



A₁ and A₂ milk caseins-comparative FTIR and spectrofluorimetry analysis

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Received: 29 June 2020; Accepted: 14 July 2021

ABSTRACT

Around 35% of the total caseins are β -caseins, which are further classified as A₁ β -caseins and A₂ β -caseins, based on differences in the amino acid composition of both. A₂ is the wild type genetic variant of β -casein while A₁ is the mutant. The present study aimed at the isolation of A₁ and A₂ casein from different cow milk sources and its characterization by using simple chemical techniques, viz. FTIR and spectrofluorimetry. The commercial milk sample from *Bos indicus* (Gir) (A₂) was obtained from Bombay Panjrapole, Mumbai and two commercially available packaged cow milk samples (pasteurized, skimmed) namely from Gokul and Mother Dairy (A₁) were also obtained for comparison analysis from the local market. The isolation of casein was performed by standard method and analyzed using SDS-PAGE, FTIR and spectrofluorimetry. There was evidence that the A₂ milk lacked histidine and rich in aromatic amino acids like tryptophan using FTIR and spectrofluorimetry techniques.

Keywords: A₁ milk, A₂ milk, BCM-7, BCM-9, FTIR, Spectrofluorimetry

Western dairy cattle breed Holstein Friesians is known to produce A₁ milk (Ciećelińska *et al.* 2012, Sridharan and Chidananda 2020) while Indian breeds like Gir, Deoni, Khillari, Nagori etc. (Dahd.nic.in.) are known to produce A₂ milk (Mishra *et al.* 2009, Ciećelińska *et al.* 2012, Sridharan and Chidananda 2020).

The bovine caseins are classified as α S1-casein, α S2-casein, β -casein and κ -casein in an approximate ratio of 4:1:4:1 respectively (Visser *et al.* 1991, Fox *et al.* 2015). The bovine CSN gene codes for β -casein having two major alleles, i.e. A₁ and A₂ (Jianqin *et al.* 2015). A₂ is the original wild type genetic variant of β -casein while A₁ is the mutant one (Jenness and Patton 1976, Siroëiã *et al.* 2017). The two milk proteins differ by one amino acid; a proline occurs at position 67 in A₂ and histidine in A₁ β -casein (Gerosa and Skoet 2012, Fox *et al.* 2015). A₁ and A₂ β -casein are processed differently by digestive enzymes (Stefanescu *et al.* 2017). A seven-amino peptide, β -casomorphin-7 (BCM 7) is released by digestion of A₁ milk whereas digestion of A₂ milk releases BCM-9 (Farrell *et al.* 2001). A correlation was observed between the prevalence of milk with A₁ β -casein proteins and various chronic diseases like Type 1 Diabetes, coronary heart disease (CHD) and, possibly, schizophrenia and autism in people with immune deficiencies (Elliott 1992, 1999; Truswell 2005, De Noni *et al.* 2009, Elsik *et al.* 2009, Lacroix and Li-Chan 2013, Ho *et al.* 2014, Jianqin *et al.* 2015, Chia *et al.* 2017, Duarte-

Vázquez *et al.* 2017, Behera *et al.* 2018).

Various genetic variation studies and chemical methods like gas chromatography coupled mass spectrometry, FTIR and spectrofluorimetry has been used to characterize milk (Calamari *et al.* 2010, Fuerer *et al.* 2020, Sebastiani *et al.* 2020) but there are minimal studies on characterization and structural differences of A₁ and A₂ milk type. The present study aims to characterization and distinguish differences in A₁ and A₂ casein molecules from commercially available milk samples by FTIR and spectrofluorimeter.

MATERIALS AND METHODS

Sample collection: The commercial milk sample from *Bos indicus* (Gir) (A₂) was obtained from Bombay Panjrapole, Mumbai. The cow breed was confirmed by the veterinarian Dr. Ramesh Pokar of Bombay Panjrapole, Mumbai. Around 500 ml of freshly packed (unpasteurized, unskimmed) milk was taken. 500 ml each of two commercially available packaged cow milk samples (pasteurized, skimmed) from Gokul and Mother Dairy (A₁) were also obtained for comparison analysis from the local market. The samples were deep-frozen until further use. High purity laboratory-grade casein powder from Merck was used as standard.

