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Isolation of β -casein and its enzymatic hydrolysis by trypsin from Deoni and Malnad *Gidda* cattle milk

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ABSTRACT

Milk proteins are well known as natural sources of bioactive peptides with a wide range of nutritional and functional properties. Among the different casein fractions, β -casein is considered an ideal substrate for enzymatic hydrolysis because of its favourable amino acid composition. This study aimed to isolate β -casein from the milk of Deoni and Malnad *Gidda* cattle and evaluate its hydrolysis using trypsin. Fresh pooled milk samples from both breeds were defatted, and whole casein was isolated by isoelectric precipitation. β -Casein was then separated using the differential urea solubility method, followed by purification and freeze-drying. Enzymatic hydrolysis was carried out under controlled conditions using trypsin, and the extent of hydrolysis was monitored by measuring the degree of hydrolysis at different incubation intervals. In both breeds, the degree of hydrolysis increased steadily with incubation time, reaching its highest value after 8 h, with only minor changes thereafter. Protein concentration showed minor changes during hydrolysis and gradually increased after the initial stage, reflecting the efficient breakdown of β -casein into soluble peptide fractions. Overall, the results demonstrate that trypsin effectively hydrolysed β -casein from both Deoni and Malnad *Gidda* cattle, producing peptide-rich hydrolysates suitable for further evaluation of their functional properties. This study provides a reliable approach for preparing β -casein hydrolysates from indigenous cattle breeds and offers a foundation for their potential use in the development of functional foods and value-added dairy ingredients.

Keywords: Bioactive peptides, degree of hydrolysis, Deoni cattle, enzymatic hydrolysis, Malnad *Gidda*

Introduction

Milk proteins are an important source of essential amino acids and biologically active compounds that contribute to human nutrition and overall health. Among these proteins, caseins account for nearly 80% of the total protein in bovine milk and play a key role in determining its nutritional, functional, and technological properties (Fox *et al.*, 2003; Fox *et al.*, 2015) [1, 2]. Casein is composed of four major fractions— α ₁-, α ₂-, β -, and κ -casein—each having distinct physicochemical and functional characteristics that influence the quality and processing behaviour of milk (Farrell *et al.*, 2004) [3]. Among these fractions, β -casein has attracted considerable interest because of its high nutritional value and its ability to release biologically active peptides during enzymatic hydrolysis (Clare and Swaisgood, 2000; Korhonen and Pihlanto, 2006) [4]. Enzymatic hydrolysis is widely used to produce bioactive peptides from milk proteins. During this process, proteolytic enzymes such as trypsin cleave specific peptide bonds, generating low-molecular-weight peptides with improved solubility and enhanced biological activities (Adler-Nissen, 1986; Banach *et al.*, 2013) [5, 6]. The extent of protein breakdown is commonly assessed by the degree of hydrolysis, an important parameter that influences the physicochemical, nutritional, and functional properties of the resulting peptide fractions (Adler-Nissen, 1986; Espejo-Carpio *et al.*, 2018) [5, 7]. Consequently, controlled enzymatic hydrolysis has become an effective approach for producing functional dairy ingredients with potential applications in health-promoting foods.

India is home to a rich diversity of indigenous cattle breeds that are well adapted to tropical climatic conditions and are known for producing milk with desirable nutritional characteristics. Among these, Deoni and Malnad *Gidda* are two important indigenous breeds of southern India. Previous studies have shown that milk from these breeds contains comparatively higher protein levels than that of many crossbred cattle and predominantly carries the A2 β -casein variant, making it a promising source for the production of bioactive peptides (Naik *et al.*, 2013; Ramesha *et al.*, 2016; Thimmesh, 2018). In addition, the unique protein composition of indigenous cattle milk has been associated with favourable functional properties and growing interest in its potential applications in functional dairy products (Brodziak *et al.*, 2020) [8].

Although the general composition of milk from indigenous cattle has been reported, information on the isolation of β -casein and its controlled enzymatic hydrolysis in Deoni and Malnad *Gidda* cattle remains limited. Understanding the hydrolysis behaviour of β -casein is an important first step in producing peptide-rich hydrolysates for the evaluation of their biological and functional properties.

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Therefore, this study aimed to isolate β -casein from the milk of Deoni and Malnad *Gidda* cattle and evaluate its enzymatic hydrolysis using trypsin. The progress of hydrolysis was monitored by measuring the degree of hydrolysis and protein content of the resulting hydrolysates, providing a foundation for future studies on the functional potential of β -casein-derived bioactive peptides.

Materials and Methods

Study location

The study was carried out at the Southern Regional Station (SRS), ICAR–National Dairy Research Institute (NDRI), Bengaluru, Karnataka, India. All laboratory analyses were performed in the Department of Dairy Chemistry.

