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RENNETABILITY, RHEOLOGICAL, GEOMETRICAL AND MICROSTRUCTURAL PROPERTIES OF BUFFALO MILK GEL DURING COAGULATION PROCESS: IMPACT OF HEAT TREATMENTS

BY

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ABSTRACT

In this paper, three different milk heating temperatures often described in the literature, 63°C/30 min, 72°C/15 s and 85°C/15 s were compared to unheated milk (control treatment), for their influences on the enzymatic hydrolysis of κ-casein (primary phase) and the entire coagulation process (primary and secondary phases) by monitoring the shear stress (SS) and torque (T) forces. The impact of milk heating was also followed by measuring the rheological and microstructural properties of the induced gels. Heating buffaloes' milk decreased the rate of rennet-induced flocculation compared to unheated milk. Heat-induced enzymatic inhibition was showed by increasing the rennet onset time from 4 to 11, 7 and 12 min for raw milk and milk heated at 63°C/30 min, 72°C/15 s and 85°C/15 s, respectively. Heat treatment at 72°C/15 s caused a slight decrease in SS (70.09 D/cm^2) compared to unheated milk (74.6 D/cm^2), while those of 63°C/30 min and 85°C/15s decreased SS significantly. Torque showed similar profile to that of SS.

The rheological properties of rennet-induced gels (intensity, sphericity, compactness, surface roughness, matrix area and pores area) were significantly affected by heat treatments ($p<0.001$). Raw milk gel exhibited the lowest intensity, roughness, compactness, sphericity and matrix area (42.6%, 0.57, 0.44, 0.58 and 44.5%, respectively). Heating milk at 63°C/30 min, 72°C/15 s and 85°C/15 s significantly ($p<0.001$) decreased the gel pores area (30.9%, 27.4% and 40.7%, respectively) compared to that of unheated milk (55.4%). Raw milk gel seemed to be homogenous and have more connections between particles in a three-dimensional network and more pores producing a porous structure. No noticeable heat-induced effects were observed on the structure of the casein micelle fraction gel of heated milk at 72°C/15 s.

INTRODUCTION

The chemical and physical changes occur in milk during cheese making determine the rheological properties and microstructural characteristics of cheese products.

The initial stage of manufacturing most varieties of cheese involves the rennet coagulation of milk; structural changes occurring during this stage have implications for the development of the physical properties of the finished cheese. There are two distinguishable phases in the milk clotting process. In the primary phase, 80 to 90% of the κ-CN molecules in the casein micelle are cleaved before casein aggregation is visible at the normal pH of milk (Dalgleish, 1993; Swaisgood, 1996; Singh & Wauguna, 2001 and Vasbinder *et al.*, 2003a). Further aggregation of micelles continues during the secondary phase to form the protein network or gel (Shalabi & Fox, 1982). Milk gels are usually classified as particle gels, although it is now recognized that they are not simple particle gels because the internal structure of the casein particle plays an important role in the rheological properties of milk gels (Horne, 1998). In milk gels, the overall visual appearance, microstructure, and rheological properties are important physical attributes, which contribute to the overall sensory perception and functionality of these products.

The casein particles also undergo rearrangement, fusion, and syneresis in the process of forming cheese curd (Dalgleish, 1993 and Green & Grandison, 1993). Casein-based gels are inherently dynamic in nature (van Vliet *et al.*, 1991; Walstra, 1993 and Mellema *et al.*, 2000). However, many of the physico-chemical events that transform the initial rennet-induced gel into cheese curd remain poorly understood (Green & Grandison, 1993).

Manufacturing of cheese is preferably carried out with unheated milk (Singh & Wauguna, 2001). However, heat treatment of cheese milk is an alternative method preventing microbiological defects in cheese (Solorza-Feria, 2000). Besides, it improves its keeping quality and achieves desirable properties in the final product (Singh & Wauguna, 2001). Upon heat treatment of milk above 60°C several processes take place, of which denaturation of whey proteins is the most obvious (Singh *et al.*, 1996 and Oldfield *et.al.*, 1998& 2000). Heat treatment of milk impaired clotting properties resulting in a weaker curd, therefore, making it less suitable for cheese manufacturing (Singh & Wauguna, 2001). In the literature, various explanations are given for this phenomenon, which either alone or in combination would cause the impaired clotting, such as 1) incomplete enzymatic hydrolysis (Vasbinder *et al.*, 2003b). 2) reduced concentration of serum calcium due to precipitation of calcium phosphate (Schreiber, 2001) and 3) stabilization of casein micelles due to the coating with charged denatured whey proteins (Steffl *et al.*, 1999; Schreiber, 2001 and Singh & Wauguna, 2001). However, conflicting results in the literature hamper complete understanding of impaired rennetability caused by heat treatment.