Isolation of casein from milk: The isolation of casein from milk was carried out as described by Kumar *et al.* (2007).

SDS-PAGE: The standard protocol of SDS PAGE was performed as described by Laemmli (1970) to separate α , β , and γ -caseins. Casein precipitate (1 g) was subjected to boiling in 100 mM tris-buffer (pH 8.0). This was followed by filtration using Whatmann filter paper no.1 and storage

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at 4°C. The solution was then cracked at 100°C for 5 min and stored at -20°C. The samples were run at 15% polyacrylamide concentration at 100 V for 1.5 h, followed by staining and destaining procedure.

FTIR: A PerkinElmer spectrum version 10.03.07 was used to do the FTIR analysis of casein samples. The dried casein precipitate samples were directly fed into the instrument to obtain the FTIR graph (Database of ATR-FTIR spectra of various materials, 2015).

Spectrofluorimetry: The casein precipitates were dissolved in 0.1 M Phosphate buffer pH 7.5 to a final concentration of 2 mg/ml. As casein precipitates had poor solubility in the buffer, the solution was heated in a water bath at 45–50°C till the solution turned almost clear. The samples were fed in the spectrofluorometer and graphs were obtained at the excitation wavelength of 280 nm and the emission wavelength was recorded from 0 to 1000 (Karoui *et al.* 2004). The analysis was done on the Cary Eclipse MY17520003 spectrofluorometer.

RESULTS AND DISCUSSION

SDS-PAGE: The casein precipitate samples were run at RT on denaturing SDS PAGE. Denaturing SDS PAGE gave well-separated bands of all casein precipitate samples. The fractions namely α , β and γ -caseins were identified using standard casein as reference. Lanes 1, 2, 3, and 4 contained Standard casein, Panjrapole casein, Gokul casein, and Mother Dairy casein respectively (Fig. 1). The dark bands in all the 4 lanes consisted of α -casein and β -casein. The bands appeared close as the difference in molecular weight between α -casein (23.6 kDa) and β -casein (24 kDa) is very less (Fig. 1). The lighter bands are of κ casein (19 kDa). Jovanovic *et al.* (2007) and Zagorchev *et al.* (2013) have separated milk on SDS PAGE which gave similar separation as observed in this study. Thus caseins can be easily separated into its components, i.e. α , β and κ on SDS-PAGE.

Fourier transformed infrared spectroscopy (FTIR): The FTIR peaks obtained for the casein precipitate of Gokul and Mother Dairy milk samples were 13 while the casein precipitate of the Panjrapole milk sample showed 11 (Table 1, Figs 2, 3). The additional FTIR peaks in the of

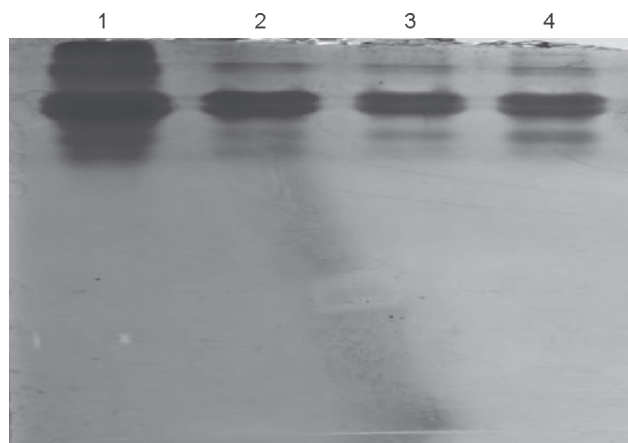


Fig. 1. SDS PAGE of isolated casein. 1, Standard casein; 2, Panjrapole casein; 3, Gokul casein; 4, Mother dairy casein.

