Effect of type and quality of milk on heat induced protein–protein interactions in *khoa*

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Abstract The present study was carried out to evaluate the effect of developed acidity and subsequent neutralization of milk (cow/buffalo) on heat induced protein–protein interactions occurring at various stages during *khoa* preparation. Protein–protein interactions were studied in terms of surface hydrophobicity ($F_{\max}$), sulfhydryl (–SH) group and SDS PAGE. As milk progressed to boiling stage, increase in $F_{\max}$ and decrease in –SH content was observed. *Khoa* prepared from cow milk had comparatively higher values for $F_{\max}$ and lower values for –SH group. $F_{\max}$ was observed to be highest in acidic samples followed by neutralized and fresh samples. While considering –SH group, maximum values were observed in neutralized samples followed by acidic and fresh samples of both milk and *khoa*. However, no visible difference was observed in SDS PAGE patterns of casein fractions isolated from different types of samples. The bands of β -lg and α -la did not resolve clearly in the *khoa* samples due to high heat

treatment involved in its preparation, indicating intense denaturation of whey proteins especially in neutralized samples where an alkaline medium resulted in strong binding between casein and whey proteins. The quality of milk also resulted in altered heat induced protein–protein interactions in *khoa*.

Keywords *Khoa* · Proteins · Sulfhydryl · Hydrophobicity · SDS PAGE

Introduction

India has retained the leading position as world's largest milk producing nation with approximate production of 156 MT in 2015–2016 (Narke 2017). A considerable share of total milk produced is utilized for preparation of traditional *mithais* (sweets) such as *peda*, *burfi*, *milk cake* and *gula-bjamun*. Depending upon the end use, *khoa* can be further classified as *Pindi*, *Dhap*, and *Danedar* which differs in composition, texture and quality. *Khoa* serves as a base material for these traditional sweets. It is a top most consumed dairy product in India (Ezhil Raj et al. 2010) and a significant percentage (~ 7) of the total milk is used for the production of *khoa* (Rasane et al. 2015). *Khoa* is a desiccated milk product prepared by continuous heating of milk in an open shallow pan to a total solid content of approximately 70%. Heating of milk brings about interactional changes within the milk constituents and denaturation of milk protein is one of them. Heat induced denaturation and coagulation of milk proteins brings about various changes in colour and consistency of the product in final stages (Davies 1940). As per Mann et al. (2008) denaturation of protective coverings of whey proteins and other colloids are the first observed changes as milk

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proceeds to boiling process. The process is further accelerated by incorporation of air and the frothing during stirring. Heating caused the modification in protein structure by exposing hydrophobic sites buried on the protein surface. It triggers the interactional changes within the milk proteins and eventually leads to protein aggregation followed by precipitation (Bonomi et al. 1988). Application of heat also caused conformational changes in β -lg by exposing reactive the thiol group. This thiol group can form disulphide bonds with other cysteine-containing proteins or proteins having di-sulfide bridges, e.g. α -la, κ -casein and α s2-casein thereby leading to protein–protein interactions in heated milk (Vasbinder et al. 2003). The heat induced association of whey proteins with casein micelle increased with heating time and temperature (Anema et al. 2004). Polyacrylamide gel electrophoresis (SDS, native, and two-dimensional PAGE) can be used to track the possible heat induced structural changes in milk proteins. Various studies confirmed the formation of heat induced complex between whey and casein proteins (Parnell-Clunies et al. 1988; Prasad and Balachandran 1989; Havea 2006; Donato et al. 2007).

In recent years, adulteration is still a burning issue in world's food society scenario and India has not been untouched by this menace. Milk and milk products have been the victims of adulteration since a long time e.g. melamine contamination in Chinese infant milk products (Xin and Stone 2008). Neutralization of milk is one of most practiced and common adulteration in history. The tropical climate and comparatively higher ambient temperature of Indian sub-continent are somehow responsible for rapid development of acidity in milk if not handled carefully. During festive seasons there is an unavoidable gap of demand and supply, neutralization of such milk makes it stable to heat processing required for preparation of sweets (Choudhary et al. 2016, 2017a, b). The economic motivation of middleman is somehow a driving factor for such frauds. Most of the research work on *khoa* in the past revolved only around standardization of manufacturing methods, chemical composition, sensory evaluation, preservation and improvement in keeping quality (Rajorhia et al. 1990; De 2004; Rehman and Salariya 2006; Choudhary et al. 2016, 2017a). *Khoa* is a protein rich product and during final stages of its manufacture, heat coagulation of milk resulted in destabilization of casein and altered salt