Structural and rheological properties of complex particle gels are now becoming reasonably well understood. What seems to be needed now is the establishment of a generic link between the interactions, structure and rheology.

The aim of this work was to use the rheological parameters to obtain experimental data about the effect of heating on renneting properties of buffaloes' milk. For that purpose, rennet aggregation and gelation phases were monitored by

measuring shear stress and torque. This was supplemented by the determination of several rheological properties and microstructure of the formed gel. Thus, better understanding of the effect of heat treatment as a key processing condition on the first and second stages of milk renneting could be obtained, which might be of interest as far as cheese making is concerned.

MATERIALS AND METHODS

Rennet gel formation

Raw buffaloes' milk was obtained from the herd of Faculty of Agriculture, Cairo University, Giza, Egypt. The chemical constitution; titratable acidity 0.16%, active acidity with pH value 6.65, total protein 4.1% and 6.5% fat content. Three equal portions of the milk were heated at 63°C /30 min, 72°C/15 s and 85°C /15 s. The heated milk samples and a control sample (unheated) were cooled to 37°C. Distilled water diluted calf rennet (four times) was used for milk coagulation as 0.4 ml (0.2 N)/180 ml milk.

Rheological properties of rennet gel

Shear stress (SS) and torque (T) at constant shear rate (12.2 1/s) were continuously monitored during coagulation process in triplicate measurements by means of rotational rheometer type Brookfield DVTII fitted with UL unit (Brookfield Engineering Lab., Inc., Stoughton, MA) connected to IBM compatible computer, equipped with Brookfield DV Gather+ 1.0 software, used to record for 25 min.

Image analysis of SE micrographes

Cubes (3x3x3mm) of the gel samples in triplicate for unheated and the three temperatures heated buffalo milk (63°C/30 min, 72°C/15 s and 85°C/15 s) were prepared for SEM examination according to El-zeny (1991). Samples were viewed through Scanning Electron Microscope (JEOL- JSM – 35.Tokyo, Japan) equipped with an IBM-compatible computer to record the images. Three photographs, for each sample, were taken and the images of at least three fields for each micrograph were analyzed by Climax Vision computer program (Climax Technologies Inc., Longueil, Qc., J4GITS, Canada). The images were digitally processed to produce binary images which were measured through the system to obtain several rheological and geometrical properties (El-zeini, 2006). These include: intensity (measures the amount of the pixels in defined volume), Sphericity (S: describes how close a shape to a perfect sphere, for a perfect sphere S=0), compactness (describes how dense the surface is depending on the distance between the pixels, the smaller the distance, the higher the compactness), surface roughness (measure the irregularity of the surfaces)and the matrix area (area of protein and entrapped fat globules, 2-dimension) expressed as a percent of the field total area. Assessment of the curd syneresis susceptibility was made by measuring the area of the pores in the protein matrix of the renneted gel.

Statistical analysis

A complete randomized block design was used to evaluate the effect of the treatments (milk pre-heating and incubation time) on the dependant variables

measured in triplicate using sub-program MSTAT (v4c, 1989, MSU, USA). Multiple Linear regression analysis was applied and "T" test and LSD were used to analyze the differences between means at $p< 0.01$.

RESULTS AND DISCUSSION

Rennet-induced gelation of heated milk

Rennet-induced gelation of heated milk was monitored by SS and T measurements. A clotting curves, showing rheological measurements (SS and T) over the period of milk renneting of raw milk, 63°C/30 min, 72°C/15 s and 85°C/15 s heated milks, are shown in Figure 1 and 3. During the early stages of the renneting reaction, the lag times increased with increasing milk heating temperature. Shear stress forces remained constant, and then began to rise approximately at 4, 11, 7 and 12 min after renneting of raw milk, 63°C/30 min, 72°C/15 s and 85°C/15 s milks, respectively, (Figure 2). The points at which shear stress began to rise are a direct measurement of the onset times rennet attacked the sensitive bonds Phe105–Met106 in κ-casein molecules. These times were generally longer in gels made of milks heated at 63°C/30 min, 72°C/15 s and 85°C/15 s by 7, 3 and 8 min, respectively, over that of raw milk ($p<0.001$). Additionally, highly significant differences ($p<0.001$) were found in SS as a respond of clotting advancing time. Values for maximum SS were 74.6, 50.4, 70.09 and 38 D/cm^2 for raw, 63°C/30 min, 72°C/15 s and 85°C/15 s milks, respectively (Figure 1). These results indicate that gels made from heated milk are weaker than that made from raw milk. Heat treatment might cause a slight change in casein micelles orientation, whey proteins denaturation and Ca^{2+} concentration (Steffl *et al.*, 1999; Allogio *et al.*, 2000 and Law & Leaver, 2000).

