Natural State Changes of Cows' and Buffaloes' Milk Proteins Induced by Microbial Transglutaminase

F.H.R. Abd-Rabo, S. M. El-Dieb, A.M. Abd-El-Fattah and S.S. Sakr

Dairy science Department, Cairo University, Giza, Egypt

Abstract: Incorporation of some amino acids in milk protein as a result of cross-linking by Microbial Transglutaminase (MTGase) was investigated. Effect of MTGase on electrophoretic patterns, microstructure, micellar hydration and sedimentable solids of milk proteins as well as the viscosity of whole and skim cows and buffaloes milk was also studied. Incubation of milk with MTGase at 40^{O} C for 1h prior to thermal inactivation (at 80^{O}C/2min) resulted in a complete incorporation of glutamine and argynine in skim cows milk protein and glysine and valine in skim buffaloes milk protein. That treatment also induced reductions in levels of monomeric caseins (αs_1-, β-, and κ-caseins) and an increase in the fractions of relatively low electrophoretic mobility. The effect of MTGase on the microstructure of treated samples was quite clear; the enzyme was capable of forming covalent linkages between protein molecules. The micellar hydration and viscosity of treated skim milk samples were markedly improved and were the highest between the samples makes it possible to produce different types of dairy products with low fat contents or a reduced content of non-fat solids. [Journal of American Science 2010; 6(9):612-620]. (ISSN: 1545-1003).

Keywords: Microbial Transglutaminase; electrophoretic pattern, microstructure, micellular hydration

1. Introduction:

Microbial Transglutaminase (Ca^{2+} independent) is a single polypeptide with a molecular weight of about 38000, consisting of 331 amino acids and it has isoelectric point pH 8.9 (Sakamoto et al. 1994; Schorsch et al. 2000; Özrenk 2006). Transglutaminase catalyses an acyl transfer reaction between γ-carboxyamide groups of peptide-bound glutamine residues (acyl donor) and the primary amino groups in a variety of amine compounds (acyl acceptor), including peptide-bound ε-amino groups of lysine residues. As a result of cross-linking of peptide-bound glutamine and lysine residues ε-(γ-glutamyl) lysine iso-peptide bonds and high-molecular weight polymers are formed. In the absence of amine substrates, transglutaminase is capable of catalyzing the deamidation and amine incorporation of glutamine residues as shown in Fig.1 (Sharma et al. 2001; Soares et al. 2004).

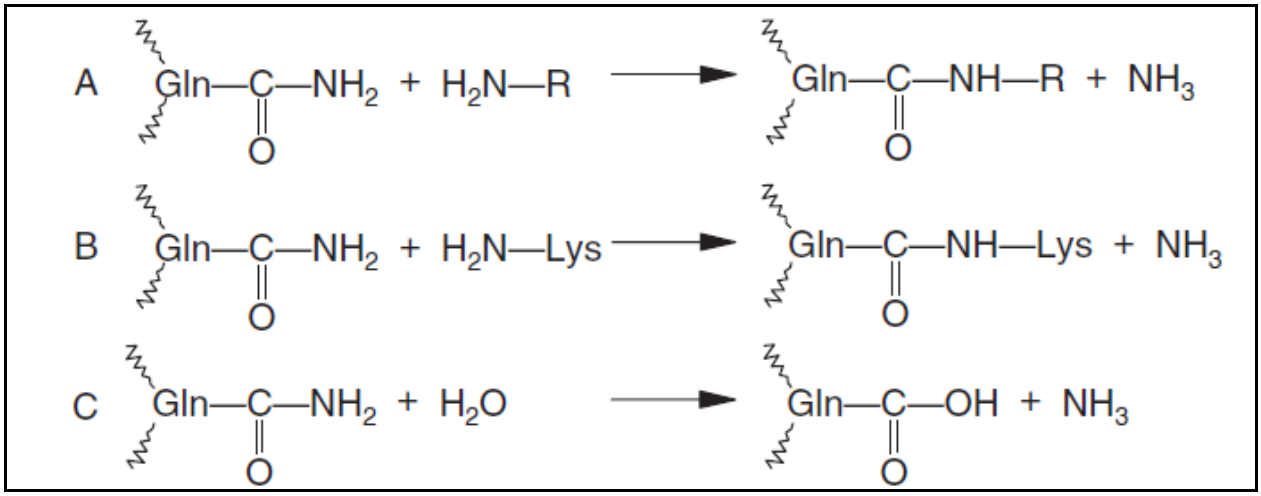

Fig. 1. : Reaction catalyzed by Transglutaminase (Jaros et al., 2006)
(A) Acyl transfer reaction; (B) Cross-linking reaction; (C) Deamidation.

