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STUDY OF PROTEOLYSIS IN CHEESE MADE FROM DEONI AND CROSSBRED MILK

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Abstract – The cheese made from Deoni and Crossbred milk were prepared and analysed for proteolysis. Soluble protein content of cheese made from Deoni milk was significantly higher (P<0.05) than cheese made from Crossbred milk at the end of 12M. The degradation of protein was more in Deoni milk Cheddar cheese compared to Crossbred milk Cheddar cheese throughout the ripening, which was revealed by RP-HPLC.

INTRODUCTION

Cheese contains high casein content which is the repository of several bioactive peptides, released by a number of proteases. During maturation, Proteolysis is the significantly notable and the most complex event (Cogan and Beresford, 2002). Especially in internal bacterial ripened cheeses, it is a key process in regulating the rate of flavour and texture development (Fox and Mc Sweeney, 2003). The primary proteolysis caused by the actions of milk enzymes (plasmin) and from rennet (pepsin and chymosin) (Forde and Fitz-Gerald, 2000). Casein Hydrolysis results in generation of peptides which are in large and medium size, further they are degraded by coagulants and enzymes obtained from starter and nonstarter bacteria. Small peptides are further degraded to free amino acids by Intracellular bacterial peptidases that are released after cellular lysis. (Sousa *et al*., 2001). Amino acids are broken down to amines, alcohols, aldehydes and ammonia, which give rise to aroma and flavor (Smit *et al*., 2005). During proteolysis, the breakdown of the protein network imparts changes in textural of cheese matrix and Amine and Carboxyl groups that are released leads to reduction in water activity by water molecules binding to it (Sousa *et al*., 2001)

The proteolytic index of matured cheeses is associated with bioactivities (Lopez Fandino *et al*., 2006). Recently, it has been recognised that various specific peptides that are released by enzymes or that are present in fermented foods may exert beneficial effects in vivo. Cheese and milk products have been found to be a rich source of peptides having positive effect on cardiovascular health, especially angiotensin-I converting enzyme inhibitors (ACE). The tripeptides, isoleucyl-prolyl-proline (Ile-Pro-Pro) and Valyl-prolyl-proline (Val-Pro-Pro), have been identified as antihypertensive agents, which inhibit the action of ACE (Ong and Shah, 2008). Numerous peptides derived from hydrolysed protein have also been shown to have antioxidant activity against the peroxidation of lipids and fatty acids. One of these antioxidant peptides, casein (f177-183) identified as β-casokinin. These two bio active peptides, i.e. antioxidative and ACE-inhibitory) are mainly present in ripened Cheddar cheese. Formation of these bioactive peptides is significantly affected by the nature of proteolysis in cheese which in turn is affected by starter culture and enzymatic activity.

MATERIALS AND METHOD

Materials

Cheddar cheese was made from Crossbred and Deoni cow milk obtained from dairy plant of ICAR-National Dairy Research Institute, Bengaluru. Cheddar cheese DVS culture was procured from

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Chr. Hansen, Denmark. Microbial rennet (FROMASE 2200 TL Granulate; derived from *Rhizomucor miehei*; milk clotting activity ≥ 2200 IMCU/g) was obtained from M/s DSM, Mumbai. All the chemicals and reagents used for chemical analyses were of AR grade.

Cheddar cheese manufacture

Preparation of Cheddar cheese was according to the standard protocol as suggested in Kosikowski and Mistry, (1997). For the preparation 50 litres of Crossbred and Deoni whole milk were standardized to get casein with fat ratio of 0.68- 0.70 with their respective skim milk and it was pasteurized at 63 °C for 30 min. The milk was then cooled to 30-31 °C and DVS culture of 0.9 g was added and incubated for 30 min. 1.5 g of microbial rennet was added for 50 litres of incubated milk. The curd was cut with cheese knives after 45 min of coagulation, and further cooked at 38-39 °C for 40 min. Whey was drained from the curd and was cheddared at 39 °C till it reached to 0.45-0.50 % lactic acid. Salt was added to the milled curd and hooped in cheese moulds, dressed and pressed overnight. The cheese blocks were turned to the other side, next day and pressed again for 24 h. These pressed cheese blocks were then removed and placed in the ageing room for next 2 days. Thus obtained surface dried cheese blocks were paraffined and aged for 12 months at 7± 2 °C. Cheese samples were collected at two month intervals for analysis.

