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Characterization of Equine Milk and Cheese Making

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Abstract

We have studied on characterization and cheese making like mineral contents, protein composition and coagulation pattern on equine milk. At first, for contents of mineral on equine milk, It was lower in equine than bovine milk. Contents of Na, Mg, P, Ca and K, the major minerals, were indicated as 18.3 mg, 0.4 mg, 33.3 mg, 80.9 mg and 134.9 mg respectively by 100 g. In the distribution of nitrogen, the ratio NPN to Nt was indicated as 9.8% while that of bovine milk was 7%. And In NCN, its percentage was indicated as 45.6% showing that Equine casein was lower than bovine. From these results, equine milk could not be applicable to cheese production since there are no coagulable nitrogen fraction such as κ-casein, as there are with bovine milk. Equine milk will be more acceptable if we accept that the phylogenic affinity is near to human. It is the same as equine from the view points that monogastric, which did not contain ruminant's casein. For the rennet coagulation, equine milk was different than bovine milk. Equine milk did not coagulated by rennet after the addition of Ca^{2+}. But when bovine κ-casein was added in the presece of rennet, and Ca^{2+} to equine milk, coagulation occurred. Such phenomenon was also observed by the use SEM. Verification of κ-casein by SDS-PAGE did not existed in equine milk. The Casein of equine milk (54.4%) is similar to human milk in that casein/whey is about 1. For equine milk, this can be explained because distance between casein and Ca is great, casein being lower, which result in reaction of casein with Ca^{2+} because it could not activated which lasting time of coagulation is too long.

Key words : equine milk, rennet, coagulation, cheese, SEM

INTRODUCTION

Milk protein is almost synthesized in secretory cell of the mammary gland. Immunoglobulin of the milk proteins is synthesized in plasmicytes. In milk, there are 2 type of proteins, one whey protein, the other casein (Solaroli *et al.*, 1993).

In particular, the ration of casein to whey protein is similar to human milk. Almost all caseins were composed of micelles with a size of 30~600 nm. Micelles vary by mammary species, season, lactation state and various treatments. The diameter of micelle is 1.5 times for bovine milk and 4 times for human milk(Gallagher, 1997).

Casein micelle is very stable structure of colloidal system because the casein micelle can be frozen and even dried, and it is composed of casein proteins and calcium phosphate (Montilla *et al.*, 1995). We know that casein protein is composed of α-, β- and κ- casein proteins. Enzymatic coagulation of casein micelles to be dependent on the concentration of the micelles in the milk, on the temperature, pH and ionic strength (Ruettimann and Ladish, 1987). In cheese making an enzyme preparation is added to the milk. rennet contains the enzyme chymosin which cleaves off the glycomacropeptide (GMP) from the κ-casein brush on the surface of the micelles (Dekruif, 1999). Milk is coagulated by rennet as coagulation enzyme. This result is that rennet cleave to the bond of Phe-Met of κ-casein in casein micelle. Measurement of rennet coagulation time is very easy and simple (Maron *et al.*, 1995). The factor that influences coagulation are enzyme concentration, temperature, pH and concentration of Ca^{2+} (Daviau *et al.*, 2000 ; Gunasekara and Ay, 1996). The rennet coagulation of milk combines an initial enzymatic hydrolysis reaction and then a subsequent enzyme independent protein

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aggregation reaction (Van Hooydonk and Walstra, 1987). The addition of Ca^{2+} decreases the rennet clotting time(Van Hooydonk and Walstra, 1987 ; Balcones *et al.*, 1996). but if the concentration of Ca^{2+} is too high, coagulation time may be increased (Patel and Reuter, 1986).

For the mineral contents of equine milk, Csapo-kiss (1995) has been reported that the mineral contents of equine milk has been lower than that of other farm animals, Ca is 48.5~135 mg/100 g, P is 21.6~120.5 g, Mg 2.9~11.8 g, Na 7.5~23.7 g and K 30.3~99 mg.

The aim of this article is to compare the characterization of equine milk to bovine milk for various possibilities on cheese making.

