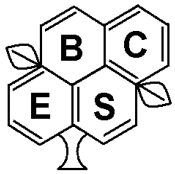

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IDENTIFICATION OF MILK SPECIES BY PROTEIN PROFILE AFTER SDS-PAGE

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ABSTRACT

Human milk as will as non-traditional of protein milk from species mares', ass's, cow, buffalo, goat, sheep and camel were investigated by SDS-PAGE polyacrylamide gel electrophoresis. The results of human milk proteins showed that whey fraction contains, lactoferrin, secretory IgA (SIgA), serum albumin and α-lactalbumin which represents 22.8, 10.99, 9.08 and 17.02 respectively. The investigation will be concern with differentiation of milk species by protein profile. The majority of proteins in human, mares' and ass's milk proteins are found in the whey fraction and represented by five proteins; α-lactalbumin, lactoferrin, Secretory IgA (SIgA), Serum albumin and Lysozyme. While the major whey proteins in the other species (cow, buffalo, goat and sheep) represented by four proteins; lactoferrin, Secretory IgA (SIgA), Serum albumin and β-lactoglobulin. The major similarity between species were identified between mares' and ass's as well as human milk proteins especially in the appearance of lysozyme and α-lactalbumin in high concentration. These observations encourage the possibility of using these milks as alternative of human milk.

Keywords: Milk protein species, lactoferrin, serum albumin, β-lactoglobulin, α-lactalbumin.

Abbreviations: SDS: Sodium dodecyl-sulphate; PAGE: Polyacrylamide gel electrophoresis; SIgA: secretory immunoglobulin; H: Heavy chain; L: Light chain.

INTRODUCTION

Dairy products are diverse and are commercially important within the food industry. Milk used to make such products is obtained from a number of species including mares', ass's, cow, buffalo, goat, sheep and camel (Hurley *et al* 2004). Milk is the main source of nutrition for the specific mammalian newborn, and provides all the essential nutrients for growth and development, e.g. proteins, minerals, carbohydrates, fatty acids, growth factors, immune modulators; therefore, the composition of milk differs by the needs of the neonate of different species species (El-Agamy *et al* 1992). For example, human milk is the fit food for human infants, but when breast-feeding is not available, cow milk is usually used as a substitute which could lead to nutritional and immunological problems. In Africa, Eastern Europe, central Asian and Egypt countries; mares' milk are used for different purposes, eyes infection and as alternatives to human milk. Mares' milk may be a curative agent for digestive and cardiovascular disease (Ochirkhuyag *et al* 2000 and Bonomi *et al* 1994). This suggests a new possibility for producing income from mares' dairy production. Consequently, mares' milk proteins need to be evaluated as human food. Recent clinical studies confirm ass's milk feeding as a safe and valid treatment of most complicated cases of multiple food intolerance (Carroccio *et al* 2000). However, information on ass's milk composition is more limited than that on mare's milk, which has also been studied as an infant food (Malacarne *et al* 2002). Meanwhile camel milk is considered one of the main components of the human diet in many parts of the world. It contains all essential nutrients as cow milk in addition to antimicrobial factors such as lysozyme, lactoferrin and immunoglobulins (El-Agamy *et al* 1998). In Europe, mares', cow, sheep and goats' milk has attained considerable economic importance as a result of widespread acceptance of traditional cheeses such as Roquefort, Feta and Manchego (Ramos and Juarez 1986). Human milk proteins were studied in the colostrum and mature of Egyptian milk and it was shown that most human milk proteins are found in whey fraction, and represents, lactoferrin, secretory IgA (SIgA), serum albumin and α-lactalbumin Afify *et al* (1997 and 2003). The aim of this investigation is to study the inter-specific relationship between different milk species as well as intra-specific within species by identifying protein

profile. Seven milk species are used in our investigation namely mares', ass's, cow, buffalo, goat, sheep, camel and human milk protein.

