -casein. These proteins contain encrypted peptide sequences that are released during enzymatic hydrolysis and are known to possess a variety of biological functions (Fox and McSweeney, 2003; Farrell *et al.*, 2004; Fox *et al.*, 2015) [7, 6].

Among the casein fractions, β -casein is particularly susceptible to proteolytic hydrolysis because of its flexible and amphiphilic structure. This characteristic makes it an excellent precursor for generating antioxidant peptides. Controlled hydrolysis using food-grade enzymes, especially trypsin, has been widely used to produce peptides with enhanced radical-scavenging activity and other beneficial functional properties (Gómez-Ruiz *et al.*, 2008; Hogan *et al.*, 2009; Espejo-Carpio *et al.*, 2018) [5].

Reactive oxygen species (ROS), which are continuously generated during normal cellular metabolism, can cause oxidative stress when produced in excess. Oxidative stress is associated with lipid peroxidation, protein oxidation, and cellular damage, all of which contribute to the development of several chronic diseases. Consequently, natural antioxidants derived from food proteins have received increasing attention as safer alternatives to synthetic antioxidants. The antioxidant potential of protein hydrolysates is influenced by several factors, including enzyme specificity, degree of hydrolysis, peptide size, and amino acid composition (Chen *et al.*, 1998; Becker *et al.*, 2004; He *et al.*, 2013).

Among the available methods for evaluating antioxidant activity, the ABTS and DPPH radical scavenging assays are widely used because they are simple, sensitive, and reproducible. These assays measure the ability of antioxidant compounds to neutralize stable free radicals, and the antioxidant capacity is commonly expressed as Trolox equivalent antioxidant capacity (TEAC) (Brand-Williams *et al.*, 1995; Re *et al.*, 1999; Becker *et al.*, 2004) [13].

Corresponding Author: Nivedita PG

M.Tech Scholar,
Department of Dairy Chemistry, ICAR–National Dairy Research Institute, Southern Regional Station, Bengaluru, Karnataka, India

Deoni and Malnad *Gidda* are two important indigenous cattle breeds known for their adaptability and the desirable nutritional quality of their milk. Although the antioxidant properties of milk-derived peptides have been investigated in several studies, comparative information on the antioxidant activity of trypsin-derived β -casein hydrolysates from these indigenous breeds is still limited (Brunda, 2019; Brodziak *et al.*, 2020) [3, 4]. Understanding the antioxidant potential of peptides derived from these breeds could support the development of functional dairy ingredients with enhanced health benefits.

Therefore, this study aimed to evaluate the antioxidant activity of trypsin-derived β -casein hydrolysates prepared from Deoni and Malnad *Gidda* cattle milk using ABTS and DPPH radical scavenging assays. The findings will contribute to understanding the antioxidant potential of β -casein-derived peptides from indigenous cattle breeds and their possible application as natural functional food ingredients.

Materials and Methods

Milk samples and preparation of β -casein hydrolysates

Pooled milk samples from Deoni and Malnad *Gidda* cattle maintained at the ICAR–National Dairy Research Institute, Southern Regional Station, Bengaluru, were used for this study. Whole casein was isolated from skim milk by isoelectric precipitation, followed by purification of β -casein using the urea fractionation method. The purified β -casein was hydrolysed with trypsin under optimized conditions at an enzyme-to-substrate ratio of 1:25, pH 8.0, and 37 °C for 0, 8, and 12 h. The enzymatic reaction was terminated by heating the hydrolysates at 90 °C for 10–15 min. The samples were then centrifuged to obtain peptide-rich supernatants, which were used for antioxidant analysis. The hydrolysis conditions were selected based on the optimization study described previously.

Determination of antioxidant activity by ABTS radical scavenging assay

The antioxidant activity of the β -casein hydrolysates was evaluated using the ABTS [2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)] radical scavenging assay following the method described by Re *et al.* (1999) [13]. A Trolox standard curve (50–250 μ M) was prepared, and appropriately diluted hydrolysate samples were reacted with the ABTS working solution. After 10 min of incubation, the absorbance was measured at 734 nm. Antioxidant activity was expressed as Trolox equivalent antioxidant capacity (TEAC; μ M TE mg^{-1} protein).