Soluble Protein

The soluble protein content of cheese was determined as per the method described by Kosikowski (1982). In this procedure, the soluble nitrogen fraction was extracted in a suitable solvent and the nitrogen content was estimated same as for the total nitrogen.

Three grams of ground cheese was accurately weighed and mixed with a small amount of Sharpe's extraction solution (at 40 °C) in a mortar. The contents were grinded to a paste and transferred to a 100 ml volumetric flask. The volume was made up to the mark with Sharpe's extraction solution. The flask was kept in a water bath at 50 to 55 °C for one hour with occasional shaking. It was then filtered through Whatman filter paper No. 42. Fifty ml of filtrate was transferred to Kjeldahl tube containing some glass beads. Two grams of digestion mixture (K_2SO_4 : $CuSO_4$ = 100: 2) and 10 ml of concentrated sulphuric acid (AR) were added to the tube. It was then digested, distilled and titrated as was carried out for total protein (% N * 6.38)

Per cent protein was calculated as follows:

$$\text{Nitrogen (\%)} = \frac{1.4007 \times (A - B) \times N \times 100}{\text{Sample weight (g)}}$$

Where, Sample weight = 1.5 g (50% of the extract was distilled) A= Sample titre value, B= Blank titre value, N= Normality of H_2SO_4.

%Total Protein = % Nitrogen x 6.38 (6.38 is the conversion factor for milk and milk products)

Composition of Sharpe's stock solution

Glacial acetic acid- 57.50 ml, Sodium acetate -136.10 g, Sodium chloride - 47.00 g, Calcium chloride -8.90 g

Volume was made up to 1000 ml with distilled water.

Composition of Sharpe's extraction solution: Sharpe's stock solution of 250 ml was taken in a 1000 ml volumetric flask and the volume was made up to the mark with distilled water.

Reverse Phase High Performance Liquid Chromatography (RP-HPLC)

Reverse phase high performance liquid chromatography (RP-HPLC) is currently used in the separation and study of peptides. The retention of peptides and proteins in the column is a function of their relative hydrophobicity. Water soluble cheese extract of cheese was resolved on RP-HPLC. RP-HPLC of the water soluble cheese extract of cheese samples were carried out as per the procedure of Pritchard *et al*. (2010) with some modifications.

Sample preparation

Water soluble extracts (WSE) of the peptides formed in all Cheddar cheese were prepared using the method developed by Kuchroo and Fox (1982), with some modification. Grated Cheddar cheese of 20 g was mixed with 60 ml of distilled water in a small mixer. The mixtures were centrifuged at 4250 g for 30 min at 4 °C (cooling centrifuge, REMI, Mumbai). The fat layers were removed and the water extracts were filtered through Whatman No.1. The pH of the extracts was adjusted to 4.6 using 1N HCl. The precipitated proteins were removed by filtering through Whatman No.1. To further remove any impurities, the water-soluble extracts were filtered through a 0.22 µm pore size syringe driven filter. The water-soluble extract was kept in deep freeze and used for HPLC

Standard Preparation

Dissolve 1 mg of sample in 1 mL of Buffer A. If there is some undissolved material, filter the sample through a 0.22-µm filter.

Solvent Preparation

Filter all solvents through a 0.22-µm filter before use. This removes particulates that could block solvent lines or the column and also serves to degass the solvent. If the HPLC instrument is not installed with on-line degassing capability, check with your instrument requirements to assess whether further degassing is required.

Operating conditions

The mobile phase and other operating conditions used during the analysis were as follows:

Mobile Phase:

Buffer A	:	0.1% (v/v) TFA in water
Buffer B	:	100% CH_3CN containing 0.1% (v/v) TFA
Flow rate	:	1 ml/min
Wavelength	:	215nm
Temperature	:	Room Temperature
Injection Vol.	:	50 µl

Procedure

The HPLC column was equilibrated with the mobile phase till baseline stabilized.

Column Equilibration and Blank Run

1. Connect the guard and the column to the solvent delivery system according to the HPLC system requirements and equilibrate under the following initial conditions.
a. Solvent: 100% Buffer A
b. Flow rate: 1 ml/min
c. Detection wavelength: 215 nm
D. Temperature: Ambient
2. Once a stable baseline is obtained, inject 10 µl of Milli-Q water (either manually or via an automatic injector). It is generally advisable to perform two blank runs to ensure proper equilibration of the column.

