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Iso-electric Focusing of Camel Milk Proteins

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ABSTRACT

The procedure for phenotyping of most genetic variants in cow milk was optimised for iso-electric focusing (IEF) of camel milk proteins and milk from individual camels of different breeds was screened. The caseins obtained from IEF bands were also investigated by N-terminal sequencing. Camel milk casein was separated at different pH and the proteins in the whey obtained were then separated by IEF. Above pH 4.3 casein bands were observed in the whey. According to the pattern of protein bands in IEF, the 103 camels screened had one of the three main groups of milk designated aa, ab and, bb. A small number of camels differed from *bb* milk type by an extra band and this group was designated as bb^{l}. The high frequency of particular milk type in some breeds suggests that their production characteristics could be related to the phenotypes.

INTRODUCTION

Genetic polymorphism in milk proteins is due to gene mutation resulting in either substitution or deletion of amino acids sequence along a peptide chain. In over 30 genetic variants identified in bovine milk, gene mutations leading to deletion of amino acids occur in only two cases whereas the more frequent gene mutations caused by substitution of amino acid are responsible for the other variants (Ng-Kwai-Hang and Grosclaude, 1992). Because milk protein genes are inherited in a simple Mendelian fashion, there is considerable interest in using them as markers for milk production and composition traits. It has been reported that κ-casein B (κ-Cn B) and β-lactoglobulin A (β-Lg A) are associated with a higher protein content in cow milk. The ratio of casein to whey proteins is increased by the presence of β-Lg B and κ-Cn B. Favourable effects of κ-Cn B, α_{s1}-Cn C and β-Cn B on rennetability and coagulum properties have also been observed, and even a positive relationship between κ-Cn B and cheese yield (Ng-Kwai-Hang and Grosclaude, 1992). Moreover, casein variants that have been associated with higher casein content in milk have also been correlated with higher amounts of the corresponding casein fraction (Jakob, 1994). Studies on goat α_{s1}-Cn have also shown convincing evidence that certain alleles of this protein are associated with higher concentration of casein in milk (Ng-Kwai-Hang and Grosclaude, 1992).

The main work on genetic polymorphism using isoelectric focusing (IEF) has been done mainly on cow and to some extent on goat milk. Only fragmentary data are available for genetic polymorphism of milk proteins in other species. Analysis of casein and whey protein fractions from milk of Somali camels by Di Stasio *et al.* (1983) reported the occurrence of polymorphism for the supposedly β-Lg, but not in the other proteins. Two variants, A and B, of α-La were later detected by Conti *et al.* (1985) in milk from Somali camels. These two forms of α-La had similar mobility in PAGE at pH 8.3, but differed in pI due to a difference in amino acid sequence at the *N*- terminal.

An IEF procedure for simultaneous phenotyping of most genetic variants in cow milk was described by Seibert *et al.* (1985). This procedure was optimised in the present investigation for IEF of camel milk proteins. In addition the occurrence of genetic polymorphism was also investigated by *N*-terminal sequencing of protein obtained from IEF bands.

MATERIALS AND METHODS

Preparation of milk for IEF

In Ol Maisor Ranch, Kenya, milk from 103 individual camels was collected. To prevent microbial growth 10 mg 2-bromo-2-nitro-1,3 propanediol (Bronopol Tablets, Preservative systems Ltd from Chemgo Organica, Basel, Switzerland) were added to 50 mL milk. The samples were immediately cooled to 4°C and transported to the laboratory within 24 h. Milk fat was removed by centrifugation (400g, 4°C, 30 min) before freezing or lyophilization.

Acid casein was prepared by adding to skim milk 10% acetic acid (10% v/v) allowing to stand 30 min at 35°C then adding 10% 1 M NaOAc and adjusting the pH to 4.3 with HCI and shaking gently and allowing to stand for 30 min before centrifugation (20,000g, 5°C, 30 min). The supernatant was retained and the casein subsequently washed twice with equal volume of buffer (400 mL water + 20 mL acetic acid (10% v/v) + 20 mL 1 M NaOAc adjusted to pH 4.3) and freeze dried.

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To prepare whey proteins, the supernatant from the acid casein was centrifuged (20,000g, 5°C, 30 min) and whey proteins were precipitated by saturation with ammonium sulphate and left overnight. The precipitate was collected by centrifugation (10,000g, 15°C, 15 min) and the sediment of whey proteins was lyophilised.