MATERIALS AND METHODS

2.1. Source of milk

Milk species were collected from different animals (mares', ass's, cow, buffalo, goat, sheep and camel as well as human) under specific conditions. The samples were collected manually under supervision into sterilized vials (20 ml) and stored in a deep freezer at -20°C until being used for analysis. Human milk proteins were collected from Frans hospital while the other milk species were collected from experimental station at faculty of agriculture, Cairo University.

2.2. Sample preparation

Mature milk samples were centrifuged at 10.400 x g at 4°C for 15 min to remove their fat components and the skim milk samples were separated and diluted with double volume of SDS gel loading buffer (0.5 M Tris-HCl, pH 6.80, 10% SDS, 20% glycerol and 0.01 bromophenol blue, 100 mM DTT), and denaturated at 95°C for 5 min.

2.3. SDS-PAGE

SDS-PAGE of mature milk sample was carried out in discontinuous systems in Tris-Glycine with SDS 0.1 % (Laemmli, 1970). The SDS- gel electrophoresis apparatus includes glass plates, combs, spacer, casting device, gel chamber and power supply unit (POOM-Phore, Germany), composition of SDS-PAGE for making resolving gels is stated in Table (1).

Protein markers with molecular weight of 205 KDa (Myosin), 116 (β-Galactosidase), 97 KDa (Phosphorelase), 84 KDa (Fructose-6-phosphate kinase), 66 KDa (Albumin bovine), 55 KDa (Glutamic dehydrogenase bovine), 45 KDa (Ovalbumin egg), 36 KDa (Glyceraldhyde-3-phosphate dehydrogenase), 29 KDa (Carbonic anhydrase), 24 KDa (Trypsinogen), 20 KDa (Trypsin inhibitor), 14.2 KDa (α-lactalbumin) from sigma were used as standards (Fig.1). Proteins were fixed in the gel by immersion for 1 h in 40% methanol (v/v) and 10% trichloroacetic acid solution and stained with Coomassie Brilliant Blue. Scanning of the proteins were carried out using Ultrascan XL laser scanner and for the data Gel-scan XL computer software was used.

Table (1): Formulation of SDS-PAGE for making resolving gels was as follows:

Solution component (30 ml)	5%	15%	Stacking gel
1.5 M Tris-HCl, pH 8.80 (ml)	7.50	7.50	—
0.5 M Tris-HCl, pH6.80 (ml)	—	—	2.50
30% Acrylamide solution (ml)	5.0	15.0	1.30
10% SDS (µl)	300	300	50
10% APS (µl)	50	50	50
TEMED (µl)	30	30	10
Water (ml)	17.12	7.12	6.0
Total volume (ml)	30	30	10

2.4. Statistical analysis

Statistical analysis of variance (t-test) within groups and between groups was conducted by using method of Miller and Miller (1992).