Determination of antioxidant activity by DPPH radical scavenging assay

DPPH radical scavenging activity was determined according to the method of Mao *et al.* (2011). A Trolox standard curve (10–80 μ M) was prepared, and the hydrolysate samples were mixed with 0.2 mM DPPH solution. The reaction mixtures were incubated at 37 °C for 30 min, and absorbance was recorded at 517 nm using an ELISA plate reader. The antioxidant activity was calculated from the Trolox standard curve and expressed as μ M TEAC mg^{-1} protein.

Statistical analysis

The experimental data were analysed using one-way analysis of variance (ANOVA) with SAS software (Version 9.3; SAS Institute Inc., Cary, NC, USA). Results are presented as the mean of three independent laboratory determinations ($n = 3$), and differences were considered statistically significant at $P < 0.05$.

Results and Discussion

Antioxidant activity of β -casein hydrolysates by ABTS radical scavenging assay

The antioxidant activity of trypsin-derived β -casein hydrolysates from Deoni and Malnad *Gidda* cattle milk, measured by the ABTS radical scavenging assay, is presented in Table 1. Enzymatic hydrolysis markedly improved the antioxidant activity of β -casein in both breeds. The antioxidant capacity increased with hydrolysis time,

reached its highest level after 8 h, and showed a slight decline after 12 h of hydrolysis.

Table 1. ABTS radical scavenging activity (μ M TE mg^{-1} protein) of trypsin-derived β -casein hydrolysates

Hydrolysis time (h)	Deoni (μ M TE mg^{-1} protein)	Malnad <i>Gidda</i> (μ M TE mg^{-1} protein)
0	1302.2	1534.2
8	2972.5	3202.4
12	2954.0	2600.4

Values are expressed as the mean of three independent laboratory determinations ($n = 3$).

In Deoni β -casein hydrolysates, ABTS radical scavenging activity increased from 1302.2 μ M TE mg^{-1} protein in the unhydrolysed sample to 2972.5 μ M TE mg^{-1} protein after 8 h of hydrolysis and remained relatively high (2954.0 μ M TE mg^{-1} protein) after 12 h. A similar trend was observed in Malnad *Gidda* hydrolysates, where antioxidant activity increased from 1534.2 μ M TE mg^{-1} protein before hydrolysis to 3202.4 μ M TE mg^{-1} protein after 8 h, followed by a slight decrease to 2600.4 μ M TE mg^{-1} protein at 12 h.

The increase in antioxidant activity during hydrolysis is likely due to the release of low-molecular-weight peptides capable of donating electrons or hydrogen atoms to neutralize ABTS free radicals. The highest antioxidant activity recorded after 8 h coincided with the optimum degree of hydrolysis, suggesting that this incubation period favoured the formation of peptides with greater antioxidant potential. Similar observations have been reported by Peña-Ramos *et al.* (2001) [12], who showed that antioxidant activity is closely related to the degree of hydrolysis and is influenced by enzyme specificity and hydrolysis conditions. Enhanced antioxidant activity following trypsin hydrolysis has also been reported for milk protein hydrolysates by Irshad *et al.* (2013) [8], Kumar *et al.* (2016), and Brunda (2019) [3].

A slight reduction in antioxidant activity after 12 h of hydrolysis was observed in both breeds. This may be attributed to excessive proteolysis, which can further degrade bioactive peptides into smaller fragments with lower radical scavenging ability. Similar findings were reported by Vastag *et al.* (2010) [15], who suggested that prolonged hydrolysis beyond the optimum incubation period may reduce antioxidant activity because of the breakdown of active peptide sequences.