IEF of camel milk proteins

Separation of casein and whey protein

To 1 M NaOAc was added an equal amount of 10% v/v acetic acid to obtain a buffer of pH 4.40. To this buffer was added either sodium acetate or acetic acid to obtain buffers of pH ranging from 3.3 to 5.2 in steps of 0.1 pH unit. Bulk camel milk was then mixed with the respective buffer (milk : buffer 1: 1) and the pH of the mixture measured. Precipitation was allowed to occur for 1 h at 20°C and the mixture was centrifuged at 1000g for 30 min. The whey was centrifuged at 1000g for 1 h at 4°C to remove any casein particles before IEF.

IEF procedure

IEF was carried out according to Seibert *el al. (1985)* except that using a mixture of 1 part Ampholine® pH 4-6 and 5 parts Pharmalyte® pH 4.2-4.9, (Pharmacia Biotech AG, Switzerland) IEF gels having a pH gradient of 4-6 were prepared. The gels of 0.2 x 10 x 240 mm were run on a 2217 Ultophor Electrofocusing Unit with 2303 Multidrive XL power supply (Pharmacia-LKB, Bromma, Sweden). Pre-focusing conditions of 1500 V, 10 W, 20 mA for 300 Vh and focusing conditions of 3500 V, 25 W, 50 mA for 3500 Vh were used.

In cow milk analysis, proteins are solubilised by a 1: 10 dilution of skim milk with sample buffer before IEF. In camel milk, a dilution of 1:4 with sample buffer was found the most suitable. In all cases, 5 *µL* samples were applied per gel lane.

Preparative IEF

Preparative IEF was carried out as in normal IEF except that the gel thickness and length were 0.1 and 24 cm, respectively. The anode was 0.2 M phosphoric acid and the cathode 0.2 M lysine. Gels were run at 10°C on a 2217 Ultophor Electrofocusing Unit with 2303 Multidrive XL power supply (Pharmacia-LKB, Bromma, Sweden). Ten mg Iyophilised or 100 µL liquid sample was solubilised in 700 µL sample buffer according to Bjellqvist et al. (1993) containing 0.1 *%* spermine and 30 µL mercaptoethanol added. In each case all the sample was applied near the anode and focused at 3000V, 50mA, l0W to 15,000Vh then 3000V, 50mA, 5W to 20,000 Vh.

Electro-elution and purification of proteins bands from IEF gels

Individual bands were cut off from preparative IEF gels and equilibrated for 15 min prior to electro-elution with 200 µL solution of 0.05 M Tris-HCl, ph 6.8, containing 6 M urea, 30% glycerol, 2 % SDS and 2% DTE followed by a 5 min equilibration in a solution where DTE was substituted with 2.5% iodoacetamide *(Görg et al 1985).*

Electro-elution was done using the Little Blue Tank™ (ISCO, Inc, Nebraska, USA) according to manufacturers' instructions. Tris-HCl 12.5 mM, 0.1 % SDS buffer at pH 8.0 was used in the anode, cathode and the sample micro-trap and for each sample trap 5 mA and 1 W were applied and the limit voltage was 200 V. Running times were 1-2 h and the sample migrated to the anode. Fractions 3 and 4 were harvested from the micro-traps and made to 2.5 mL using the electro-elution buffer. Small molecular weight products were removed in a pre-packed Sephadex G-25 M PD-10 column (Pharmacia Biotech AG, Switzerland) by elution using the same buffer. The sample was then freeze dried and the dye and SDS removed using Solvent System A according to Koningsberg and Henderson (1983). *N*-terminal sequence of proteins was then determined.

N-terminal sequence

N-terminal sequencing was by Adman degradation (Matsudaira, 1989) using an automated device.

RESULTS AND DISCUSSION

Acid precipitation of camel milk casein

To determine the pH at which casein and whey proteins can be separated, casein was precipitated at different pH between 3.55 and 5.30 at 20°C. The proteins of whey obtained were then separated by IEF (Fig. f). Above pH 4.3 both casein and whey proteins bands were present in the TEF patterns. Therefore, it was concluded that the best separation of casein and whey proteins of camel milk occurred at pH 4.3. These results show the precipitation of casein in camel milk can not be performed in the same way as in cow milk, in which casein is precipitated by acidification of milk at 20°C to pH 4.6 (Eigel *et al. 1984).*

This low pI of camel milk casein has implications on the determination of casein in camel milk. In the literature casein in camel milk has been precipitated at the same pH as casein in cow milk (Farah and Farah-Riesen, 1985; Larsson-Razniciewicz and Mohamed, 1986). In our findings, if camel casein is precipitated at pH 4.6, a proportion of casein will remain in the whey and the non casein nitrogen (NCN) is overestimated leading to the subsequent underestimation of the casein content. This may explain the low casein content in camel milk compared to that of cow milk cited in literature (Bayoumi, 1990; Farah, 1996).