MTGase can be used to modify proteins to improve their nutritional and functional properties (Huang et al. 1995; Motoki and Seguro, 1998). For this reason, MTGase technology is going to be an essential tool for protein modification in both food and non-food processing in the future (Motoki and Siguro, 1998). So, the natural state changes of cows and buffaloes milk proteins induced by MTGase were investigated.

2. Materials and Methods:

Materials

Whole and skim cows and buffaloes milk were obtained from the Dairy science Department, Faculty of Agriculture, Cairo University. A microbial preparation of Transglutaminase (EC: 2.3.2.13) obtained from *Streptoverticillium mobaraense*, commercially available as (ACTIVA® YG) was used with no extra purification. The enzyme (declared activity of $1000Ug^{-1}$) was a gift from Ajinomoto Europe sales GmbH, Hamburg, Germany.

Experimental procedure

Raw milk samples were analyzed for total solids (TS), total nitrogen (TN) and protein % (Tab. 1.).

Tab. (1) : Total solids and protein contents of whole and skim Cows' and Buffaloes' milk

Milk sample	T.S%	T.N%	Protein%
Whole Cows' milk	12.6	0.5831	3.7
Skim Cows' milk	9.4	0.6704	4.3
Whole Buffaloes' milk	15.6	0.6100	4.0
Skim Buffaloes' milk	11.6	0.6872	4.4

Treated samples were prepared according to the following flow chart:

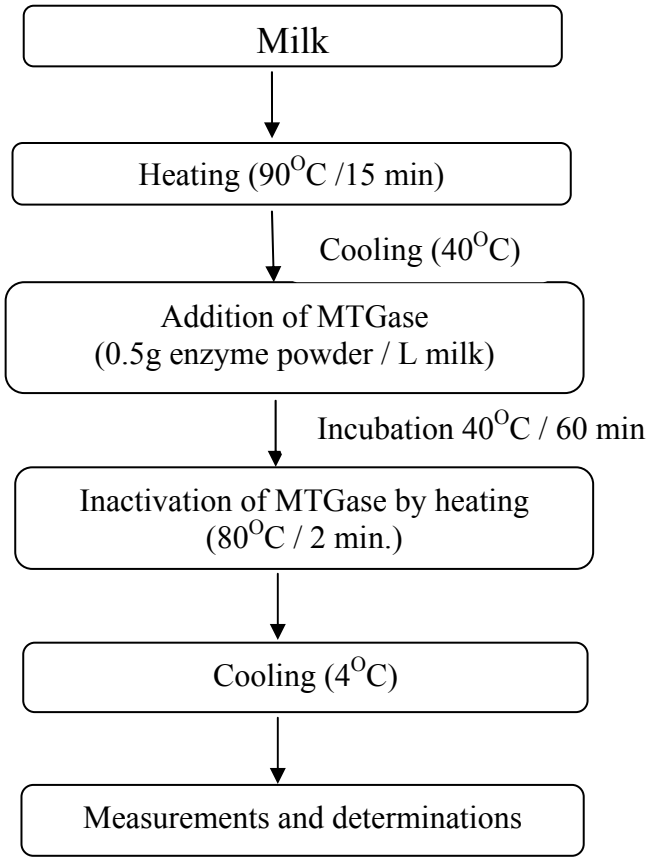

Fig. 2. : Preparation of treated milk samples

Determination of free amino acids

Free amino acids were determined after extraction by using diluting buffer. Automatic amino acid analyzer (AAA 400 INGOS Ltd, lab-Faculty of Agriculture-Cairo University) was used for the determination.

Electrophoretic patterns determination

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) technique was employed. Modified technique by Abdel-Ghany (1984) was applied to obtain the electrophoretic patterns of milk proteins. The bands were quantified using phoretix 1D quantifier, (Linear Dynamics, England).