Gokul and Mother Dairy casein precipitate samples were 2955.52/cm and 1414.71/cm. The rest of the other peaks were the same in all the casein samples except for a few deviations in the frequency of the peaks. The broad range of the spectrum is covered by -OH bonds which were seen at 3278.32/cm. The presence of the carboxyl group at around 3280/cm is either due to lack of precipitate washing after milk acidification or due to stabilizers used in commercial packaged milk for durability. The casein was characterized by vibrational bands at 2917.74 and 2850.53/cm of CH₂ groups, i.e. symmetric and asymmetric stretching bonds of alkyl groups. This is usually present in organic compounds (Siroèiã *et al.* 2017, Barth 2000, Calamari *et al.* 2010) as observed in the present study. These vibrations indicated the presence of amino acids with a higher concentration of CH₂ groups such as lysine and arginine. The vibrational bands at 1741.64/cm indicate the presence of carbonyl groups (C=O) in casein molecules that form strong hydrogen bonds with its surroundings (Siroèiã *et al.* 2017). Furthermore, a vibration peak of carbonyl groups was observed in the interval between 1300 and 1000/cm as well as at 1633.77 and 1539.06/cm, the area of amide stretching (NHCO) similar to the carbonyl groups. Siroèiã *et al.* (2017) recorded comparable vibrational peaks at 1652 and 1585/cm in casein samples. In the present study, the frequency variation seen may be due to the change in the number of hydrogen bonds. Asymmetric CH₃ vibrations of

Table 1. FTIR Peaks of the milk casein samples

Peaks in Gokul and Mother Dairy sample	Peaks in Panjrapol sample
3278.32/cm	3281.11/cm
2955.52/cm	-
2917.74/cm	2917.88/cm
2850.53/cm	2850.32/cm
1741.64/cm	1741.44/cm
1633.77/cm	1634.57/cm
1539.06/cm	1539.55/cm
1455.67/cm	1455.76/cm
1414.71/cm	-
1239.88/cm	1239.56/cm
1173.72/cm	1173.98/cm
1096.87/cm	1095.87/cm
624.22/cm	627.08/cm

Table 2. Spectrofluorimetry peaks of the milk casein samples

Peaks in Standard Casein	Peaks in Gokul sample	Peaks in Mother Dairy sample	Panjrapol sample
260.00 nm	260.00 nm	260.00 nm	260.00 nm
270.93 nm	270.93 nm	270.93 nm	270.93 nm
297.07 nm	297.07 nm	297.07 nm	297.07 nm
311.07 nm	311.07 nm	311.07 nm	311.07 nm
-	-	-	332.96 nm
335.93 nm	-	-	-
361.07 nm	361.07 nm	361.07 nm	361.07 nm

threonine at 1455.76/cm were noted. Barth (2000) observed δ CH vibrations of threonine at 1455/cm in proteins including casein samples. The variable intensity of vibrational bands in the samples points toward a difference in the amount of threonine in the samples. A sharp peak at 1173.72/cm pointed towards medium to strong P-O-CH₂R vibrations (Database of ATR-FTIR spectra). The peak at 1096.87/cm indicated pyrrolidone ring stretching. The vibrational peak at 1090/cm signifies a band shift of proline (Barth 2000, Mary *et al.* 2009) which also observed in the present study. The vibrational bands at 624.22/cm seen on the spectrum indicated vibration of CH₂ bond and their presence in the (-CH₂)_n groups. The comparable vibrational bands at 718/cm was observed by Siroëiã *et al.* (2017). A lower frequency vibration band noted in the present study may be due to reduced (-CH₂)_n groups in the samples. 2955.52/cm peak signified a cyclic 5 carbon molecule, which could probably be an amino acid-like histidine which contains an imidazole ring (Database of ATR-FTIR spectra). A peak at 1414.71/cm also pointed towards the probable presence of histidine due to strong C=C and C=N in-plane vibrations of substituted pyrroles (Database of ATR-FTIR spectra, Baker *et al.* 2014, Baum *et al.* 2016). Therefore, these peaks may correspond to the presence of histidine (Fig. 2) in Gokul and Mother Dairy casein precipitate samples a pointer of A₁ milk. It has been noted that histidine is absent in A₂ milk of Indian indigenous cow, *B. indicus* (Pearce 1975, Mishra *et al.* 2009, Lacroix and Li-Chan 2013, Baum *et al.* 2016, Stefanescu *et al.* 2017). Thus, the absence of these peaks in Panjrapole casein precipitate could be an indication of A₂ milk.