balance (Rajorhia et al. 1990). Limited information is available regarding the effect of developed acidity and subsequent neutralization of milk on heat induced protein–protein interactions at various stages during the preparation of *khoa*. Therefore, this study was designed to evaluate the consequence of developed acidity and subsequent neutralization of milk on heat induced protein–protein interactions in *khoa* at various stages of preparation i.e. raw, boiled milk and *khoa* stage (*Pindi khoa*).

Materials and methods

Materials

Sodium bicarbonate, Comassie brilliant blue, Bromophenol, Glycerol, 1-anilinonaphthalene-8-sulfonate (ANS) and Ethylenediamine tetra acetic acid (EDTA) were procured from Sigma Aldrich, St. Louis, Missouri, USA. Dinitrophenyle hydrazine and Resorcinol were procured from Rankem, RFCL Ltd., New Delhi, India. Acrylamide, *N,N'*-methylene-bis-acrylamide, *N,N,N',N'*-tetramethylethylenediamine (TEMED) and Ellman's reagent from Thermo Fisher Scientific India Pvt. Ltd., Delhi, India. Sodium dodecyl sulphate (SDS) from Sisco Research Laboratories (SRL) Pvt. Ltd., Mumbai, India.

Methods

Present study was carried out at ICAR-National Dairy Research Institute, Karnal. Freshly pooled good quality cow and buffalo milks were obtained from the Experimental dairy of the Institute. The gross composition of fresh cow and buffalo milk is presented in Table 1. Natural acidity in milk was developed by incubating fresh milks at 30 °C in an incubator (Narang Scientific Works Pvt. Limited, Delhi, India) up to 0.18% lactic acid (LA) by evaluating acidity hourly until it reached up to 0.18% LA. Calculated amount of neutralizer (Sodium bicarbonate) was added at required rate to adjust the acidity to 0.14% LA (~ acidity of fresh milk). The fresh, acidic and neutralized cow and buffalo milk samples were used to prepare *khoa* according to the method of De (2004). During preparation of *khoa*, samples were drawn at three different

Table 1 Chemical characteristic of cow and buffalo milks

Parameters	Percentage (%)					
	Fat	Protein	Lactose	Ash	SNF	pH
Cow milk	3.93 ± 0.15	3.63 ± 0.05	4.79 ± 0.10	0.74 ± 0.02	8.67 ± 0.21	6.46 ± 0.11
Buffalo milk	7.56 ± 0.12	4.34 ± 0.05	5.10 ± 0.11	0.82 ± 0.02	9.79 ± 0.21	6.62 ± 0.14

Data represented as mean ± SEM, n = 3

stages i.e. raw milk stage, boiled milk stage and final stage (*khoa*) to evaluate the heat induced protein–protein interactions at every stage. The proximate composition of *khoa* in terms of fat, lactose, ash, total solids, pH, acidity, moisture and protein of *khoa* was determined by the method described by Choudhary et al. (2016). The gross composition of three types of *khoa* prepared from buffalo and cow milk is presented in Table 2.

Surface hydrophobicity

Surface hydrophobicity of milk and *khoa* samples was determined by the method of Yuksel et al. (2010). Analysis of hydrophobic sites was carried out in milk samples diluted 1:10 (v/v) with 50 mM phosphate buffer (pH 7.0). In case of *khoa*, 2 g of the product was made into slurry with 5 ml water and volume was made up to 10 ml in a volumetric flask (This reconstituted 10 ml *khoa* sample is equivalent to 10 ml of milk). One ml of reconstituted *khoa* sample was diluted 1:10 (v/v) with phosphate buffer as in the case of milk. The fluorescent probe used was 1-anilino-naphthalene-8-sulfonate (ANS). Relative fluorescence of the samples was measured by using a fluorescence spectrophotometer at excitation wavelength 390 nm, emission wavelength 480 nm with slit width 5 nm. Ten μM of ANS concentration was prepared in phosphate buffer and used for ANS titration. Final concentration of ANS in the protein solution was between 0 and 140 μM during titration. The main aim of this parameter was to measure the maximum fluorescence which corresponded to level of saturation of the fluorescent marker (ANS) binding. Fluorescence of the samples was also measured before ANS titration and designated as blank.