Sample Injection and Analysis

Once a stable baseline is obtained, inject 50µl of the sample was injected and run with linear Gradient 0.2- 60% of solvent B to 50 min followed by 0.2% Solvent B to 55 min. The flow rate was adjusted to 1 ml/min and the bound peptides were eluted from the column at room temperature. Elutions of peptides were monitored at 215 nm and the peptide profiles of the samples were observed on the monitor.

Statistical analysis

The statistical analysis was executed using the statistical software SPSS 16.0. (Stat Soft Polska Sp. Z o. o., Kraków, Poland). Student's t-test and analysis of variance (ANOVA) with Tukey's post-hoc test was applied for testing the significance of difference between two or more treatments, respectively at 5% level of significance. Prism Graph-pad version 8.1.2 was used for graphical representation of the data.

RESULTS AND DISCUSSION

Changes in Soluble protein content of Cheddar cheese prepared from Crossbred and Deoni milk during maturation

The percentage of soluble protein indicates the extent of cheese maturation. The amount of soluble protein content in CMCC and DMCC on 0th day was 2.11 and 2.25%, respectively (Fig. 1)

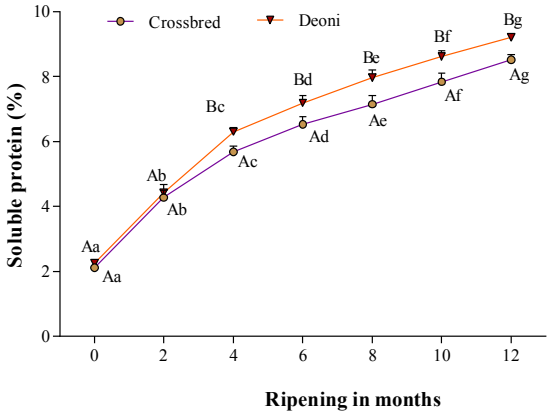

Fig. 1. Effect of type of Milk on soluble protein of Cheddar cheese during maturation

Results are expressed as Mean ± S.D.; means with different Capital letter superscripts (A,B,) differ significantly ($p<0.05$) between the Cheddar cheeses (columns); means with different small letter superscripts (a, b,..) differ significantly ($p<0.05$) across the ripening months (rows); n=3.

The gradual non-significant ($p > 0.05$) increase in soluble protein in both the cheeses during initial 2 months of maturation may be attributed to the action of rennet. The soluble protein content increased significantly ($p < 0.05$) in both the cheeses from 4th month onwards of maturation and it

reached to 8.51 and 9.21 % in CMCC and DMCC, respectively at the end of 12 months. The amount of soluble protein was about 37.97 and 39.10 % of total protein in CMCC and DMCC, respectively. The progressive elevation of soluble protein content at various stages of ripening had also been noticed in many studies (Meira *et al.*, 2012). Overall, the concentration of soluble protein was relatively higher in DMCC compared to CMCC during ripening due to higher content of casein in Indigenous milk than Crossbred milk. Higher content of soluble protein in DMCC showed that soluble peptides formed by various proteolytic enzymes like plasmin, chymosin, starter and non-starter proteases, peptidase are mainly responsible for this increase in soluble protein (Rynne *et al.*, 2004) and rapid rates in DMCC during ripening due to also higher casein content. Higher proteolysis in Cheddar cheese made from buffalo milk compared to cow milk has been reported due to higher casein content in buffalo milk (Imtiaz *et al.*, 2012).

Changes in peptide profile of Cheddar cheese made from Crossbred and Deoni milk

Proteolysis pattern and peptide profiling in cheeses during ripening were assessed using RP-HPLC.