Spectrofluorometer: The casein precipitate of Standard casein, Gokul, Mother Dairy, and Panjrapole samples showed similar emission spectra with differences in the peak intensity (Supplementary Fig. 4). The prominent peak seen in Standard casein precipitate was 335.93 nm and in Gokul, casein precipitate was 270.93 nm and 361.07 nm. The Mother Dairy casein precipitate did not show any prominent peaks. The casein precipitate of the Panjrapole milk sample gave four prominent peaks at 260 nm, 297.07 nm, 311.07 nm, and 332.96 nm (Table 2).

Tyrosine and tryptophan are used experimentally because their quantum yields (emitted photons/ excited photons) are high enough to give a good fluorescence signal. For Trp residue, there is strong Stokes shift dependent on the solvent, meaning that the maximum emission wavelength of Trp will differ depending on the Trp environment (De Noni *et al.* 2009, Lacroix and Li-Chan 2013).

The peaks at 335.93 nm and 332.96 nm observed in Standard casein and Panjrapole casein precipitates signify the presence of casein at room temperature rich in tryptophan. All the samples showed the presence of a peak at 361 nm with varied intensity. This is a characteristic emission spectrum of tryptophan when the excitation wavelength is set to 230 or 280 nm. The combination of fluorescence assigned to tryptophan (emission spectra using excitation wavelength at 295 nm) was applied in a front-

face fluorescence study of milk by Duarte-Vázquez *et al.* (2017) gave the similar emission spectra. The intensity of the peak at 361 nm is directly proportional to the amount of tryptophan present (Yang *et al.* 2015, Taniguchi, and Lindsey 2018) as seen in the present casein samples under investigation.

The peak in the range of 330 to 340 nm is indicative of β -casein rich in tryptophan at various temperatures in milk samples both buffalo and cow (Pearce 1975, Dufour and Riaublanc 1997) as observed in the present investigation. The peaks at 311.07 nm, 297.07 nm, and 260.00 nm are characteristic emission spectrum of tryptophan in milk. Similar peaks at 311 nm, 292 nm, and 260 nm were observed in other milk samples (Dufour and Riaublanc 1997, Taniguchi and Lindsey 2018). Tyrosine shows emission maxima at around 270 nm (Yang *et al.* 2015, Taniguchi and Lindsey 2018). Tyrosine and/or phenylalanine presence was confirmed by a peak at 270 nm as phenylalanine is a precursor of tyrosine. The presence of tyrosine is confirmed in the samples under study with varying amounts of tyrosine residues. The presence of phenylalanine is also confirmed by peaks at 270 nm which is also the range of phenylalanine fluorescence (Taniguchi and Lindsey 2018). The milk has been classified on basis of origin using spectrofluorimetry by scientists like Dufour and Riaublanc (1997) and Karoui *et al.* (2004). Though standard casein, Gokul, Mother Dairy, and Panjrapole casein precipitates showed similar results, there was a difference in the intensity of spectral peaks in the Panjrapole casein precipitate (A₂) which had higher peak intensity (Supplementary Fig. 4.). This may be attributable to higher amounts of aromatic amino acids especially tryptophan in Panjrapole casein precipitate.

Even though standard casein, Gokul casein, and Mother Dairy casein precipitate (A₁) demonstrated similar results, there was certainly a change in structure in Panjrapole casein precipitate (A₂), which was confirmed by FTIR spectroscopy and spectrofluorometer.

There is evidence that the Panjrapole casein precipitate (A₂) does not have detectable histidine. It also has a higher amount of aromatic amino acids like tryptophan. These essential amino acids play a critical role in determining the 3D conformation of the casein molecule, any changes in them may result in an altered biological activity. This may further help in better postulation regarding the health benefits of A₂ milk over A₁ milk. The various spectroscopic studies along with LC-MS may help in faster and reliable detection, and characterization of A₁ and A₂ milk samples.

ACKNOWLEDGEMENTS

We thank Dr. Ramesh Pokar for the identification of cow breed and arranging the milk sample. We are also grateful to the Department of Chemistry, University of Mumbai for providing the FTIR instrumentation.

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