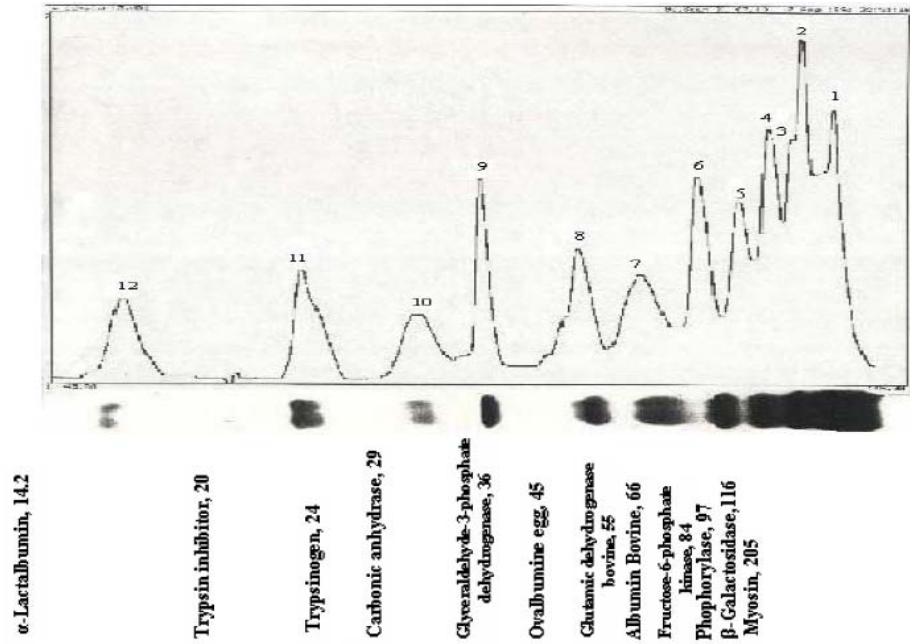

Fig (1): Scanning of protein profile of protein marker after SDS-PAGE separation.

RESULTS AND DISCUSSION

Identification of milk species were studied through the investigation of protein profile of each species after using SDS-PAGE. From the obtained results; we could classify protein profile of the species as the following groups:

1- The couple of cow and buffalo milk protein
2- Camel milk protein
3- The couple of mares' and ass's milk protein
4- The couple of goat and sheep milk protein
5- Human milk protein

Protein patterns of first four species milk groups were compared with major constituent of human milk protein patterns, as well as protein markers, to show its similarity between protein profiles of the different species (Fig. 2).

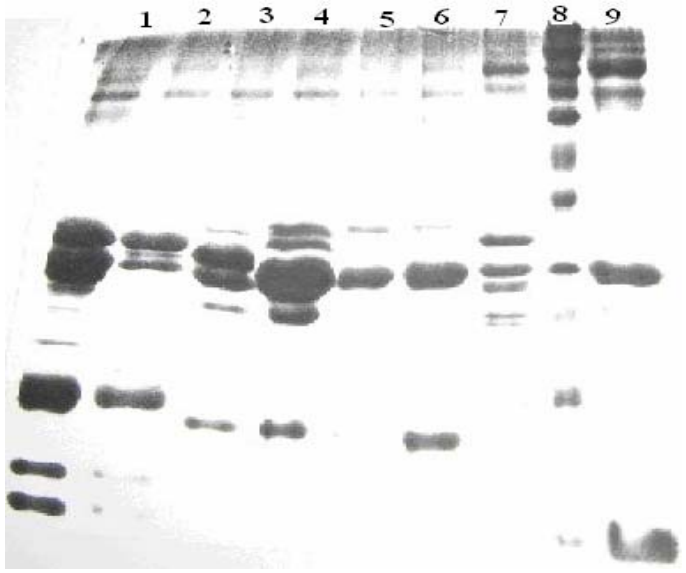

Fig (2): Electrophoretic separation of milk protein species in Tris-glycine buffer pH 8.3 with 0.1% SDS. (Lane 1= Mares', lane 2= Ass, lane 3= Cow, lane 4= Buffalo, lane 5= Goat, lane 6= Sheep, lane 7= Camel, lane 8= Protein marker, lane 9= Human).

1- Cow and buffalo milk proteins

Electrophoretic analysis of protein patterns of cow and buffalo referred to the presence of lactoferrin 84 KDa, serum albumin, 66 KDa; 35 KDa (buffalo) and 34 KDa, casein subunits of 27, 28, 29 KDa and β-casein 30 KDa, while α-lactoalbumin was not detected or traces. It is very important to note that cow and buffalo contain protein band with molecular weight 34 KDa which represents 6.2 and 5.9% respectively. In addition, protein band with MW 35 KDa was identified in buffalo (10.5%) and disappeared from cow (Table 2) as confirmed by El-Agamy (2000).