Microstructure of casein micelles

Microstructure of casein micelles were examined using Transmission electron microscope (TEM), JEOL JEM.1400 el. The method of Garcia-Risco et al. (2000) was used for sample preparation.

Micellar hydration and Sedimentable solids

Beckman L7-65 ultracentrifuge with Rotor type 70.1 Ti (Serial No. 93U, 33 53 made in USA) at 110.000xg (37.000 rpm) / 4^{O} C was used to precipitate milk casein micelles. The method suggested by Huppertz et al. (2004) was used to determine the micellar hydration and sedimentable solids of casein micelles.

Measurement of milk viscosity

Brookfield DV II + pro viscometer (Brookfield lab. USA) was used to determine the apparent viscosity of milk samples at 25^{O} C±1 by using UL S00 spindle. The reading was detected after stability.

Statistical analysis

A randomize complete block design was used for analysis all data with three replications for each parameter. The treatment means were compared by least significant differences (L.S.D.) test as given by Snedecor and Cochran (1976).

3. Results and Discussion:

Data obtained in Tab. 2 show the percent of some amino acids in milk protein as a result of cross-linking by MTGase in treated samples. Glutamine and Arginine were completely incorporated in skim Cows′ milk protein, while Glysine and Valine were in skim Buffaloes′ milk protein. In contrast to free amino acids, the concentration of ammonia increased as shown in the same Table. The percent increase was 298.6% and 291.9% in skim and whole Cows' milk and 165.9% in skim Buffaloes' milk.

MTGase can modify most food proteins such as egg yolk, milk caseins, α-lactalbumin (α-lac.) and β-lactoglobulin (β-lg.) as well as many other albumins by means of amine incorporation, cross-linking and deamidation (Jaros et al., 2006, Fig.1).

As shown, the MTGase is capable of incorporating amino acids or peptides covalently into proteins. This reaction can improve nutritive values of food proteins, because covalently incorporated amino acids or peptides behave like amino acid residues in a protein. For instance, caseins and soya proteins in which Methionine and Lysine respectively are limiting factors could be improved by such a MTGase reaction. It is also obvious that the incorporation of amino acids is accompanied by an increase in the ammonia (NH3) content (Motoki et al. 1986; Motoki and Seguro, 1998; Sharma et al. 2001).

In this point preliminary experiments were run to follow the MTGase reactions in whole and skim Cows′ and Buffaloes′ milk.

Electrophoretic pattern of milk proteins as affected by MTGase

The electrophoretic pattern of whole and skim cows and buffaloes milk with and without MTGase is shown in Fig. 3. The rate of cross linking is quite clear in treated samples in comparison with untreated samples. Incubation of milk with MTGase at 40^{O}C for 1h. resulted in a noticeable reduction in κ-casein and a significant reduction in the levels of monomeric α_{s1}- and β-caseins, β-lactoglobulin and α-lactalbumin as determined by SDS-PAGE. Such reduction (Tab. 3) in monomeric caseins is due to MTGase induced cross-linking between protein molecules. These results agree with those of Nonaka et al. (1997) and Sharma et al. (2001). They mentioned that, milk caseins, **α**-lactalbumin and β-lactoglobulin as well as many other albumins have been shown to be excellent substrates for MTGase – induced cross-linking. Also, Hinz et al. (2007) found that the apparent results of cross-linking of κ-casein or β-casein were considerably faster than that of α_s1-casein.

Transglutaminase induced reductions in levels of monomeric caseins were accompanied by the appearance of fractions of relatively low electrophoretic mobility (High molecular weight bands shown in Fig. 3. The level of polymers increase was 283 and 311 % in treated whole and skim Cows' milk and 70.64 and 77.62 % in treated whole and skim Buffaloes' milk respectively. These results are in agreement with Wróblewska et al. (2009) results. They mentioned that the addition of MTGase caused partial transformation of proteins into high molecular polymers.