Sulphydryl content

Sulphydryl groups in milk and *khoa* samples were measured using the protocol of Beveridge et al. (1974). Raw and boiled milk samples were acidified at 20 °C with 1 N HCl to pH 4.6 in order to fractionate casein from whey. In case of *khoa*, 10 ml of reconstituted *khoa* samples was acidified to pH 4.6 in order to fractionate casein from whey. Whey protein fractions were lyophilized. Lyophilized whey protein was reconstituted in tris–glycine buffer [TGB, 10.4 g tris, 6.9 g glycine, 1.2 g ethylene diaminetetraacetic acid (EDTA)/l, pH 8.0] to obtain 10% whey protein solution. Ellman's reagent (5–5'-dithiobis-2-nitrobenzoic acid (DTNB) was prepared in tris glycine buffer (TGB) at a concentration of 4 mg/ml. 0.5 ml of protein solution was added to 2.5 ml of 8 M urea in TGB and vortexed. Twenty μl of ellman's reagent was added, vortexed and absorbance read at 412 nm. Sulphydryl content was expressed as $\mu\text{moles SH/g}$ of protein.

Table 2 Gross composition and physico-chemical characteristics of fresh, acidic and neutralized buffalo milk *khoa* (a) buffalo milk *khoa* (b) cow milk *khoa* (Choudhary et al. 2016)

Sample parameters	Fat (%)	Protein (%)	Lactose (%)	Moisture (%)	Ash (%)	Total solids (%)	pH	Acidity (% LA)
(a) Buffalo milk <i>khoa</i>								
FBMK	35.13 $\pm$ 1.06 ^A	17.56 $\pm$ 0.17 ^A	20.59 $\pm$ 0.78 ^B	23.94 $\pm$ 0.41 ^B	2.74 $\pm$ 0.025 ^B	76.06 $\pm$ 0.41 ^B	6.48 $\pm$ 0.003 ^B	0.57 $\pm$ 0.003 ^B
ABMK	35.50 $\pm$ 1.53 ^A	17.70 $\pm$ 0.14 ^A	17.66 $\pm$ 1.02 ^A	22.43 $\pm$ 0.55 ^A	2.64 $\pm$ 0.034 ^A	77.57 $\pm$ 0.55 ^C	6.38 $\pm$ 0.005 ^A	0.62 $\pm$ 0.002 ^C
NBMK	35.41 $\pm$ 1.35 ^A	17.76 $\pm$ 0.29 ^A	17.03 $\pm$ 1.32 ^A	25.44 $\pm$ 0.70 ^C	2.85 $\pm$ 0.040 ^C	74.56 $\pm$ 0.70 ^A	6.68 $\pm$ 0.005 ^C	0.55 $\pm$ 0.005 ^A
(b) Cow milk <i>khoa</i>								
FCMK	26.46 $\pm$ 0.50 ^A	18.30 $\pm$ 0.12 ^A	24.87 $\pm$ 0.18 ^B	29.65 $\pm$ 0.62 ^B	3.24 $\pm$ 0.017 ^B	70.35 $\pm$ 0.62 ^B	6.40 $\pm$ 0.005 ^B	0.63 $\pm$ 0.002 ^B
ACMK	26.13 $\pm$ 1.08 ^A	18.43 $\pm$ 0.29 ^A	21.53 $\pm$ 0.44 ^A	27.07 $\pm$ 0.37 ^A	3.17 $\pm$ 0.012 ^A	72.92 $\pm$ 0.37 ^C	6.22 $\pm$ 0.005 ^A	0.68 $\pm$ 0.006 ^C
NCMK	25.19 $\pm$ 0.80 ^A	19.03 $\pm$ 0.04 ^A	20.80 $\pm$ 0.50 ^A	30.75 $\pm$ 0.30 ^C	3.46 $\pm$ 0.019 ^C	69.25 $\pm$ 0.30 ^A	6.51 $\pm$ 0.003 ^C	0.58 $\pm$ 0.006 ^A