MATERIALS AND METHODS

Materials

1) Bovine and Equine Milk

Bovine milk was obtained from university ranch of Chonbuk National University and equine milk was received from Walgok Farm, located in Chonbuk Jangsoo (Thoroughbred). The obtained samples were skimmed by centrifuge 5,000 rpm /20 min.

Methods

1) Determination of Minerals

(1) Minerals (Na, Mg, Ca and K)

1 mL of samples were diluted in 20 ml of 5% lanthan solution to avoid precipitation of organic matter, then the solution was filled up 200 mL with distilled water (Humbert, 1976).

Lanthan Solution as a spectral buffer

La_2O_3 - 118 g

HCl - 250 mL

Distilled water -fill up 1,000 mL

This solution passed in ICP/MS (AGILENT-7500A, USA) and each mineral was expressed mg/100 g.

Operating conditions are shown in Table 1.

Table 1. Operating conditions for ICP-MS system

Inductively coupled plasma	
Forward power	1.2 kW
Reflected power	<2 W
Coolant flow rate	15 L/min
Auxiliary flow rate	1 L/min
Carrier gas flow rate	1 L/min
Sample uptake rate	0.4 rps
Sampling depth	7.7 mm
Mass spectrometer	
Interfacce pressure	3.34×10^{2} Pas
Quadrupole chamber pressure	3.0×10^{-5} Pas
Sampler orifice	1 mm
Skimmer orifice	0.4 mm
Data acquisition	
Detecotr	ETP
Channel dwell time	5 ms
Integration time per channel	0.1 s

(2) Determination of Phosphorus

This method followed Lee (1981) which originated from Fiske and Subbarow (1925).

At first, 10ml of the sample were digested along with 30 mL of H_2SO_4. Digested sample was filled to 200 mL with distilled water, and then we were mixed with the following substances to reveal the color. This solution was measured absorption at A740 nm. The concentration of phosphorus had been expressed at mg/100 g.

→ 3 mL of digested sample

→ 1 mL of 20% H_2SO_4

→ 1 mL of NH molybdate

→ 0.5 mL of 20 % sodium sulfite (Na_2SO_4)

→ 0.5 mLof 1% hydroquinone ($C_6H_4(OH)_2$)

→ fill up to 10 mL distilled water

Standard calibration for the phosphorus was followed with K_2HPO_4 from 0 to 30 ppm.

2) Distribution of Nitrogen

(1) Total Nitrogen (NT)

Distribution of each nitrogen was represented as a % in total nitrogen and the Kjeldahl method was used.

(2) Non Caseinic Nitrogen (NCN)

20 mL of sample was mixed slowly with equal volume of 2M acetate buffer solution (pH 4.6), let stand 30 min and filtered and collected 5 mL of filtrate was used for determination by Kjeldahl method.

(3) Non Proteic Nitrogen (NPN)

20 mL of sample was mixed slowly with an equal volume

of 24% TCA solution then filtered, and 5 mL of filtrate was used for determination of nitrogen by use of Kjeldahl method.

3) Rennet Coagulation Time (RCT)

(1) Measurement of Rennet Coagulation Time

There are many physical methods that serve as indicators of coagulation, but we know that the simple and correct method is suggested by Sommer and Matsen (1935), Berridge (1945) and Lee and Park (1989).

For this experiment we used the RCT method by measuring the first appearance of film on the wall of a test tube in the presence of rennet (1:100000, KF tech, Korea) with the bovine or equine milk in a water bath at 35℃. In some cases of the equine milk, we added bovine κ-casein (C0406, Sigma, USA) with $CaCl_2$ to shorten RCT for the purpose of induction for enzymatic coagulation.

(2) Changes during Coagulation by Scanning Electron Microscopy (SEM)

For the SEM, all samples were post-fixed by glutaldehyde. After post-fixation, they were dried on the slide glass and fixed by osmium, then dehydrated and a subsititution with isoamyl acetate, was followed gold coating by through the use of a JFC-1100 Eion sputter using votage 20 kV, 5 min. These samples were observed under 20 kV by JSM-6400 (JEOL, Japan).