Tab.2. Free amino acids in cows and buffaloes milk as affected by Microbial Transglutaminase

Amino acids %	Treatments					
	C1	T1	T2	C2	T3	T4
Nutritionally nonessential amino acids						
Glutamine	0.0327^{b}	0.0296^{c}	-	0.0946^{a}	-	0.0049^{d}
Glysine	0.0134^{a}	0.0007^{c}	0.0009^{c}	0.0039^{b}	-	-
Alanine	0.0573^{a}	0.0072^{c}	0.0035^{d}	0.0143^{b}	-	0.0052^{d}
Tyrosine	0.1845^{a}	0.0142^{c}	0.0049^{d}	0.0375^{b}	-	0.0316^{c}
Nutritionally essential amino acids						
Lysine	0.0254^{a}	0.0045^{c}	0.0016^{d}	0.0127^{b}	-	0.0060^{c}
Thrionine	0.0354^{a}	0.0036^{c}	0.0026^{c}	0.0115^{b}	-	0.0107^{b}
Valine	0.0310^{a}	0.0099^{b}	0.0018^{c}	0.0112^{b}	-	-
Methionine	0.0277^{a}	0.0064^{c}	0.0006^{e}	0.0155^{b}	-	0.0038^{d}
Leucine	0.1133^{a}	0.0339^{c}	0.0042^{e}	0.0553^{b}	-	0.01497^{d}
Histidine	0.0465^{a}	0.0040^{c}	0.0026^{c}	0.0086^{b}	-	0.0078^{b}
Arginine	0.4001^{a}	0.2053^{b}	-	0.2038^{bc}	-	0.2020^{c}
Other						
Amonia	0.0296^{d}	0.1160^{b}	0.1180^{a}	0.0123^{e}	-	0.0327^{c}

C1: whole Cows' milk without MTGase. C2: whole Buffaloes' milk without MTGase.
T1: whole Cows' milk with 0.05% MTGase. T3: whole Buffaloes' milk with 0.05% MTGase.
T2: skim Cows' milk with 0.05% MTGase T4: skim Buffaloes' milk with 0.05% MTGase.
(-): Data not shown
a, b, c, d means in the same raw with different superscripts differed significantly at ($P< 0.05$).

Microstructure of milk proteins as affected by MTGase

Transmission electron micrographs of whole and skim cows and buffaloes milk with and without transglutaminase are shown in Fig. 4. The effect of Transglutaminase on the microstructure is quite clear; the enzyme was capable of forming covalent linkages between protein molecules. The addition of MTGase allowed the gel to form much more quickly compared with samples without MTGase. In other words a noticeable high molecular weight polymer as a result of cross-linking of peptide-bound glutamine and lysine residues in treated samples were formed. Data obtained also showed that the aggregates of protein molecules in treated skim Cows' milk were the highest between treatments.

These results are in agreement with Hinz a, b et al. (2007) who mentioned that cross-linking of food proteins by MTGase can modify their gelatin and rheological properties. Schorsch et al. (2000) also reported that gels with different characteristic and some unusual and interesting features in terms of strength, kinetics of gelatin and synersis behavior can be formed using Transglutaminase.