Torque profile was similar to that of SS (Figure 3 & 4). These results indicated that heating milk prior to gelation affects the primary and secondary phases of rennet action (Anema, 2000; Oldfield *et al.*, 2000 and El-Zeini, 2001). Heat treatment of milk results in a complex mixture of native whey proteins, whey proteins aggregates and casein micelles covered with appendages of whey proteins (Vasbinder *et al.*, 2003a and Vasbinder *et al.*, 2004). At higher temperatures as 85°C/15 s, more denaturation of whey proteins occurs which causes a decreased rate of clotting and flocculated rather poorly (Vasbinder& de Kruif, 2003 and Vasbinder *et al.*, 2001). There are various possible explanations for the effect of the heating on the gelation speed. One of them is shifting the enzyme onset time to higher values for heated milk gels (Dalgleish, 1993 and Vasbinder *et al.*, 2003b). Another one is that heat treatment of milk affects the electrophoretic mobility of the protein structures (Jang & Swaisgood, 1990). Additionally, calcium salts of milk are heat-sensitive component (Allogio *et al.*, 2000 and Metwally & El-Zeini, 2004).

Rheological and geometrical data

Six rheological parameters (intensity, roughness, compactness, sphericity, matrix area and pores area) were measured in renneted gels made of raw and heated buffaloes' milk at 63°C/30 min, 72°C/15 s and 85°C/15 s and shown in Figure 5. Heat treatment of milk resulted in significant ($p<0.001$)

changes in intensity, roughness, compactness, sphericity and matrix area for all gels tested and correlated well with gel intensity, roughness and sphericity. While, low correlations were maintained with gel compactness and matrix area (Table 1). Raw milk gel exhibited the lowest intensity, roughness, compactness, sphericity and matrix area (42.6%, 0.57, 0.44, 0.58 and 44.5%, respectively) which might be due to the presence of whey proteins and casein in the native forms. Heating the milk prior to cheese making brought about significant increase ($p<0.001$) in intensity, roughness and sphericity of the gels. At higher temperatures (85°C/15 s), compactness and matrix area of the gel (0.903 and 59.22%, respectively) were lower than those of milk heated at 63°C/30 min (0.78 and 69.03%, respectively) and 72°C/15 s (0.64 and 73.26%, respectively). The increase in the compactness and decrease in matrix area of the gel made of milk heated at 85°C/15 s showed that rennet coagulation was prevented either by lowering the concentration of soluble calcium as a result of milk heating or by the disulfide interactions between whey proteins and κ-casein (Jang & Swaisgood, 1990; Anema & Lie, 2003 and Metwally & El-Zeini, 2004). The increased gel surface roughness is partially caused by alteration in the surface properties of the whey protein-coated casein micelles (Ghosh *et al.*, 1996 and El-Zeini, 2001) and partially by the disulfide interactions occurring during the gel state (Anema, 2000). The highest sphericity was reported with gel of milk heated at 85°C/15 s (0.99) and the lowest with that of raw milk (0.58). These results indicated that heating of milk prior to renneting is the main factor causing an inhibition of rennet coagulation and altering the properties of the induced gels (Dalgleish, 1993).

Measuring the pores area is an indirect way to predict the gel syneresis. Heating milk at 63°C/30 min, 72°C/15 s and 85°C/15 s decreased the pores areas significantly ($p<0.001$) for all gels (30.9%, 27.4% and 40.7%, respectively) compared to that (55.4%) made of unheated milk (Figure 5). Raising the milk heating temperature to 85°C/15 s increased the gel pores area than those made of the other two heated milks. The decrease in the number of reticulation bonds may lead to a decrease of syneresis (Metwally & El-Zeini, 2004). Accordingly, the heat induced reduction of syneresis can be ascribed to β-lactoglobulin/κ-casein complex which interferes with micelle—micelle attractions responsible for the gel syneresis (Anema, 2000 and El-Zeini, 2001). On the other hand, water binding capacity of denatured whey protein is high and contributes to the water being retained in the curd (Pearce *et al.*, 1986).