(3) SDS-Polyacrylamide Gel Electrophoresis (PAGE)

The application method was suggested by Laemmli and Favre (1973) whereby the gel is made of separation gel (T: 15.4 %, C: 2.7%, 0.38M Tris-HCl buffer containing 0.1% sodium dodecylsulfate (SDS), pH 8.8) and a concentration gel (T: 4.9%, C: 2.7%, 0.03% (p/v)SDS 0.125M Tris-HCl buffer containing 0.03% (p/v) SDS, pH 6.8).

Samples (2 mg/mL) are placed 0.12M Tris-HCl buffer containing 2% SDS and 5% 2-mercaptoethanol, pH 6.8. Samples were heated at 100℃/3 min before adding glycerol 50% (v/v) containing bromophenol blue. Electric buffer contain Tris 0.05M, glycine 0.4M and 0.1% (p/v) SDS. The electrostatic condition was executed during 2 h 30 min in 60 mA, 500 V and 30 W.

- *Fixation, Staining and Destaining*

Fixation was executed 12% TCA during 2 hrs. and then stained with 0.1% Comassie blue R-250 containing ethanol 50% and 2% TCA during 2 hrs, Destaining was carried out in 30% ethanol with 7.5% acetic acid.

RESULTS AND DISCUSSIONS

Determination of Minerals

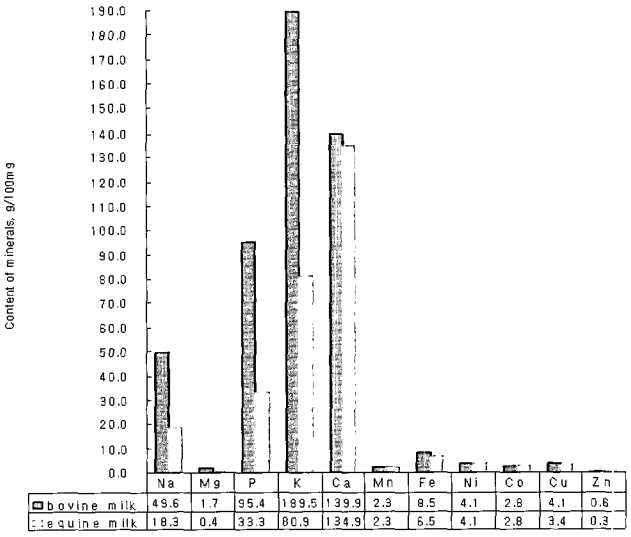
Minerals maintain the life and function of cells in body composition. Determined minerals concentration shown as a histogram in Fig. 1.

The indicated concentration of Na, P, K and Ca in bovine milk indicated was higher than that of equine and it is same in Mn, Co and Zn. But there are a few differences in Fe, Cu, Mg and Zn between two species.

Choi (1999) has been reported that minerals in bovine milk were 48 mg for sodium, 12 mg for magnesium, 120 mg for calcium, and 157 mg for potassium. These results are similar to those determined in our research. And Csapo-Kiss (1995) has shown that minerals in equine milk were 7.5~23.7 mg for sodium, 0.29~135 mg for magnesium, 21.6~120.5 mg for phosphorus, 48.5~135 mg for calcium, and 30.3~99 mg for potassium per 100 g. These are also similar to our research.

The determination of several minerals by ICP/MS before neutron activation analysis has various advantages such as precision, low sample volume consumption, excellent detection limits, consistant sample quality throughout, and a wider dynamic range than spectrophotometry or atomic absorption spectrometry.

There is no distinct evidence yet for the superiority of



	Na	Mg	P	K	Ca	Mn	Fe	Ni	Co	Cu	Zn
bovine milk	49.6	1.7	95.4	189.5	139.9	2.3	8.5	4.1	2.8	4.1	0.6
equine milk	18.3	0.4	33.3	80.9	134.9	2.3	6.5	4.1	2.8	3.4	0.3

Fig. 1. Histogram for minerals concentration(mg/100g) determined by ICP/MS.

equine milk as a human food from the point of mineral composition.