Data are presented as mean $\pm$ SEM (n = 3)

ABMK acidic buffalo milk *khoa*, FBMK fresh buffalo milk *khoa*, NBMK neutralized buffalo milk *khoa*, ACMK acidic cow milk *khoa*, FCMK fresh cow milk *khoa*, NCMK neutralized cow milk *khoa*

A–C Means within column with different upper case superscript are significantly different ($p < 0.05$) from each other

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) of milk and *Khoa* proteins

SDS-PAGE was performed according to the method described by Simon (2004) on 12.5% gel concentration. Casein samples were prepared from milk and *khoa* samples by the method of Gupta and Ganguli (1965). Lyophilized casein samples were directly dissolved in sample buffer containing 20 µl mercaptoethanol/ml to obtain a concentration of 1 mg/ml. In case of *khoa*, lyophilized casein samples were dissolved in sample buffer and kept for 24 h at room temperature. Proteins were denatured by keeping it in a boiling water bath for 4 min before being injected for electrophoresis. Cooled the contents and centrifuged at 600 rpm. Twenty µl of this sample was injected in the wells of the gel. The working solutions were filtered and stored at 6 °C. Solutions were brought to room temperature before use.

Statistical analysis

Data reported were expressed as mean values with standard errors. Mean and standard error mean (SEM) were calculated using Microsoft excel (2007) (Microsoft Corp., Redmond, WA). Significant difference between values was verified by one way or two way analysis of variance and comparison between means was made by critical difference value (Snedecor and Cochran 1994).

Results and discussion

In present study effect of developed acidity and subsequent neutralization of milk was assessed on protein–protein interactions occurring in *khoa* during various stages of its

preparation. The chemical composition of milks used for *khoa* preparation is presented in Table 1. It was observed that buffalo milk has remarkably higher fat, protein, lactose, ash and SNF content as compared to cow milk which is responsible for comparatively higher yield and better texture of *khoa* as compared to cow milk *khoa*.

Surface hydrophobicity

Surface hydrophobicity of milk and *khoa* samples was analyzed in terms of $F_{\max}$ using ANS titration curve. $F_{\max}$ represented the maximum fluorescence that could be attained under the given conditions and also the maximum numbers of surface sites allowable, to which ANS could be bound (Yuksel et al. 2010). One percent milk or homogenized *khoa* samples in phosphate buffer were used for ANS titration. $F_{\max}$ values of fresh, acidic and neutralized cow and buffalo milk and their respective *khoa* samples at various stages i.e. raw, boiled and *khoa* stages are presented in Table 3 and Fig. 1 Significant difference ($p < 0.05$) was observed in $F_{\max}$ values of fresh, acidic and neutralized cow and buffalo milk samples at raw as well as boiled milk stage. However, non-significant difference ($p < 0.05$) was observed in the resulting *khoa* samples. It was observed from the titration curves (Fig. 1) that as the ANS concentration increased, fluorescence intensity increased up to 307.82, 344.12, 317.81 and 332.12, 388.14, and 343.84 in case of fresh, acidic and neutralized raw buffalo and cow milk samples, respectively. Similarly, in case of boiled milk, $F_{\max}$ increased with the addition of ANS up to maximum level. Carbonaro et al. (1996) reported that interactions between κ -casein and β -lg cause changes in structure of dominant casein which further increases surface hydrophobicity of milk protein.