On the other hand β-lactoglobulin with MW of 18 KDa was identified in four species cow, buffalo, goat and sheep and represents 10.2, 12.7, 8.1 and 15.3% respectively. These results are in agreement with the presence of β-lactoglobulin in cow's milk and at the same time are in agreement with those obtained by Burr *et al* (1997). On the other hand protein band with MW of 24 KDa were identified in either cow and buffalo and represents 13%. The percentages of each protein components were scanned by densitometer and are shown in Table (2) and illustrated in Fig (2). The detailed information's about identification of the other milk species were studied through the investigation of protein profile of each species after SDS-PAGE as follows:

Table (2): Percentage of protein components of different milk species

Protein Species	Mare	Ass	Cow	Buffalo	Goat	Sheep	Camel	Human	Mean	SD
Lactoferrin 84 KDa	11.3	18.5	18	15.5	18.5	16.8	23.1	22.8	18.06	3.82
Serum albumin 66 KDa	9	9.2	8.9	3.9	9.5	6.6	6.9	9.08	7.89	1.95
SIg A_H 60 KDa	2	—	7	—	5.7	7.8	—	5.7	5.64	2.22
55 KDa	—	—	—	—	—	—	3.9	—	3.9	—
45 KDa	—	—	—	—	—	—	6.7	—	6.7	—
35 KDa	—	—	—	10.5	14.3	9.6	—	—	11.47	2.50
34 KDa	—	—	6.2	5.9	—	—	12	—	8.03	3.44
β-Casein (1) 30 KDa	10.5	14.9	13.8	13.9	17.9	14.2	13.5	12.56	13.91	2.09
Casein (2) 29KDa	9.4	4.3	11.2	13	15	14	10.5	4.94	10.29	3.95
SIg A_L 28 KDa	13.5	12.6	7.3	5.6	11	8	8	5.29	8.91	3.10
Casein (3) 27 KDa	8.0	—	4.4	6	—	—	—	3.59	5.50	1.95
24 KDa	2.9	—	13	13	—	4	5.5	—	7.68	4.95
23 KDa	—	—	—	—	—	3.7	4.4	—	4.05	0.50
22 KDa	3	0.9	—	—	—	—	2.7	—	2.20	1.14
21 KDa	—	—	—	—	—	—	2.8	—	2.8	—
β-Lactoglobulin 19 KDa	17.5	16.6	—	—	—	—	—	—	17.05	0.64
β-Lactoglobulin 18 KDa	—	—	10.2	12.7	8.1	15.3	—	—	9.88	5.84
Lysozyme 17 KDa	6.3	12.5	—	—	—	—	—	1.84	6.88	5.35
α-Lactalbumin 14.2 KDa	6.6	10.5	—	—	—	—	—	17.02	11.37	5.27
SIgA (L+H)	15.5	12.6	14.3	5.6	16.7	15.8	8	10.99	12.44	3.98
[A]*Major whey protein	48.7	63.3	—	—	—	—	—	61.73	57.21	9.13
[B]*Major whey protein	—	—	51.4	37.7	52.8	54.5	—	—	49.10	7.71

[A]*Major whey protein of (human, mare, ass) Lactoferrin, Serum albumin, SIgA (H+L), Lysozyme and α-Lactalbumin. [B]*Major whey protein of (cow, buffalo, goat, sheep, camel) Lactoferrin, Serum albumin, SIgA (H+L) and β- Lactoglobulin.

2- Camel milk protein

Protein profile of camel milk proved the presence of two major protein bands which consider distinctive with MW of 45 and 55 KDa. These two proteins were identified as unknown proteins as reported by Zhaung *et al* (2005). On the other hand, these two proteins were

identified as a fraction of IgA, which gave after reduction two-fractions one heavy chain with MW of 55.5 KDa and one light chain (22.5 KDa) as reported by El-Hatmi *et al* (2007). These patterns were different from that of bovine SIgA which had heavy chain of 60 KDa and a light chain of 28 KDa as cited by Butler (1974). It could be concluding that the complete reduction of IgA with mercaptoethanol produce two major bands of 55.5 and 22.5 KDa. These two fractions appeared as 60 KDa and 28 KDa in cow and 28 KDa in camel. The protein band with MW of 34 KDa was identified in camel milk represents 12 % compared with cow 6.2 % and buffalo 5.9% as showed in Table (2) and Fig (2).