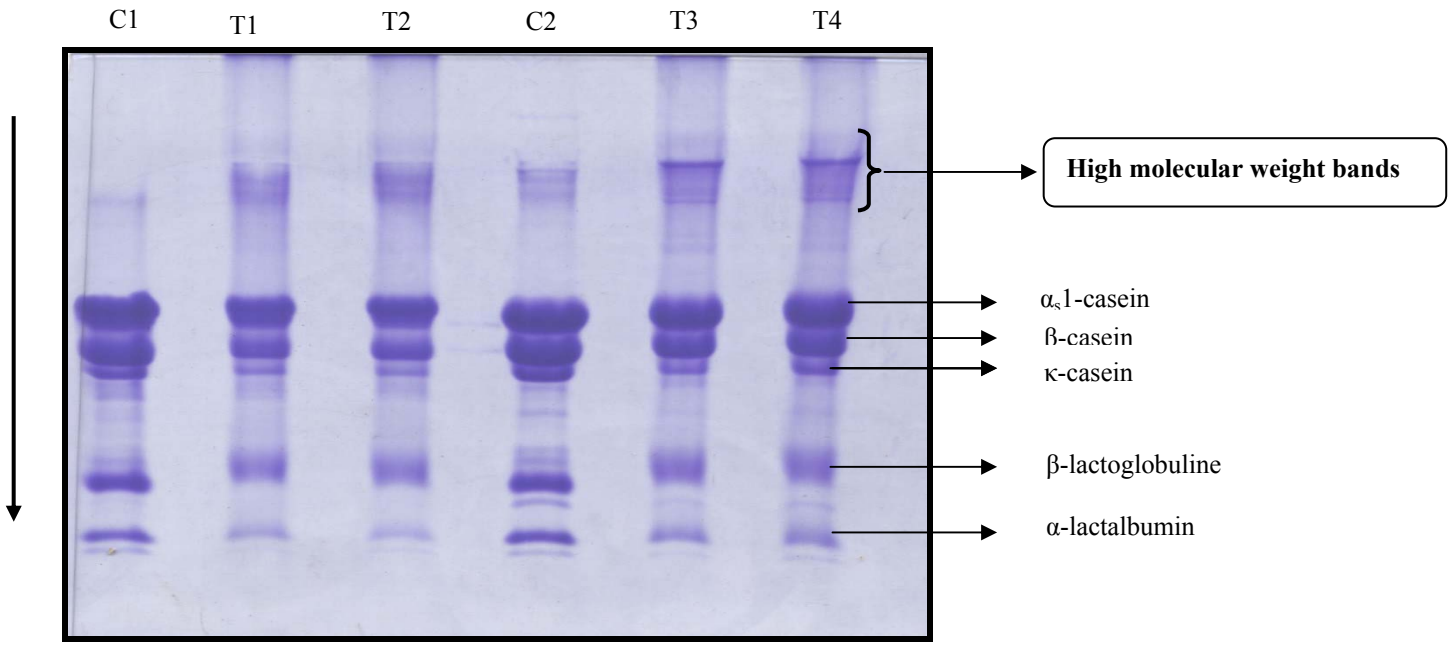

Fig. 3.: Sodium dodecyl sulphate-PAGE electrophoretograph of whole Cows' and Buffaloes' milk samples.

C1: whole Cows' milk without MTGase.
T1: whole Cows' milk with 0.05% MTGase.
T2: skim Cows' milk with 0.05% MTGase
C2: whole Buffaloes' milk without MTGase.
T3: whole Buffaloes' milk with 0.05% MTGase.
T4: skim Buffaloes' milk with 0.05% MTGase.

Micellar hydration and sedimentable solids of milk proteins

Pertaining to the effect of MTGase addition, Tab.4. shows a significant increase in the level of hydration of the cross- linking protein polymer and decrease in the level of sedimentable solids in comparison with untreated samples. The percent increase in hydration level was 29.9 and 54.3% in treated whole and skim cows milk and 29.5 and 32% in treated whole and skim buffaloes milk respectively.

Motoki and Seguros (1998) mentioned that yoghurt is a milk gel formed by acidic fermentation with lactic starter, but it may suffer from problems of serum separation with a change in temperatures or physical impacts and this can be solved by adding MTGase which improves the water holding capacity of the gel. They also mentioned that MTGase reaction makes it possible to produce dairy products, such as ice cream and cheese with low fat contents or a reduced content of non-fat-solids. Lorenzen (2000) also reported that the water binding capacity and viscosity-enhancing properties of sodium caseinate were increased above average as a result of enzymatic modification.

Viscosity of skim and whole cows and buffaloes milk as affected by MTGase

As shown in Tab. (5) the viscosity of milk was influenced by several factors and can be enhanced significantly by an enzymatic treatment. There was a significant increase in the viscosity mean of treated samples as compared with the control treatment being 1.62, 2.09 and 1.85 c.p for C1, T1 and T2 and 1.85, 3.07 and 2.02 c.p for C2, T3 and T4 respectively. As for the effect of rpm, there was a significant increase in the viscosity mean as the rpm increased. At rpm from 120-140 the viscosity mean was 1.70, 1.80 and 1.86 c.p for Cows' milk and 201, 2.16 and 2.18 c.p for Buffaloes' milk samples respectively.

On the other hand, the viscosity value in relation to milk total solids was higher in treatments T2 and T4 than the others. Thus, it can be concluded that through using MTGase the skim milk can be used as a substitute for whole milk in products of dairy products with good structure, low fat content or a reduced content of non-fat solids.