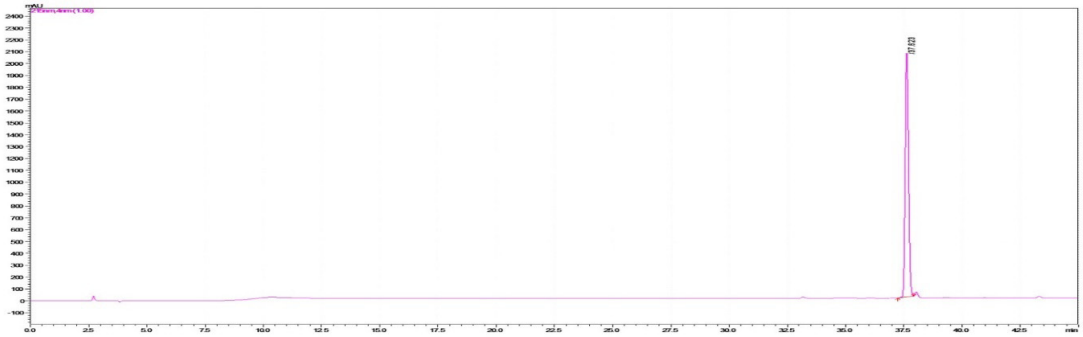

Fig. 2. RP-HPLC chromatograms of Standard human insulin

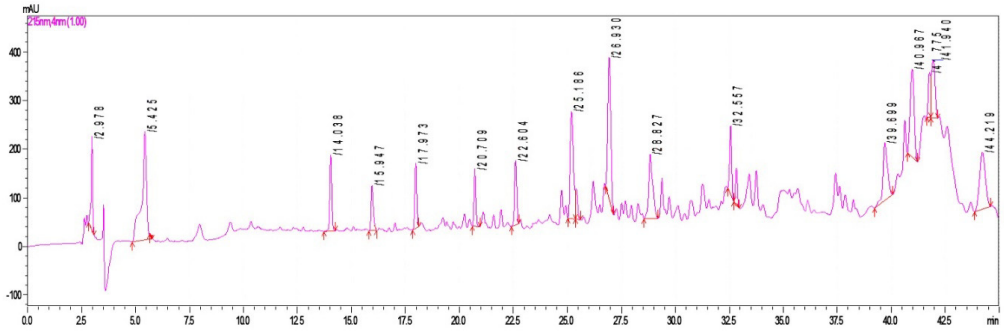

Fig. 3 (a)

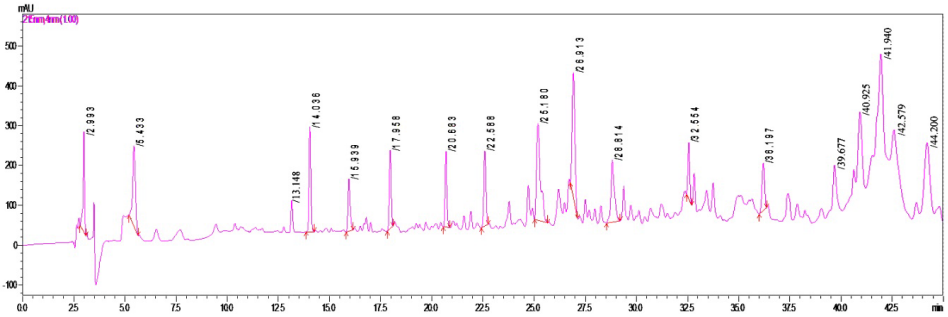

Fig. 3 (b)

Fig. 3. RP-HPLC chromatograms peptide profile of fresh Cheddar cheese made from (a) Crossbred milk and (b) Deoni milk

Total peptide analyses were carried out for 55 min, but a 37.58-minute run was taken into account as a reference standard of Human insulin with molecular weight ~ 6 kDa eluted at a retention period of 37.58 min (Fig. 2). It was assumed that all peptides eluted prior to 37.58 min could be the peptides of lower molecular weight less than 6 kDa. Comparison was shown between the RP-HPLC chromatograms of cheese made from Crossbred and Deoni milk (Fig. 3 a and b for fresh Cheddar cheese, Fig. 4 a and b at 6M)

Peptide profiles between the cheese made from crossbred and Deoni milk displayed identical patterns on 0th day, but changed with the progression of maturation, suggesting that with further proteolysis and differences existed in peptide profiles between them. As maturation progressed, a substantial increase in the amount and concentration of peptides for both cheeses was noted. However, there were marked differences between cheeses made from Deoni milk as compared with crossbred milk in the peptide profile pattern. More number of peaks (23) with higher intensity was visible in the cheese made from Deoni milk as compared to cheese made from Crossbred milk (20 peak) after 6 month of maturation.