Milk proteins from individual camels

Milk samples from 103 individual camels, belonging to Somali, Turkana, Somali x Turkana, Somali x Pokot and Pakistan breed, were screened by the modified IEF method. According to the pattern of protein bands, each of the camels screened had one of the three main milk types *aa, ab* or, *bb* shown in Fig. 2. A small number of camels differed from milk type *bb* by only an extra band b^1 this group was designated as bb^1. The major differences in bands between camels are shown in Table 1. The observed frequencies for the three main types of milks are shown in Table 2. The frequencies for the three main types of milk for the camel population sampled were 0.47 for *aa*, 0.12 for *bb,* 0.40 for *ab.* The low frequency for milk type *bb* can be attributed to the fact that bulls that were carrying either genes of *aa* or *ab* sired most of the animals as indicated in Table 3. Turkana breed had a high frequency for *aa* of 0.82 compared to 0.43 for Somali breed.

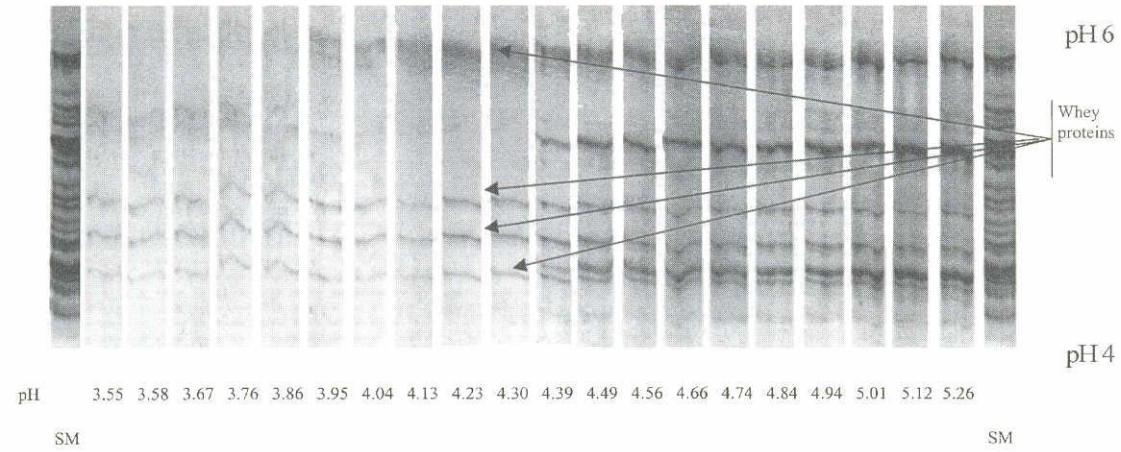

Fig 1. IEF of whey proteins from pooled camel skim milk (SM) precipitated at different pH. The whey proteins are indicated, the other bands are casein.