3- Mares' and Ass's milk proteins

The result shows that whey proteins of mares' milk contains lactoferrin MW 84 KDa, serum albumin MW 66 KDa, β-lactoglobulin MW 19 KDa, lysozyme MW 17 KDa and α-lactalbumin MW 14.2 KDa were detected. The relative percentages of the above proteins were 11.3%, 9%, 17.5%, 6.3% and 6.6% with means 18.06 %, 7.89 %, 17.05 %, 6.88 % and 11.37 % respectively.

The band with Mw 60 KDa represents 2% of total whey protein belongs to immunoglobulins (Curadi *et al* 2000), while the casein fraction, of approximate molecular weight in the range 27 to 30 KDa, showed a different sensitivity to observe for mare's milk (Ochirkhuyag *et al* 2000). The presence of α-like casein, β-like casein, κ-like casein and γ-like caseins in ass's milk has been reported by Fantuz *et al* (2001). It must be noted that the observed percentage of β-lactoglobulin 18 KDa was much lower than that in bovine milk undetected or traces, where β-lactoglobulin 19 KDa can account for up to 17.5 and 16.6 % for mares' and ass's respectively of total whey protein similar to that reported by Schryver *et al* (1986). Moreover, a β-lactoglobulin level in ass's milk was equal to or lowers than that in mare's milk which related to (Malacarne *et al* 2002). Civardi *et al* (2002) found low β-lactoglobulin content in mare's milk when compared with bovine or even ass's milk. These findings together with the low casein content are probably related to the hypoallergenic characteristics reported for both ass's milk and mare's milk (Carroccio *et al* 2000). β-lactoglobulin is in fact the probable major milk allergen in infants and small children, whereas casein is considered the predominant allergen in adults (Carroccio *et al* 1999). A major

difference in whey protein composition between mare's and ass's milk is evident when lysozyme percentage is considered; the percentage of lysozyme in ass's whey protein (12.5%) was in fact much higher than in mare's milk (6.3%). On the other hand, only traces were found in bovine milk as reported by (Malacarne *et al* 2002). The large amount of lysozyme in ass's milk is confirmed by Civardi *et al* (2002), therefore, ass's milk represents an optimal growth medium for certain strains of useful lactic acid bacteria.

The availability of using animal milk as alternative to human milk, some studies concluded that goat (Bevilacqua et al., 2001), mare and ass (El-Agamy *et al* 1997 and Carroccio *et al* 2000) and camel milk (El-Agamy 2007) could be considered as proper alternatives to human milk, due to hypoallergenic properties of their proteins. On the other side, another studies showed that milk of goat, sheep and buffalo could not be useful in all cases as alternatives to human milk, because they can be as allergic as cow milk (Restani *et al* 2002 and El-Agamy 2007).

4- Goat and Sheep milk proteins

Electrophoretic analysis of goat and sheep milk protein proved the presence of lactoferrin 84 KDa (18.5 and 16.8 %), serum albumin 66 KDa (9.5 and 6.6 %), $SIgA_H$ 60 KDa (5.7 and 7.8 %), protein with MW of 35 KDa (14.3 and 9.6 %), β-casein 30 KDa (17.9 and 14.2 %), casein 2 of 29 KDa (15 and 14 %), $SIgA_L$ with 28 KDa (11 and 8%), in addition to β-lactoglobulin 18 KDa (8.1 and 15.3 %) compared with cow and buffalo. On the other hand sheep have protein band with 23 and 24 KDa represents 4 and 3.7 % respectively, and similar to that by Martin *et al* (2004). β-lactoglobulin protein have been detected in all species mares', ass's, cow, buffalo, goat and sheep except camel and human. The MW of β-lactoglobulin detected either as 18 KDa (cow, buffalo, goat and sheep) or as 19 KDa (mares' and ass's) as cited by Jenness (1970). β-lactoglobulins constitute a family of protein showing typical evolutionary divergence but their biological function remains an enigma. The most important function of this protein in the milk of species is that it transfers large amounts of immunoglobulin to their young via colostrums (Butler 1974 and Afify *et al* 2003).