Since DMCC had higher number of peaks and intensity as compared with CMCC, it can be concluded that cheese made from Deoni milk exhibited increased rate of proteolysis. RP-HPLC peptide profile differences may be attributed to variations in protein fractions of milk obtained from different species and enzymatic activity such plasmin, chymosin, peptidases on these fractions

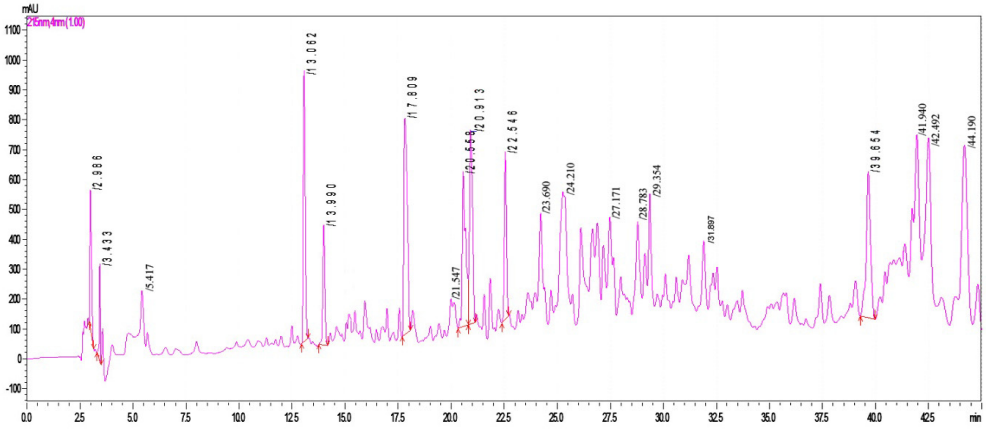

Fig. 4 (a)

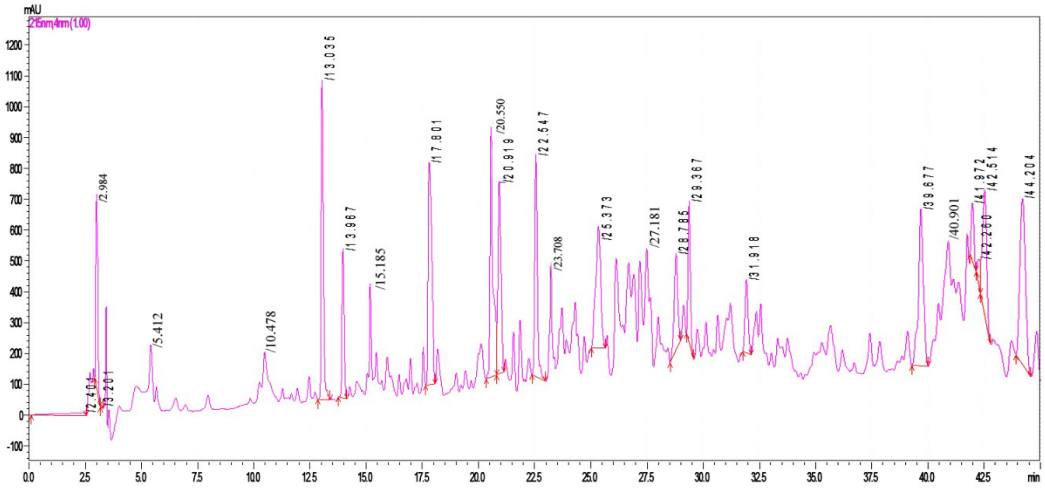

Fig. 4 (b)

Fig. 4. RP-HPLC chromatograms peptide profile Cheddar cheese made from (a) Crossbred milk and (b) Deoni milk at 6M of ripening

Imtiaz *et al.* (2012) (McSweeney, 2004). The WSN chromatograms revealed only small qualitative differences between 6 and 10M and the present peptide peaks either improved or declined in their intensity with the maturation of cheese (Law *et al.*, 1982). In the course of maturation time, degradation of peptide and protein resulted in raise in the peak number. Based on the ripening time, peptides with large and intermediate size were obtained from caseins by activity of residual rennet, plasmin, proteolytic and microbial enzymes present in the cheese. Further, secondary microflora converted these components to shorter peptides and amino acids (Gupta *et al.*, 2009). Pritchard *et al.* (2010) studied three Australian Cheddar cheeses which are available commercially and recorded that watersoluble extract had identical peptide profiles. Earlier studies (Ong and Shah 2008) showed that due to the increase in maturation period, more peptides were released into cheeses. In all Cheddar cheeses, maximum elevation in the peptide number upto 180 days of maturation was noticed.

CONCLUSION

The unique findings and results in comparison of cheese prepared from Deoni milk and Crossbred milk**.** The pattern of proteolysis was higher in DMCC compare to CMCC was confirmed quantitively by soluble nitrogen content and qualitatively by RP-HPLC were considerably higher number of main peaks with greater intensity in DMCC than CMCC.

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