Microstructure

Rennet casein structure evolution follows subsequent steps from casein molecules, which are attacked by rennet enzyme at the sensitive bond phe-meth 105-106, to particles (para-κ-casein) to flocs connected with Ca^{++} bridges and finally to a network holding all the milk components giving the curd gel. In the gel that was formed from raw milk seemed to be homogenous and have more connections between particles in a three-dimensional network and more pores producing a porous structure (Figure 6a). Gels made of milk pre-heated at 63°C/30 min consist of a network of small aggregates of micelles, alternated by thicker nodes of the same material, with less pores spread out through the matrix

compare with raw milk gel (Figure 6b). Slight noticeable heat-induced effects are observed on the structure of the casein micelle fraction gel of heated milk at the temperature 72°C/15 s (Figure 6c), casein micelles form a matrix consisting of their clusters and short chains and entrapped fat globules. Void spaces in the matrix are filled with the liquid milk serum (whey) and were less than those of raw and 63°C/30 min milk heated gels. Casein micelle clusters gradually shrink and the protein matrix becomes compacted. The gel clusters were more compacted (dark areas) than all other gels. That is due to a rearrangement in the internal structure of the coagulum took place at the end of the coagulation process [Lucey, 1995]. Differences in microstructure became noticeable between unheated and 85°C/15 s heated milk gels. Micrographs of the 85°C/15 s heated milk gel showed that casein micelles began to react with one another to form micellar aggregates, which tended to increase as a function of time (Figure 6d). Casein micelles had lost their individuality, spherical shape and boundary and appeared to be more deformed and fused than those in the unheated milk and heated at 63°C/30 min and 72°C/15 s. Casein micelles were arranged in large aggregates of particles with few connections. These microstructural differences caused by milk heating are coincidental to the differences in the rheological properties of the gels. This may be due to deformation took place in casein micelles structure as a function of heat treatment (El-Zeini, 2001).

CONCLUSIONS

Heat treatment of milk prior to cheese manufacture affects almost all aspects of coagulation process and curd rheological properties. By studying the effect of heat treatments on the renneting characteristics of raw and heated milks at 63°C/30 min, 72°C/15 s and 85°C/15 s, indicated that changes in the curd are related to changes in the casein micelles that occur during heating. Heat altered casein micelles orientation and denatured whey proteins which interacted with casein micelles. Therefore, heating of milks formed complex gels, unlike unheated milk, which had more intense, rough and compact structures. The protein matrix in the complex gels appeared to have less pores than those in gels made from the control gel of unheated milk. The surface roughness appears to be related to changes in the structure of the casein micelles as they interacted with denatured whey protein particles which spread through κ-casein molecules. In general, heat treatment of milk prior to cheese making affects not only the enzymatic phase, but also secondary phase of the coagulation process.

The rennet gel of heated milk that was most qualitatively similar to that of the control (unheated milk) was that one made from the milk heated at 72°C/15 s. Whilst, the curd that was least similar to the control gel was the one made from the milk heated at 85°C/15 s. So, this treatment was not preferable for making cheese. Therefore, heating milk at 72°C/15 s is the most suitable treatment for making cheese. Also, measuring SS and T can be a useful technique to monitor the rennet gelation process.

Table (1): Statistical analysis of rheological and microstructural parameters of buffaloes' milk renneted gel measured as a function of temperature and incubation time.

Factor	Source	F Value	Prob	LSD	Multi.R	Equations
Intensity(%)	Temperature (A)	1.7×10^5	0.001	0.1787	0.967	Y= 31.71 + 1.6075e+001
Sphericity		1.1×10^4	0.001	0.007	0.96	Y= 0.5075 + 1.34e-001
Roughness		1.06×10^3	0.001	0.02527	0.935	Y= 0.5 + 1.2687e-001
compactness		4.664×10^3	0.001	0.0079	-	Y= 0.51925 + 7.3667e-002
matrix area%		6.83×10^4	0.001	0.16720	-	Y= 49.4275 + 4.824e+000
Pores area%		5.16×10^4	0.001	0.1895	-	Y= 50.4999 – 4.7480e+000
Shear Stress (SS)	Temperature(A)	18.4×10^6	0.001	0.03677	0.788	Y= -17.722 – 3.2 e-001
	Time (B)	22.3×10^6	0.001			Y= - 17.722 – 2.9 e+000
	AB	14.5×10^5	0.001			
Torque (T)	Temperature(A)	1.5×10^3	0.001	5.185	0.792	Y= -23.15 – 4.8 e-001
	Time (B)	2.03×10^3	0.001			Y= -23.15 + 3.7 e+000
	AB	1.2×10^2	0.001			