Among the two breeds, β -casein hydrolysates from Malnad *Gidda* consistently exhibited higher ABTS radical scavenging activity than those from Deoni, particularly after 8 h of hydrolysis. This suggests that breed-related differences in β -casein composition may influence the release of antioxidant peptides during enzymatic hydrolysis.

Antioxidant activity of β -casein hydrolysates by DPPH radical scavenging assay

The DPPH radical scavenging activity of trypsin-derived β -casein hydrolysates from Deoni and Malnad *Gidda* cattle milk is presented in Table 2. Similar to the ABTS assay, antioxidant activity increased markedly after enzymatic hydrolysis, with the highest values recorded after 8 h.

Table 2. DPPH radical scavenging activity (μ M TEAC mg^{-1} protein) of trypsin-derived β -casein hydrolysates

Hydrolysis time (h)	Deoni (μ M TEAC mg^{-1} protein)	Malnad <i>Gidda</i> (μ M TEAC mg^{-1} protein)
0	10.59	10.66
8	19.70	21.10
12	19.40	18.40

Values are expressed as the mean of three independent laboratory determinations ($n = 3$).

In Deoni β -casein hydrolysates, DPPH radical scavenging activity increased from 10.59 μ M TEAC mg^{-1} protein before hydrolysis to

19.70 μM TEAC mg^{-1} protein after 8 h, and remained almost unchanged (19.40 μM TEAC mg^{-1} protein) after 12 h. Malnad *Gidda* hydrolysates followed a similar pattern, with antioxidant activity increasing from 10.66 μM TEAC mg^{-1} protein in the unhydrolysed sample to 21.10 μM TEAC mg^{-1} protein after 8 h, before declining slightly to 18.40 μM TEAC mg^{-1} protein at 12 h.

The improvement in DPPH radical scavenging activity reflects the formation of peptides enriched with hydrophobic and aromatic amino acid residues that readily donate hydrogen atoms to stabilize DPPH radicals. The maximum activity observed after 8 h indicates that controlled hydrolysis promoted the generation of peptides with enhanced antioxidant properties. Similar trends have been reported for bovine casein hydrolysates produced using trypsin and other proteolytic enzymes (Irshad *et al.*, 2013; Kumar *et al.*, 2016; Brunda, 2019) [8, 3].

The slight decline in antioxidant activity after prolonged hydrolysis suggests that excessive proteolysis may alter peptide composition and reduce the concentration of bioactive antioxidant sequences. Peña-Ramos *et al.* (2001) [12] also reported that the antioxidant properties of protein hydrolysates are influenced not only by the degree of hydrolysis but also by peptide size and amino acid composition.

Comparison of the two indigenous breeds showed that Malnad *Gidda* β -casein hydrolysates exhibited slightly higher DPPH radical scavenging activity than those from Deoni at the optimum hydrolysis time. This finding further supports the potential of Malnad *Gidda* milk as a source of antioxidant peptides and highlights the suitability of β -casein hydrolysates from indigenous cattle for use as natural antioxidant ingredients in functional dairy products.

Conclusion

This study showed that trypsin hydrolysis effectively enhanced the antioxidant activity of β -casein isolated from the milk of Deoni and Malnad *Gidda* cattle. Both the ABTS and DPPH assays demonstrated a steady increase in antioxidant activity with increasing hydrolysis time, reaching the highest values after 8 h of enzymatic hydrolysis. A slight decline in activity after 12 h suggests that prolonged hydrolysis may lead to further breakdown of bioactive peptides, reducing their antioxidant potential.

β -Casein hydrolysates from Malnad *Gidda* cattle consistently exhibited higher antioxidant activity than those from Deoni cattle, indicating a greater capacity for generating antioxidant peptides. Overall, the findings highlight the potential of β -casein from indigenous cattle milk as a natural source of antioxidant peptides with possible applications in functional foods and nutraceuticals. In addition, this study provides useful baseline information that supports the value addition and wider utilization of milk from indigenous cattle breeds in the development of health-promoting dairy products.

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