Collection of milk samples

Fresh pooled whole milk samples were collected from healthy lactating Deoni and Malnad *Gidda* cows maintained at the Livestock Research Centre (LRC), SRS, ICAR–NDRI, Bengaluru. A total of 10 pooled milk samples were collected from each breed. Each pooled Deoni sample represented milk obtained from 10 healthy lactating cows, whereas each pooled Malnad *Gidda* sample represented milk from 5 healthy lactating cows. All animals were maintained under identical feeding and management conditions. Immediately after collection, the milk samples were cooled to approximately 4 °C, transported to the laboratory under refrigerated conditions, and centrifuged to obtain skim milk for subsequent casein isolation.

Isolation of Whole Casein

Whole casein was isolated from skim milk by isoelectric precipitation at pH 4.6. The precipitated casein was recovered by centrifugation, washed repeatedly with distilled water to remove residual lactose and whey proteins, dialysed against distilled water, freeze-dried, and stored at 4 °C until further analysis (Fox *et al.*, 2003; Fox *et al.*, 2015) [1, 2].

Isolation of β -casein

β -Casein was separated from whole casein using the differential urea solubility method. The isolated β -casein was extensively dialysed against distilled water to remove residual urea, freeze-dried, and stored at 4 °C until enzymatic hydrolysis (Fox *et al.*, 2015) [2].

Enzymatic hydrolysis of β -casein

Purified β -casein isolated from Deoni and Malnad *Gidda* milk was hydrolysed using trypsin under controlled conditions. A 5% (w/v) β -casein solution was prepared and hydrolysed at an enzyme-to-substrate ratio of 1:25, pH 8.0, and 37 °C. Hydrolysis was carried out for 0, 2, 4, 6, 8, 10, and 12 h. The enzymatic reaction was terminated by heating the reaction mixture at 90 °C for 10–15 min. The resulting hydrolysates were used for the determination of degree of hydrolysis and protein concentration.

Determination of degree of hydrolysis

The degree of hydrolysis (DH) of the β -casein hydrolysates was determined using the *o*-phthalaldehyde (OPA) spectrophotometric method described by Nielsen *et al.* (2001) [9]. Hydrolysate samples collected at different incubation intervals were reacted with freshly prepared OPA reagent, and the absorbance was measured at 340 nm using a UV–Visible spectrophotometer. The degree of hydrolysis was expressed as the percentage of peptide bonds cleaved during enzymatic hydrolysis. L-serine and distilled water served as the positive and negative controls, respectively.

Protein estimation of β -casein hydrolysates

Protein concentration in the β -casein hydrolysates was determined by the Lowry method using bovine serum albumin (BSA) as the standard (Lowry *et al.*, 1951) [10]. The hydrolysed samples were centrifuged to obtain peptide-rich supernatants and diluted appropriately before analysis. An aliquot (0.2 mL) of each sample was mixed with 2.0 mL of freshly prepared alkaline reagent and allowed to stand at room tem-

perature for 10 min. Subsequently, 0.2 mL of 1 N Folin–Ciocalteu reagent was added, and the reaction mixture was incubated for 30 min at room temperature. Absorbance was measured at 750 nm using a UV–Visible spectrophotometer. Protein concentration was calculated from a BSA standard curve (25–250 $\mu\text{g mL}^{-1}$) and expressed as mg mL^{-1} of hydrolysate.

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 mean $\pm$ standard deviation (SD) of three independent experimental determinations ($n = 3$), and differences were considered statistically significant at $P < 0.05$.

Results and Discussion

Degree of hydrolysis of β -casein hydrolysates

The enzymatic hydrolysis of β -casein isolated from Deoni and Malnad *Gidda* cattle milk was carried out using trypsin to evaluate the extent of protein breakdown and the formation of peptide-rich hydrolysates. The degree of hydrolysis (DH), which reflects the extent of peptide bond cleavage during enzymatic digestion, is presented in Table 1.

Table 1. Degree of hydrolysis (%) of β -casein hydrolysates from Deoni and Malnad *Gidda* cattle milk during trypsin hydrolysis.

Hydrolysis time (h)	Deoni β -casein hydrolysates (%)	Malnad <i>Gidda</i> β -casein hydrolysates (%)
0	2.12 $\pm$ 1.20	1.06 $\pm$ 0.59
2	8.46 $\pm$ 0.87	9.55 $\pm$ 0.001
4	10.99 $\pm$ 0.46	11.16 $\pm$ 0.76
6	14.75 $\pm$ 0.087	11.96 $\pm$ 1.25
8	15.55 $\pm$ 0.60	15.80 $\pm$ 0.97
10	15.53 $\pm$ 0.98	15.70 $\pm$ 0.04
12	15.38 $\pm$ 0.54	15.60 $\pm$ 1.20

Values are expressed as mean $\pm$ SD of three independent laboratory determinations performed on pooled milk samples.

The degree of hydrolysis increased steadily with increasing incubation time in both breeds, indicating continuous enzymatic cleavage of β -casein by trypsin. At the beginning of the reaction (0 h), the DH values were 2.12 $\pm$ 1.20% for Deoni and 1.06 $\pm$ 0.59% for Malnad *Gidda* β -casein. Hydrolysis progressed gradually and reached maximum values after 8 h, recording 15.55 $\pm$ 0.60% and 15.80 $\pm$ 0.97%, respectively. Beyond this point, only minor changes were observed at 10 and 12 h, suggesting that the hydrolysis reaction had nearly reached completion.