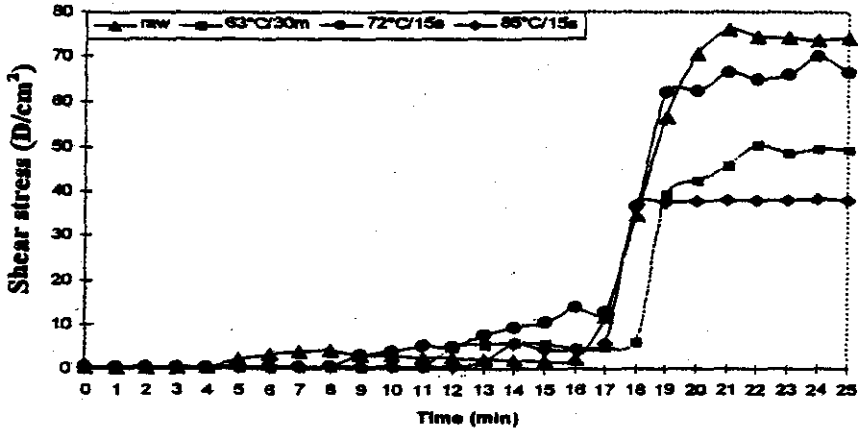

Fig. (1): Shear Stress (D/cm^2) profiles as a function of time for rennet gels made from raw and heated buffaloes' milk at 63°C/30 min, 72°C/15s and 85°C/15s.

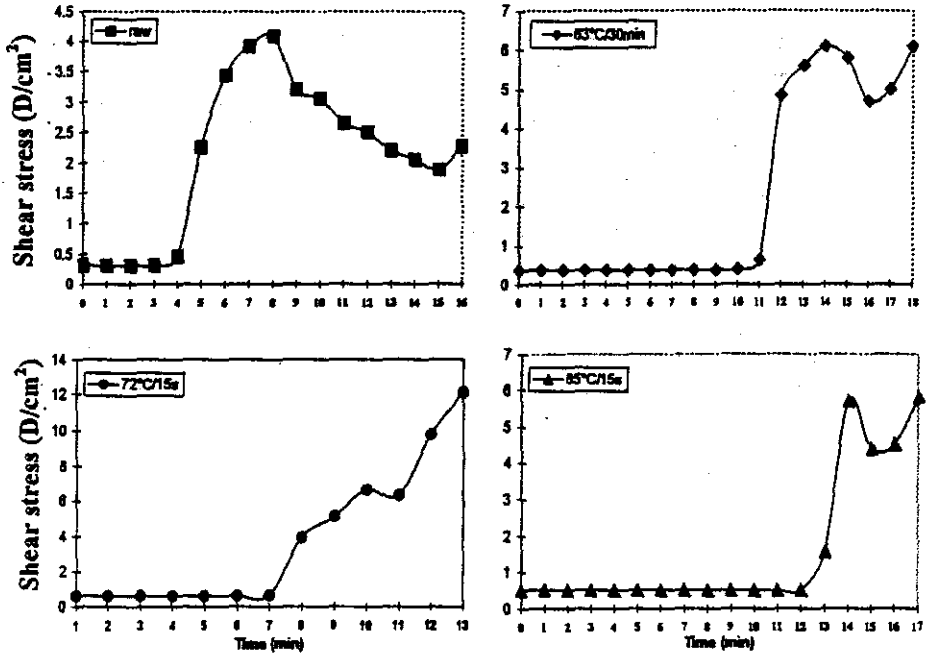

Fig. (2): Shear Stress (D/cm^2) profiles at the initial stage of gelation as a function of heat treatment of milk for rennet gels made from raw and heated buffaloes' milk at 63°C/30 min, 72°C/15s and 85°C/15s.