Table 3 $F_{\max}$ values of milk at various stages of *during* preparation of *khoa* (a) Buffalo Milk (b) Cow milk

Sample stages of analysis	$F_{\max}$ (%)		
	Raw milk	Boiled milk	<i>Khoa</i>
(a) Buffalo milk			
FBMK	307.82 ± 1.56 ^{cA}	366.13 ± 1.81 ^{bA}	3.96 ± 0.08 ^{aA}
ABMK	344.12 ± 2.66 ^{cC}	391.38 ± 0.82 ^{bC}	5.03 ± 0.08 ^{aA}
NBMK	317.81 ± 1.75 ^{cB}	376.44 ± 0.55 ^{bB}	4.69 ± 0.10 ^{aA}
(b) Cow milk			
FCMK	332.12 ± 1.87 ^{bA}	356.45 ± 1.55 ^{cA}	4.41 ± 0.16 ^{aA}
ACMK	388.14 ± 1.22 ^{bC}	413.25 ± 2.32 ^{cC}	5.33 ± 0.05 ^{aA}
NCMK	343.84 ± 1.79 ^{bB}	370.64 ± 1.49 ^{cB}	4.81 ± 0.23 ^{aA}

Data are presented as mean ± SEM (n = 3)

FBMK fresh buffalo milk *Khoa*, ABMK acidic buffalo milk *Khoa*, NBMK neutralized buffalo milk *Khoa*, FCMK fresh cow milk *Khoa*, ACMK acidic cow milk *Khoa*, NCMK neutralized cow milk *Khoa*

^{A–C}Means within column with different upper case superscript are significantly different ($p < 0.05$) from each other

^{a–c}Means within row with different lower case superscript are significantly different ($p < 0.05$) from each other

Fig. 1 ANS titration curves of fresh, acidic and neutralized milk, **a** buffalo milk, **b** cow milk, at various stages during preparation of *khoa*. (i) Raw milk (ii) boiled milk (iii) *Khoa*. Where (i) *FBM* fresh buffalo milk, *ABM* acidic buffalo milk, *NBM* neutralized buffalo milk; *BFBM* boiled fresh buffalo milk, *BABM* boiled acidic buffalo milk, *BNBM* boiled neutralized buffalo milk; *FBMK* fresh buffalo milk *Khoa*, *ABMK* acidic buffalo milk *Khoa*, *NBMK* neutralized buffalo milk *Khoa*. (i) *FCM* fresh cow milk, *ACM* acidic cow milk, *NCM* neutralized cow milk; *BFCM* boiled fresh cow milk, *BACM* boiled acidic cow milk, *BNCM* boiled neutralized cow milk; *FCMK* fresh cow milk *Khoa*, *ACMK* acidic cow milk *Khoa*, *NCMK* neutralized cow milk *Khoa*

However, further addition of ANS did not increase the fluorescence intensity; which indicated that the protein solution had attained the saturation point. In case of different milk and *khoa* samples higher values of $F_{\max}$ were observed for acidic samples followed by neutralized and fresh samples, these variations could be due to the structural modification of protein during acidification and neutralization of milk. Fluorescence intensity was drastically reduced in *khoa* samples (Fig. 1) in comparison to raw and boiled milk samples; this could be due to protein–protein interactions as influenced by the heating of milk. Lower surface hydrophobicity values were observed in raw milk as hydrophobic groups were buried inside the native structure of protein molecule. Milk, when heated at higher temperature ($> 70^{\circ}\text{C}$) results in unfolding of protein molecules which causes the exposure of hydrophobic sites and thus an increase in surface hydrophobicity (Parnell-Clunies et al. 1988). However, with the increase in severity of heat treatment, the protein–protein aggregation phenomena and structural collapse occurs resulting in decrease of surface hydrophobicity (Bonomi et al. 1988). Alais (1984) reported that the very first impact of heat treatment on milk protein was denaturation of serum proteins followed by their aggregation with casein micelle. Our results correlated well with Bonomi et al. (1988) who reported an increase in $F_{\max}$ at 85 and 90°C and a decrease at 120°C as compared to raw milk. Similarly, our results were supported by the work of Pagliarini et al. (1990) who reported lower surface hydrophobicity values for in-bottle sterilized milk as compared to UHT milk. Carbonaro et al. (1996) reported a decrease in surface hydrophobicity of whey protein with heat exposure and ascribed this phenomenon to protein–protein interactions. Parnell-Clunies et al. (1988) analyzed surface hydrophobicity in casein and whey fractions of vat ($85^{\circ}\text{C}/10\text{--}40\text{ min}$), HTST ($98^{\circ}\text{C}/0.5\text{--}1.87\text{ min}$) and UHT heating ($140^{\circ}\text{C}/2\text{--}8\text{ s}$) processed milks and observed a negative correlation between surface hydrophobicity and whey protein denaturation.