Tab. 3. Band density of Cows' and Buffaloes' milk protein fractions as affected by Microbial Transglutaminase

Treatments	α-lactalbumin		β -lactoglobulin		κ-casein		β-casein		α-casein		High molecular weight polymers	
	%	Change %	%	Change %	%	Change %	%	Change %	%	Change %	%	Change %
						Cows' milk						
C1	8.65[a]	-	18.32[a]	-	7.14[a]	-	19.29[a]	-	27.76[a]	-	7.05[c]	-
T1	5.96[b]	-31.10	15.54[b]	-15.17	5.66[a]	-20.73	15.36[b]	-20.37	22.97[b]	-17.23	27.00[b]	+283
T2	5.06[b]	-41.50	16.56[ab]	-9.61	5.68[a]	-20.45	15.70[b]	-18.61	23.58[b]	-15.06	29.00[a]	+311
						Buffaloes' milk						
C2	8.93[a]	-	23.00[a]	-	7.38[a]	-	17.28[a]	-	23.68[a]	-	14.34[b]	-
T3	7.83[a]	-12.32	15.98[b]	-30.52	5.65[a]	-23.44	14.31[a]	-16.84	19.69[a]	-16.85	24.47[a]	+70.64
T4	8.15[a]	-8.73	15.83[b]	-31.17	5.69[a]	-22.90	12.96[a]	-25.00	21.88[a]	-7.60	25.47[a]	+77.62

C1: whole Cows' milk without MTGase. C2: whole Buffaloes' milk without MTGase.
T1: whole Cows' milk with 0.05% MTGase. T3: whole Buffaloes' milk with 0.05% MTGase.
T2: skim Cows' milk with 0.05% MTGase T4: skim Buffaloes' milk with 0.05% MTGase.
a, b, c means in the same column with different superscripts differed significantly at ($P< 0.05$)

Tab. 4. : Micellar hydration and Sedimentable solids of milk proteins

Treatment	Micellar hydration (gH_2Og^{-1} solids)		Sedimentable solids ($g100\ g^{-1}$ milk)	
	Amount	Change %	Amount	Change %
		Cows' milk		
C1	1.37[c]	-	5.10[a]	-
T1	1.78[b]	+29.9	4.99[a]	-2.2
T2	1.99[a]	+45.3	3.79[b]	-25.7
		Buffaloes' milk		
C2	1.22[b]	-	7.43[a]	-
T3	1.58[a]	+29.5	7.27[b]	-2.2
T4	1.61[a]	+32	5.33[c]	-28.3

C1: whole Cows' milk without MTGase. C2: whole Buffaloes' milk without MTGase.
T1: whole Cows' milk with 0.05% MTGase. T3: whole Buffaloes' milk with 0.05% MTGase.
T2: skim Cows' milk with 0.05% MTGase T4: skim Buffaloes' milk with 0.05% MTGase.
a, b, c, d means in the same column with different superscripts differed significantly at ($P< 0.05$).