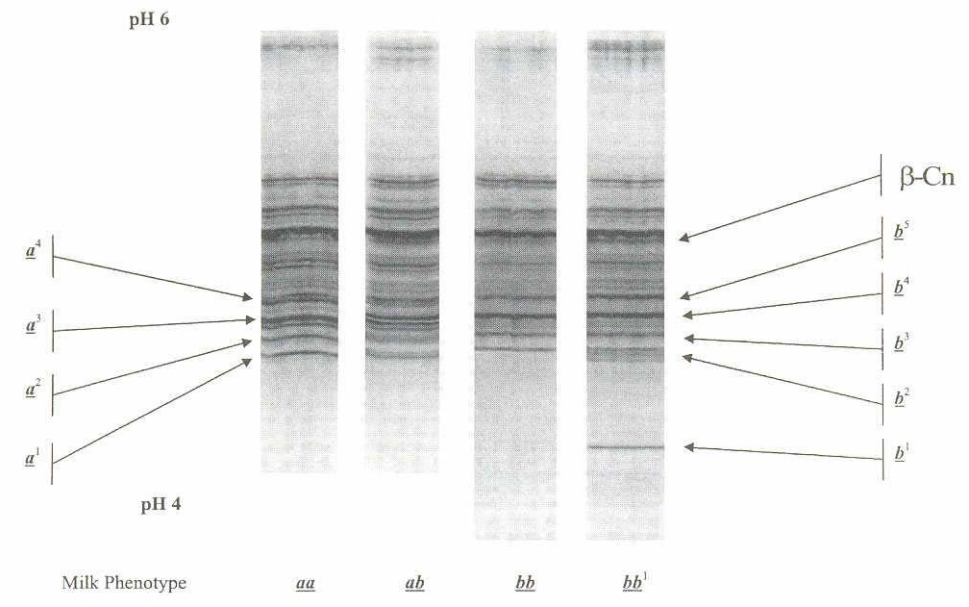

Fig. 2. IEF of different types of camel whole milk.

Table 1. Distribution of the IEF Bands in the different Groups of Camel Milk Proteins

Bands[a]	Group			
	aa	*ab*	*bb*	*bb*^{1}
a^1	+	+		
a^2	+	+		
a^3	+	+		
a^4	+	+		
b^1				+
b^2		+	+	+
b^3		+	+	+
b^4		+	+	+
b^5		+	+	+
β-Cn	+	+	+	+

[a] See Fig. 2 for the location of the hands in IEF.

Cross breeding of Turkana and Somali breed resulted in a *aa* frequency of 0.43 that was very similar to that of the Somali breed.

Table 2. Frequency of Three Main Milk Types in Camel Breeds

Camel breed	Number	Milk		
		aa	*bb*	*ab*
Somali x Turkana	49	0.429	0.143	0.429
Somali	30	0.433	0.133	0.433
Turkana	11	0.818	0.000	0.182
Pakistani	3	0.333	0.333	0.333
Somali/Pokot	6	0.500	0.000	0.500

Milk type *bb* was not found in Turkana and Somali x Pokot breed while Somali and Somali x Turkana breed had the similar frequency for type *bb*. It has been observed that Turkana breed had a lower milk yield, a higher content of fat, total solids and lactose in the milk, than the Somali and Somali x Turkana breed (Wangoh, 1997). These characteristics of Turkana breed

Table 3. Frequency in Daughters of Bulls

Bull identity[a]	Number of daughters	Frequency				Possible bull genotype
		aa	*ab*	*bb*	*bb*^{1}	
Unknown	34	0.56	0.26	0.15	0.03	-
17S Somali	26	0.46	0.54			*aa*
110S Somali	12	0.58	0.42			*aa*
Buna Somali	8	0.25	0.50	0.25		*ab*
Karandil Somali	6	0.17	0.67	0.17		*ab*
115S Somali	5	0.00	0.60	0.20	0.20	*bb*
Pakistan[b]	5	0.20	0.20	0.20	0.40	*bb*^{1} (predominantly)

[a] This is the identity given to the bulls at Ol Maisor Ranch; only bulls with more than five daughters are included in the list, all other bulls had one daughter each.
[b] This represents all the Pakistani camels sampled which are not from the same bull

Table 4. N-Terminal Sequences of Protein Bands

Milk type	Band	Sequence	Casein	
			Homologue[a]	Match %
aa	*a*^{1}	Arg, Glu, Met, Tyr, Asp, Leu, Lys	None	
	a^2	Arg, Pro, Lys, Tyr, Pro, Leu, Arg, Tyr, Pro	Human α_{s1}-Cn	85.2
			Bovine α_{s1}-Cn	59.3
			Pig α_{s1}-Cn	85.2
	a^3	Arg, Met, Lys, Tyr, Pro, Leu, Arg, Tyr, Pro	Human α_{s1}-Cn	73.7
	a^4	Same *a*^{2}		Same as *a*^{2}
			Same as *a*^{2}	
bb	*b*^{1}	Arg, Glu, Lys, Glu, Glu, Phe, Lys, Thr, Ala	None	
	b^2	Arg, Glu, Val, Tyr, GIll	Human α_{s1}-Cn	75
			Bovine α_{s1}-Cn	61.5
			Pig α_{s1}-Cn	61.5
	b^3	Arg, Pro, Lys, Tyr, Pro, Leu, Arg, Tyr, Tyr	Human α_{s1}-Cn	72.2
			Bovine α_{s1}-Cn	59.3
			Pig α_{s1}-Cn	59.3
	b^4	Arg, Pro, Pro, Gin, Pro, Leu, Arg	Human α_{s1}-Cn	68.1
	b^5	Same as *a*^{2}	Same as *a*^{2}	Same as *a*^{2}
ab	*b*^{3}	Same as *a*^{2}	Same as *a*^{2}	Same as *a*^{2}
	b^4	Arg, Ala, Lys, Glx Arg, Tyr	None	
	β-Cn	Arg, Glu, Lys, GIll, GIll, Phe, Thr, Lys, Thr	Rat β-Cn	67.5