Sulfhydryl content

Total Sulfhydryl content (–SH) was estimated in both cow and buffalo milks during preparation of *khoa* at various stages i.e. raw and boiled milks, and *khoa* stage and the results are presented in Fig. 2. It was observed that total –SH content of fresh, acidic and neutralized samples were significantly different ($p < 0.05$) from each other and the –SH content decreased significantly ($p < 0.05$) with the intensity of heating, which might be due to the conversion of –SH group into –SS bonding between κ -casein and β -lg resulting in protein–protein interactions. Among the three types of samples i.e. fresh, acidic and neutralized milk and *khoa*, total –SH was highest in fresh followed acidic and neutralized samples. Lower values in acidic milk might be due to the fact that low pH causes modification in whey protein structure resulting in lower availability of –SH groups while in case of neutralized samples the conversion of –SH bond into –SS bond increased with increase in pH up to 6.9 (Wanatabe and Klostemeyer 1976). Presence of –SH groups in isolated casein sample from boiled milk and *khoa* is due to heat induced entrapped whey proteins indicating heat induced casein–whey interactions. Vakaleris and Pofahl (1968) reported 3 times higher measurable –SH groups in high heat skim milk cheese as compared to pasteurized milk cheese. Since pasteurized milk curd is entirely casein, which has theoretically almost zero or very low

measurable –SH groups therefore any significant amount of –SH in pasteurized cheese curd was due to the presence of whey proteins trapped in the curd. Monahan et al. (1995) reported that even in the absence of heating, alkaline pH values affect the denaturation and sulphhydryl-mediated polymerization of whey proteins. Guyomarc'H (2006) reported that the heat-treatment of skim milk at alkaline pH generated aggregates of denatured whey proteins and κ -casein in the serum phase of milk, rather than on the surface of the casein micelles which might be the reason for lower sulfhydryl content in neutralized samples. Otte et al. (1999) also reported that the level of whey protein denaturation was largely affected by pH of milk and at lower pH values, the denaturation rate increased. However, Onwulata et al. (2006) reported that alkaline conditions increased denaturation in the extruded whey protein isolate. Cao et al. (2015) reported up to 8.48 μ moles of –SH/g of protein in evaporated milk with protein content of 17.99% which was in range of protein content in *khoa* samples i.e. 17–19%. Our results were supported by Parnell-Clunies et al. (1988) who reported that –SH content was negatively correlated with whey protein denaturation.

SDS PAGE

Figure 3 depicts the electrophoretic patterns of casein proteins of buffalo and cow milk and *khoa* samples.

Fig. 2 –SH content of fresh, acidic and neutralized milk during preparation of *khoa*. **a** Buffalo milk, **b** cow milk. Data are presented as mean $\pm$ SEM ($n = 3$). ^{a-c}Different lowercase letters denote significant difference ($p < 0.05$) between groups (raw milk, boiled milk and *Khoa*). Error bars show the variations of three determinations in terms of standard error of mean. ^{A-C}Different uppercase

letters denote significant difference ($p < 0.05$) across subgroups [(FBM, ABM and NBM) and (FCM, ACM and NCM)]. *FBM* fresh buffalo milk, *ABM* acidic buffalo milk, *NBM* neutralized buffalo milk, *FCM* fresh cow milk, *ACM* acidic cow milk, *NCM* neutralized cow milk