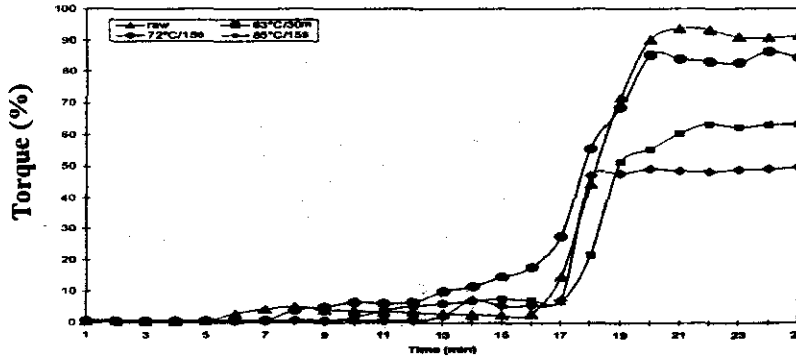

Fig. (3): Torque (%) profiles as a function of time for rennet gels made from raw and heated buffaloes' milk at 63°C/30 min, 72°C/15s and 85°C/15s.

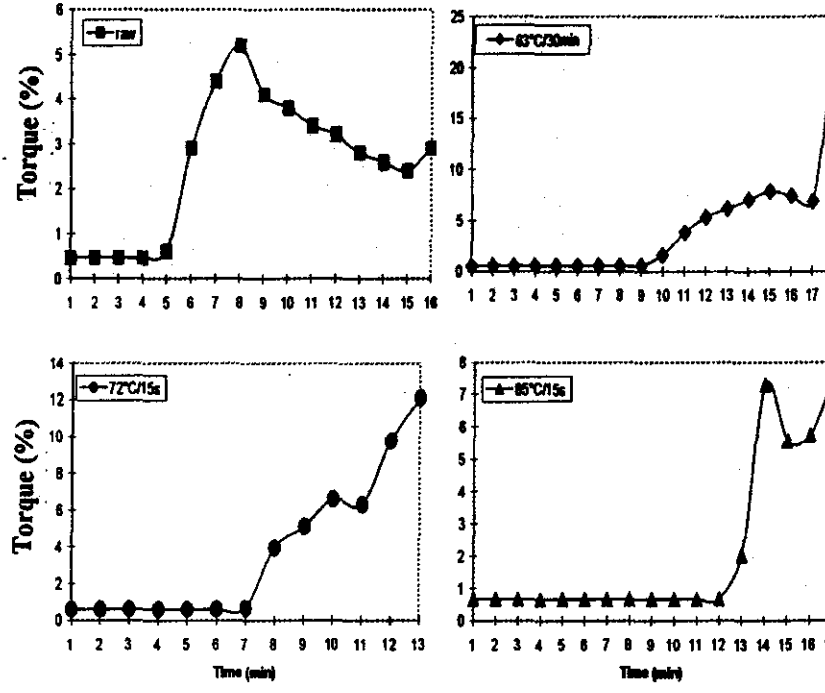

Fig. (4): Torque profiles at the initial stage of gelation as a function of pre-heat treatment of milk for rennet gels made from raw and heated buffaloes' milk at 63°C/30 min, 72°C/15s and 85°C/15s.

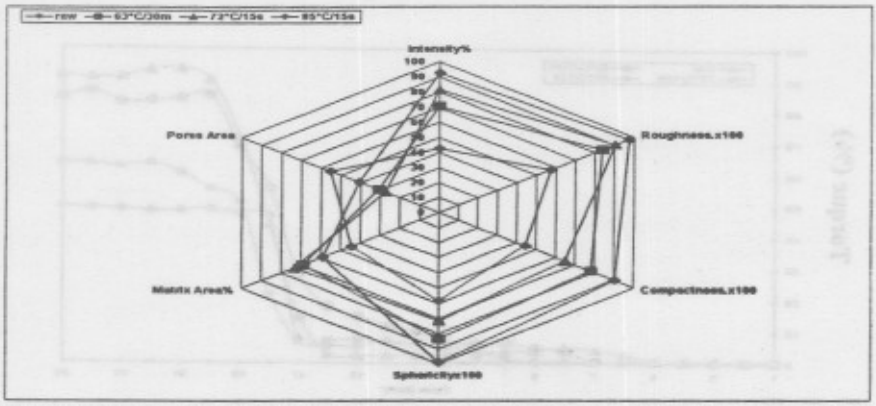

Fig. (5): Effect of buffalo milk heated at 63°C/30 min, 72°C/15s and 85°C/15s on rennet gel rheological parameters.