Distribution of Nitrogenous Substances

The result of the N distribution for bovine and equine milk is shown in Table 2. Total nitrogen in bovine milk was 0.417 %, compared to 0.603% in equine milk. The concentration of total nitrogen in equine milk is higher than bovine, which means more proteinous. This is a confirmed by a the nearly double body weight at birth an equine's offspring (Csapo-Kiss, 1995). The ratio of NPN to Nt is 7% for bovine milk and 9.8% for equine milk. On the other hand, the ration of NCN to Nt was represented 16.7% for bovine milk and 45.6% in equine milk. This could be a result of phylogenical characteristics between ruminant and monogastric, as equine milk has a casein or coagulables fraction at pH 4.6 which can not be easily coagulate by rennet.

The results above in the ration of casein and whey was similar to Bouman and Vander Schee (1978) and Doreau (1999) which indicated the same results as ours.

From the these results equine milk can not be applicable for cheese production as there are no coagulable nitrogen fraction such as the κ-casein found in bovine milk. Any equine milk will be more acceptable if we acknowledge the phylogenic affinity to human milk which is similar to equine in view points of monogastric which did no contain ruminant's caseins.

Rennet Coagulation Time (RCT)

Coagulation with rennet, evaluated by appearance of film on the wall of test tube by naked eye, is know to be a simple and correct away to establish on enzyme reaction. This is an important factor in cheese manufacturing. In addition, it can be detected heated milk by the change of casein micelles by heating, which could not generated coagulation. In this experiment, we can verify that casein of equine milk can not be coagulated for cheese with above reason. Fig. 2 represents the photo of observed rennet coagulation in tube of bovine and equine milk with rennet and without Ca. According to this photo, we can easily observe coagulation of bovine milk and the Ca addition shortened the coagulation time.

But in equine milk, rennet coagulation did not taken place, even after letting it stand 2 hrs with the rennet plus addition of Ca, due to equine κ-casein. The first phase of coagulation appearance is hydrolysis $Phe_{105}-Met_{106}$ bond of κ-casein. Equine casein is similar to monogastric animal casein (Iametti *et al.*, 2001) in that ruminant and coagulation not be occurred as well as bovine milk. But we can provoke the coagulate with the addition of separated bovine κ-casein into equine milk. The rested problem is that the time of coagulation should occurred with inappropriate time in several hours.

This would be more ameliorate by ordinate the environmental condition such as ionic concentration, pH, etc. in presence of polyphosphate which could form κ-casein network.

Another important situation or factor is the RCT related to ratio of Ca/Nt which is 0.29 in the case of bovine milk. Equine milk RCT is 0.22 according to Fig. 1 and Table 2. It is well known that increase when the ratio of Ca/Nt (0.23) during the coagulation time prompts the reaction in bovine milk (Alais, 1975). Our equine milk has lower content of Ca/Nt than bovine milk, so it should coagulate more slowly. All of the publications refer to the lower concentration of Ca/Nt. For equine milk, this could explain why the distance of casein and Ca is so far because casein is low. Which result in reaction of casein with Ca could not activated which relented time of coagulation is long.

Table 2. Distribution of Nitrogens (%)

Sample \ N fraction	NT	NPN	NCN	NPN/NT (%)	NCN/NT (%)
Bovine milk	0.471	0.033	0.079	7.0	16.7
Equine milk	0.603	0.059	0.275	9.8	45.6

1) SDS-PAGE

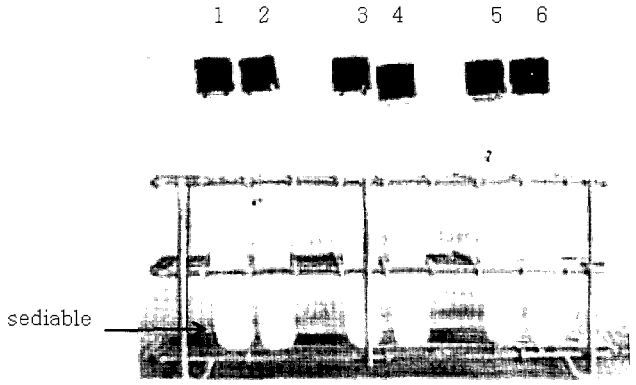


Fig. 2. Coagulation of the bovine and equine milk by rennet and rennet after added bovine κ-casein.