α-Lactalbumin and lysozyme protein in different milk species:

Indeed, α-lactalbumin has been found in all milk species as cited by Jenness (1982). The α-lactalbumin and lysozyme appear to be related to each other on the basis of identity of many residues and location of disulphide bond (Brew *et al* 1970). The two proteins were identified in mares' and ass's as well as in human milk which shows similarity relationship with a mean of 11.37 % for α-lactalbumin and 6.88 % for lysozyme. The α-lactalbumin is very important since it's identified as calcium and zinc metalloprotein (Ren *et al* 1993).

CONCLUSION

The SDS-PAGE analysis showed that major protein profile of human milk are lactoferrin 84 KDa, Serum albumin 66 KDa, SIgA heavy chain 60 KDa, β-casein 30 KDa, casein (2) 29 KDa, SIgA light chain 28 KDa, casein (3) 27 KDa, Lysozyme 17 KDa and α-lactalbumin 14.2 KDa. The majority of human, mares' and ass's milk proteins are found in the whey fraction and represented by five proteins; α-lactalbumin, lactoferrin, Secretory IgA (SIgA), Serum albumin and Lysozyme. While the major whey protein in the other species (cow, buffalo, goat and sheep) represented by four proteins; lactoferrin, Secretory IgA (SIgA), Serum albumin and β-lactoglobulin 18 KDa.

The similarity between the different species and human milk protein after SDS-PAGE separation could be seen between human, mares' and ass's concerning the appearance of lysosyme and α-lactalbumin in both. The relationships between milk species (cow, buffalo); (goat and sheep) have a distinctive protein band of β-lactoglobulin with MW 18 KDa instead of lysosyme (17 KDa) and α-lactalbumin (14.2 KDa) which differ from human milk protein. Finally the application of SDS-PAGE for identification of milk species gave great opportunity to identify such species in the field of protein chemistry. The major similarity between milk species were identified between mares' and ass's milk protein especially in the appearance of lysozyme and α-lactalbumin in high concentration. These observations encourage the possibility of using these milks as alternative of human milk, since these milks have hypoallergenic properties. Since milk will received from the nontraditional sources, the economic as well as allergenic properties and biological evaluation

of these proteins should emphasis with more investigation in the future.

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التعرف على تركيب بروتينات الالبان عن طريق التفريد الكهربى بالاكتروفوريسيس باستخدام جيل بولي اكريلاميد المحتوي علي الصوديوم دوديسيل سلفات

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لقد تمت مقارنه بروتين لبن انثى الإنسان مع ألبان الحيونات مثل (أنثى الحصان، الحمار، الأبقار، الجاموس، الأغنام، الماعز و الجمال) عن طريق التفريد الكهربى (الألكتروفوريسيس). و لقد وجد أن بروتين الإنسان يحتوى على البروتينات الآتية (اللاكتوفورين، بروتين المناعة الأفرازى أ، البيومين السيرم و الفا-لاكتو البيومين). و تضح ان هناك تشابها بين ألبان (الأبقار، الجاموس، الأغنام و الماعز) فى احتوائهم على أربعة بروتينات و هم (اللاكتوفورين، بروتين المناعة الأفرازى أ، البيومين السيرم و البيتا لاكتوجلوبيولين) . كما لوحظ وجود تشابه بين بروتينات ألبان كلا من (أنثى الإنسان، أنثى الحصان و أنثى الحمار) و ذلك فى احتوائهم على خمس بروتينات و هم (الفا-لاكتو البيومين، اللاكتوفورين، بروتين المناعة الأفرازى أ، البيومين السيرم و الليسوسيم). و اتضح ان التشابه الكبير بين بروتينات ألبان أنثى الحصان و أنثى الحمار و بين بروتينات ألبان أنثى الإنسان كان مميزا خاصة فى بروتينات الالفا-لاكتو البيومين و الليسوسيم دون الحيوانات الاخرى. هذا التشابه الكبير بين بروتينات ألبان (أنثى الإنسان، أنثى الحصان و أنثى الحمار) يشجعنا على استخدامهم كبدائل لبروتينات لبن أنثى الإنسان.