The gradual increase in DH reflects the progressive cleavage of peptide bonds in β -casein, leading to the release of smaller peptides and free amino groups. The plateau observed after 8 h is likely due to the reduced availability of susceptible peptide bonds and the accumulation of hydrolysis products, which can limit further enzyme activity. Similar behaviour has been reported during trypsin-mediated hydrolysis of milk proteins, where the hydrolysis rate slows after prolonged incubation because of substrate depletion and product inhibition.

The maximum DH values obtained in this study are comparable with those reported by Kumar *et al.* (2016) [11], who observed a degree of hydrolysis of approximately 17% during trypsin hydrolysis of camel milk casein after 8 h. Likewise, Qi *et al.* (2007) [12] reported progressive changes in peptide size and molecular weight distribution during enzymatic hydrolysis of bovine casein, reflecting continuous cleavage of peptide bonds throughout the reaction. A similar trend was observed in the present study, with hydrolysis increasing steadily up to 8 h before reaching a plateau. Comparable observations have also been reported by Vastag *et al.* (2010) [13], who found that further hydrolysis beyond the optimum incubation period produced only marginal in

creases because most susceptible peptide bonds had already been cleaved.

Overall, the hydrolysis pattern was very similar for β -casein from both Deoni and Malnad *Gidda* cattle, indicating that trypsin hydrolysed the proteins from both breeds with comparable efficiency under the conditions employed.

Protein concentration of β -casein hydrolysates

The protein concentration of β -casein hydrolysates obtained during trypsin hydrolysis is presented in Table 2. Protein concentration was determined using the Lowry method after centrifugation to obtain peptide-rich supernatants. Samples collected at 0 h were analysed without centrifugation and served as the control.

Table 2. Protein concentration (mg mL^{-1}) of β -casein hydrolysates from Deoni and Malnad *Gidda* cattle milk during trypsin hydrolysis

Hydrolysis time (h)	Deoni β -casein hydrolysates (mg mL^{-1})	Malnad <i>Gidda</i> β -casein hydrolysates (mg mL^{-1})
0	36.00 ± 0.21	35.40 ± 1.20
2	33.90 ± 0.007	32.80 ± 0.50
4	34.30 ± 0.31	33.20 ± 0.79
6	34.90 ± 0.054	33.40 ± 0.043
8	35.20 ± 0.65	34.32 ± 0.42
10	35.40 ± 0.75	34.90 ± 0.08
12	35.60 ± 0.86	35.10 ± 0.73

Values are expressed as mean $\pm$ SD ($n = 3$ independent laboratory determinations).

Only slight changes in protein concentration were observed during the hydrolysis period. In Deoni β -casein hydrolysates, the protein concentration decreased initially from $36.00 \pm 0.21 \text{ mg mL}^{-1}$ at 0 h to $33.90 \pm 0.007 \text{ mg mL}^{-1}$ after 2 h, followed by a gradual increase to $35.60 \pm 0.86 \text{ mg mL}^{-1}$ at 12 h. A similar trend was observed in Malnad *Gidda* β -casein hydrolysates, where the protein concentration increased progressively from $32.80 \pm 0.50 \text{ mg mL}^{-1}$ at 2 h to $35.10 \pm 0.73 \text{ mg mL}^{-1}$ at the end of the hydrolysis period.

The gradual increase in protein concentration corresponded well with the increase in the degree of hydrolysis. As enzymatic digestion progressed, trypsin cleaved β -casein into smaller, soluble peptides that remained in the supernatant after centrifugation and were subsequently detected by the Lowry assay. This indicates efficient conversion of β -casein into peptide-rich hydrolysates throughout the hydrolysis process.

Similar observations have been reported for enzymatically hydrolysed milk proteins, where increasing hydrolysis results in greater concentrations of soluble peptides and improved functional properties. Overall, β -casein from both Deoni and Malnad *Gidda* cattle exhibited comparable patterns of protein release during hydrolysis, demonstrating that milk from both indigenous breeds is a suitable source for producing peptide-rich β -casein hydrolysates.

Conclusion

This study demonstrated that β -casein isolated from the milk of Deoni and Malnad *Gidda* cattle can be effectively hydrolysed using trypsin under controlled conditions. The degree of hydrolysis increased steadily with incubation time and reached its maximum after 8 h in both breeds, indicating efficient enzymatic breakdown of β -casein. A corresponding increase in the protein concentration of the hydrolysates was also observed, reflecting the progressive formation of soluble peptide-rich fractions during hydrolysis.

The similar hydrolysis patterns exhibited by β -casein from both Deoni and Malnad *Gidda* cattle indicate that the proteins respond comparably to trypsin digestion under the experimental conditions used. Overall, the results suggest that β -casein from both indigenous cattle breeds is a suitable substrate for producing peptide-rich hydrolysates. These findings provide a useful foundation for future studies aimed at evaluating the biological activities and functional properties of β -casein-derived peptides and their potential application in functional foods and value-added dairy products.

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