Table 3. Explanation for rennet coagulation on Fig. 2.

Sample No.	Milk	Enzyme	Ca^{2+}	κ-casein	Coagulation
1	Bovine milk	rennet	-	-	○
2	Bovine milk	rennet	○	-	○
3	Equine milk	rennet	-	-	×
4	Equine milk	rennet	○	-	×
5	Equine milk	rennet	-	-	×
6	Equine milk	rennet	○	○	○

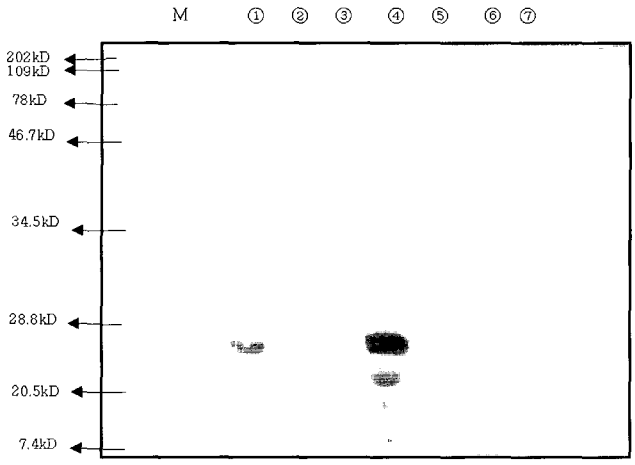


Fig. 3. SDS-PAGE of the caseins by rennet of equine and bovine milk. M; Marker, 202 kD - Myosin, 109 kD - β-galactosidase, 78 kD - Bovine serum albumin, 46.7 kD - Ovalbumin, 34.5 kD - Carbonic anhydrase, 28.8 kD - Soybean trypsin inhibitor , 20.5 kD - Lysozyme, 7.4 kD - Aprotinin. ① α-casein; ② β-casein; ③ κ-casein; ④ casein of bovine; ⑤ whey of bovine; ⑥ casein of equine; ⑦ whey of equine.

The change of rennet casein fraction by the use of SDS-PAGE as observed in Fig. 3 showed that bovine casein was markedly separated but equine milk was not separated κ-casein.

All casein in bovine milk existed Fig. 3, ④ according to standard casein (Fig. 3, ①, ② and ③) while casein of equine milk κ-casein was not observed in Fig 3, ⑥. In the case of equine κ-casein existing interior casein micelles κ-casein may be exist between α- and β-casein (Adam, 1998). The possibility existence of κ-casein as a hypothesis in human milk has still yet to be confirmed, so there are conflicting hypothesis (Brignon, *et al.*, 1985 ; Malacarne *et al.*, 2002).

Anyway it is clear that the milk of monogastric could not be coagulable with rennet without other treatment such as addition to κ-casein, etc.

Recently, the study of equine κ-casein has been reported that equine κ-casein was identified and characterised (Egito *et al.*, 2002 ; Iametti *et al.*, 2001). The ration of κ-casein in equine mink is lower than that of bovine and human milk (Egito, *et al.*, 2001).

Camel milk is the same problem of equine milk, so there is coagulation helper agent which help coagulation of camel milk.

2) Observation of SEM in Bovine and Equine Milk

A very effective way to understand and conform the phenomenon of milk coagulation with rennet is through the use of an electron microscope, which can evaluate visually and more obviously than biochemical method.

We have visualized the raw state of bovine and equine milk that represents the longer (diameter-255 nm) global shape of casein or proteinic micelle in equine milk than those of bovine (diameter-182 nm)(Egito *et al.*, 2002), results in agreement with the precedent set forth in other results (Fig. 4, photo 1 and 2).