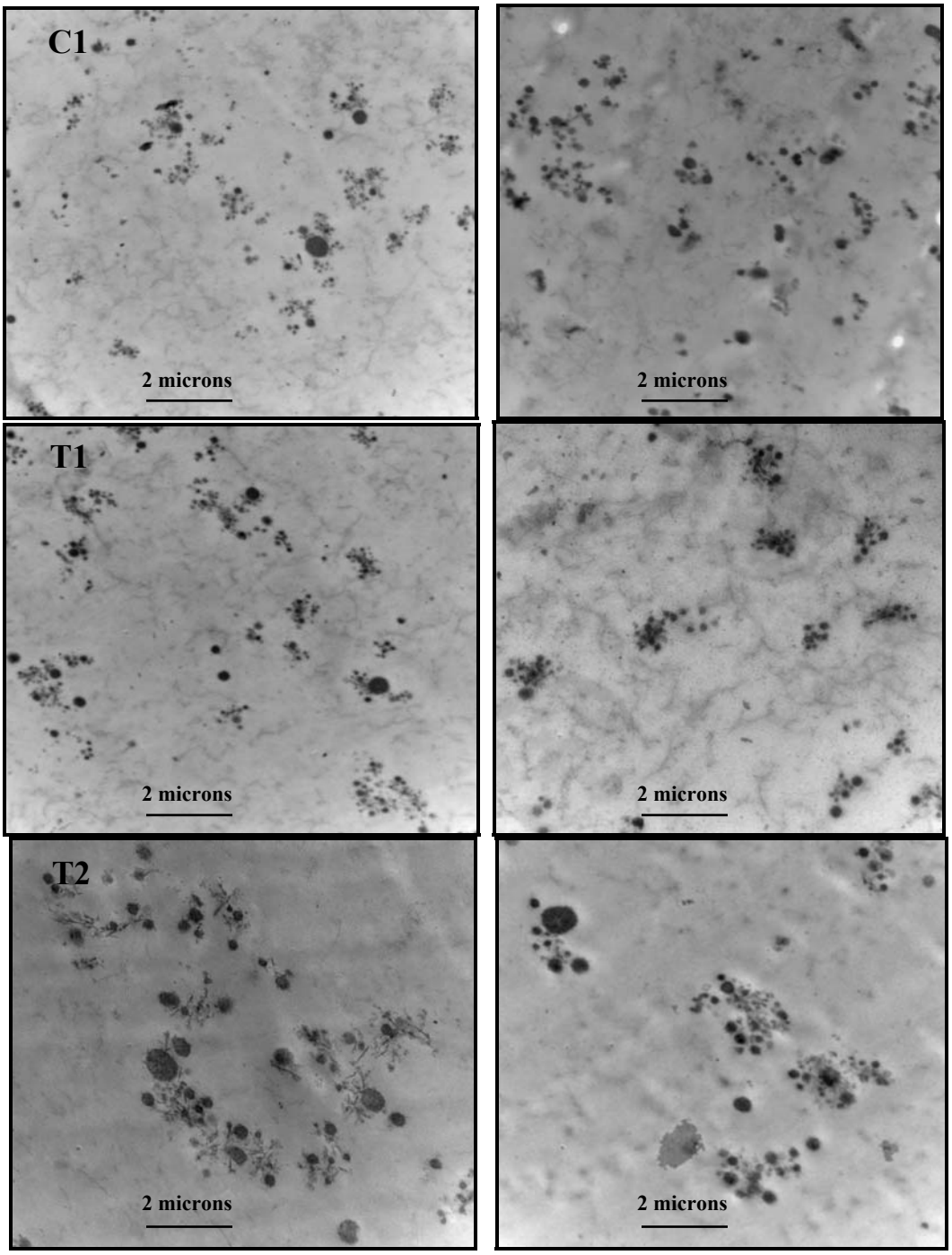

Fig. 4. : Transmission electron micrographs of milk proteins.

C1: whole Cows' milk without MTGase.
C2: whole Buffaloes' milk without MTGase.
T1: whole Cows' milk with 0.05% MTGase.
T3: whole Buffaloes' milk with 0.05% MTGase.
T2: skim Cows' milk with 0.05% MTGase
T4: skim Buffaloes' milk with 0.05% MTGase.

Tab. 5. : Viscosity of skim and whole cows and buffaloes milk with and without Microbial Transeglutaminase at different rpm

rpm	C1	T1	T2	Mean
		Cows' milk		
120	1.51^{f}	2.09^{a}	1.71^{d}	**1.70^{c}**
135	1.63^{e}	2.09^{a}	1.87^{c}	**1.80^{b}**
140	1.72^{d}	2.09^{a}	1.98^{a}	**1.86^{a}**
Mean	**1.62^{c}**	**2.09^{a}**	**1.85^{b}**	
		Buffaloes' milk		
	C2	**T3**	**T4**	
120	1.84^{c}	3.04^{a}	2.01^{b}	**2.10^{c}**
135	1.84^{c}	3.08^{a}	2.02^{b}	**2.16^{b}**
140	1.86^{c}	3.09^{a}	2.02^{b}	**2.18^{a}**
Mean	**1.85^{c}**	**3.07^{a}**	**2.02^{b}**	

C1: whole Cows' milk without MTGase. C2: whole Buffaloes' milk without MTGase.
T1: whole Cows' milk with 0.05% MTGase. T3: whole Buffaloes' milk with 0.05% MTGase.
T2: skim Cows' milk with 0.05% MTGase. T4: skim Buffaloes' milk with 0.05% MTGase.
a, b, c means in the same raw with different superscripts differed significantly at (P 0.05).

Corresponding author
F.H.R. Abd-Rabo
Dairy science Department, Cairo University, Giza, Egypt

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