Fig. 3 SDS-PAGE pattern of fresh, acidic and neutralized buffalo and cow milks and their *khoa* casein separated on 12.5% gel at various stages, **a** buffalo milk, **b** cow milk. **a** Lane 1 molecular weight markers ranging from 3.5 to 205 kDa; lane 2 fresh raw buffalo milk; lane 3 acidic raw buffalo milk; lane 4 neutralized raw buffalo milk; lane 5 boiled fresh buffalo milk; lane 6 boiled acidic buffalo milk; lane 7 boiled neutralized buffalo milk; lane 8 fresh buffalo milk *Khoa*; lane 9 acidic buffalo milk *Khoa*; lane 10 neutralized buffalo milk *Khoa*. **b** Lane 1 molecular weight markers ranging from 3.5 to 205 kDa; lane 2 fresh raw cow milk; lane 3 acidic raw cow milk; lane 4 neutralized raw cow milk; lane 5 boiled fresh cow milk; lane 6 boiled acidic cow milk; lane 7 boiled neutralized cow milk; lane 8 fresh cow milk *Khoa*; lane 9 acidic cow milk *Khoa*; lane 10 neutralized cow milk *Khoa*

Casein proteins of raw milk resolved into four major bands which were assigned their respective labels (α -S₁, α -S₂, β -casein, κ -casein) based on comparison of their

molecular weight with molecular weight markers (lane 1). However, casein protein samples prepared from boiled milk resolved only into α -S₁, α -S₂, β -casein, κ -casein and

β -lg. No visible difference was observed in the SDS pattern of fresh, acidic, neutralized cow and buffalo milk samples. However, in case of their respective *khoa* samples, particularly for *khoa* from neutralized cow and buffalo milk, bands of β -lg and α -la did not resolve clearly indicating intense denaturation of whey proteins under alkaline conditions resulting in strong binding between casein and whey proteins since whey protein denaturation increases under alkaline conditions (Onwulata et al. 2006). It was concluded from SDS PAGE that casein samples from raw milk resolved into basic casein fractions while the casein samples from boiled milk also contained β -lg fraction of whey protein, this extra band evolved due to the heat induced protein–protein interaction occurring between β -lg and κ -casein resulting in a disulfide bonded protein complex. Addition of β -mercaptoethanol to the sample buffer in SDS PAGE resulted in breakdown of disulphide bond and thus the appearance of β -lg fraction in casein sample. During *khoa* preparation, intense heating of milk resulted in agglomerated protein complex of casein and whey protein. Hence, it was evident that heating of milk during preparation of *khoa* resulted in protein–protein interactions. Doi et al. (1981) also confirmed the formation of heat induced complex between β -lg and κ -casein after heating at 90 °C for 10 min. SDS PAGE of casein–whey protein complex (in the presence of 2-mercaptoethanol) resulted in resolution of κ -casein and β -lg.

Parnell-Clunies et al. (1988) conducted SDS PAGE of casein fraction of heated milk and indicated the presence of a high molecular weight component in the gel. Similarly, Prasad and Balachandran (1989) conducted PAGE of protein fractions in freshly prepared sterilized concentrated buffalo milk. They observed changes in number of bands and their mobility in the gel matrix of polyacrylamide gel. They also indicated distinct changes in aggregation and disaggregation of casein micelles through the appearance and disappearance of different peaks in elution profile. Havea (2006) conducted two dimensional PAGE of di-sulphide linked protein aggregates in milk protein concentrate powders and observed that these aggregates consisted of κ -casein, β -lg and some α s1-casein indicating protein interactions. Reports are also available on the effect of addition of whey protein or of purified κ -casein to the milk samples. The addition of whey proteins caused an increase in the amount and the size of the complexes, however, addition of κ -casein to milk had little or no effect on the complex formation nor did the added κ -casein could react with the whey protein in milk (Donato et al. 2007).

Conclusion

The quality of milk affected the heat induced protein–protein interactions at various stages during preparation of *khoa*. Development of natural acidity in milk and subsequent neutralization thereafter significantly affected protein–protein interactions in terms of surface hydrophobicity ($F_{\max}$ and Sulfhydryl content). Presence of whey protein in isolated casein also indicated protein–protein interaction. No visible difference was observed in the SDS PAGE pattern of casein samples isolated from raw and boiled milks of fresh, acidic and neutralized cow and buffalo milk. However, intense casein–whey protein association was observed in SDS PAGE pattern of casein isolated specifically from *khoa* sample prepared from neutralized milk indicating that alkaline conditions favoured heat induced casein–whey protein interactions.

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