In photo 2 and 4 of Fig. 4a it is very clear that equine milk could not be coagulated with only rennet., which differentiate the formation of typical coagulation because of the absence of κ-casein in equine milk (Alais, 1975; Humbert, 1979; Walstra and Jenness, 1984). From these reasons we want to approach more similar environment of condition with compare to bovine milk by addition of sufficient Ca^{2+} to equine milk which (Fig. 4, photo 5) represent the phase of approached micelle with more great density but not a phase of typical enzymatic coagulum differ from photo for 8 then because more distinctly represent typical coagulum in phto 6.

From this we can be conclude that the addition of Ca could preliminarily favorize the enzymatic coagulative condition and (photo 5) that the addition of κ-casein with Ca^{2+} to equine milk could provoke the formation of coagulum comparable to bovine milk, but the density of coagulum will be looser (photo 6) than of bovine (data is not shown because bovine milk coagulation is generally well known and casein of bovine milk consist of α-, β-, κ-casein so it is not necessary to add κ-casein into bovine milk).

It can be also bring out the addition of bovine milk or ovine milk to equine milk which could be coagulate the equine milk and which could explain the added κ-casein,

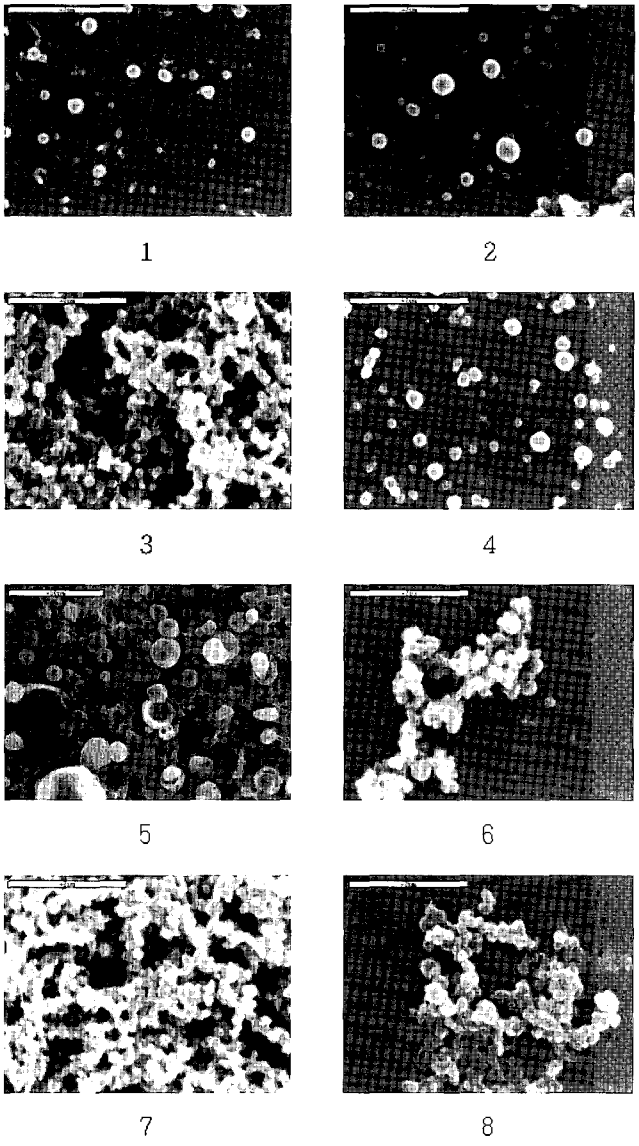


Fig. 4. Coagulation of bovine and equine milk without calcium by scanning electron microscopy. 1. Raw bovine milk, 2. Raw equine milk, 3. Bovine milk+rennet, 4. Equine milk+rennet, 5. Equine milk+rennet+Ca^{2+}, 6. Equine milk+ κ -CN+rennet+Ca^{2+}, 7. Acidification of bovine milk to near isoelectronic point (pH 4.6), 8. Acidification of equine milk to near isoelectronic point (pH 4.2).

bovine and ovine milk form the coagulable network with several environmental condition.

Difference in density of casein micelle between the two milks also indicated in photo 7 and 8, which show the looser formation of equine milk than bovine at isoelectric points.

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