[a]European Mo1ecular Biology Laboratory, Swissprot protein sequence data base searches.

could be related to high frequency for *aa* and a low one for *bb.*

However, more results are needed to establish a relationship between the protein alleles and milk composition and yield.

Band *b*^{1} that distinguished *bb*^{1} from *bb* was always present as an extra band and was not found in the other milk types. The frequency for *bb*^{1} was 0.33 in the combined *bb* and *bb*^{1} population and 0.04 in the sample population.

The *bb*^{1} milk type was only found in Somali and Pakistani breed. Its frequency in Somali and Pakistani breed was 0.07 and 0.67, respectively. It is recognised that Pakistani camels yield more milk than all other camel breeds in general. For the native Kenyan camels, Somali camels have the highest milk yield. The reason might be the presence of milk type *bb*^{1}.

The possible genotype of the bulls could be deduced from the type of milk of the 103 daughters (Table 3). We are aware of the fact that such a deduction normally requires a much larger number of observations. The milk type the 103 daughters suggest that, two bulls were *aa,* two *ab* and one *bb* type. The Pakistani bulls were predominantly *bb*^{1}. It should be noted that Pakistani camels were imported to Kenya to cross breed with local camels and pass on their renowned milk producing ability. It was also noticed that bulls of genotype *aa* or *ab* sired most of the camels. Since this milk-type appears to be related to low milk yield, this trait may not be the overriding criteria on which the selection of bulls for breeding is based. In general, the pastoralists look for, in order of importance, beauty, physical condition, and performance of parents in selecting a male stud for breeding and the stud is used until its reproductive life (Farah, 1996).

Preparative IEF, mass determination and *N*-terminal sequencing

The protein patterns obtained by preparative IEF from the four types of milks are the same as shown in Fig. 2. Each of these bands was excised from the preparative gel, electro-eluted and cleaned as described and subsequently the *N*-terminal sequence of the protein from each band determined. The result of European Molecular Biology Laboratory, Swissprot protein, database searches and the respective sequences are shown in Table 4. In milk type *aa,* a^2 and a^4 had the same *N*-terminal sequence. a^3 differed from both a^2 and *a4* in the *N*-terminal sequence. The highest homology of the *N*-terminal sequence of a^2, a^3 and a^4 were found with α_{s1}-Cn of human, pig and bovine (Table 4). Therefore it can be assumed that a^2, a^3 and a^4 are N-terminal sequences of camel α_{s1}-Cn. The substitution of methionine in a_3 for proline in a_4 can indicate a genetic variant of α_{s1}-Cn. For milk type in *bb,* the difference of *N*-terminal sequences between b^3 and b^5 was found to be the substitution of tyrosine for proline. In b^4, proline and lysine substituted the lysine and tyrosine in b^3 and b^5. b^2 had an *N*·terminal sequence that differed markedly from that of b^3,b^4 and b^5, but it also had some homology to human, bovine and pig α_{s1}-Cn. Moreover the β-Cn had some homology to rat β-Cn.

CONCLUSIONS

The procedure for simultaneous phenotyping of most genetic variants in cow milk was optimised for IEF of camel milk proteins and milk from individual camels was screened. According to the pattern of milk protein bands in IEF, the camels screened were assigned to different milk types. The high frequency of particular milk types in some breeds suggests that their production characteristics could be related to the types. Studies on a large number of camels are needed, therefore, to establish a relationship between the protein variants and milk composition and yield. This would help the breeders to improve the performance of camels.

From the present study, there is some evidence of genetic polymorphism of milk proteins in camel milk. Confirmation of this is needed by complete amino acid sequencing of proteins obtained from IEF bands.

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