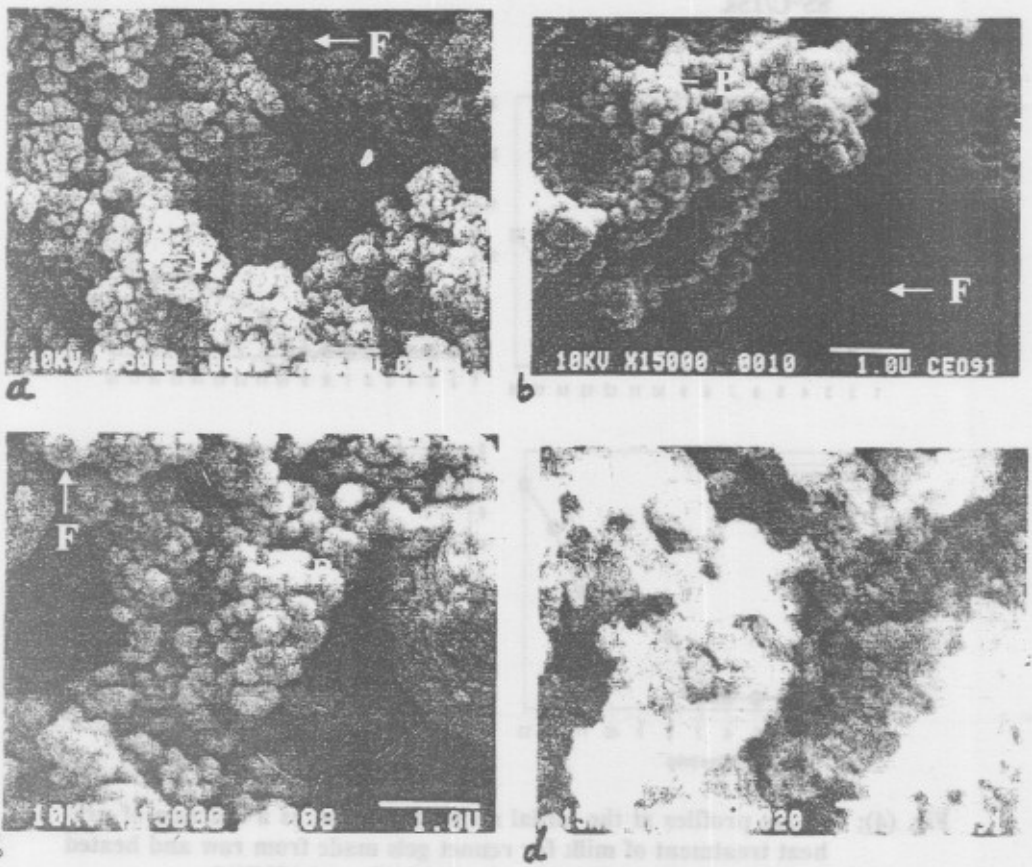

Fig. (6): Microstructure of rennet gels made from raw buffalo milk (a), milk heated at 63°C/30 min(b), 72°C/15s (c) and 85°C/5s (d)

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الخواص التجبنية والريولوجية والهندسية والتركيب البنائى الدقيق لجل اللبن الجاموسى اثناء عملية التجبن : تاثير معاملات اللبن الحرارية

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فى هذا البحث تم مقارنة اللبن الجاموسى المعامل بحرارة ٦٣°م/٣٠ ق، ٧٢°م/١٥ث، ٨٥ °م/١٥ث مع اللبن الجاموسى الخام (الكنترول) من حيث التأثير على التحلل الانزيمى للكابا- كازين (المرحلة الانزيمية للتجبن) وكذلك عملية التجبن بالكامل (المرحلة الانزيمية وغيرالانزيمية) عن طريق قياس قوى ضغط القص Shear Stress (ss) والعزمTorque (T) وقد تم ايضا متابعة تأثير المعاملات الحرارية بقياس الخواص الريولوجية والتركيب البنائى الدقيق للجيل المتكون.

اظهرت الدراسة ان تسخين اللبن الى ٦٣°م/٣٠ ق، ٧٢°م/١٥ث، ٨٥ °م /١٥ث ادى الى خفض معدل التجمع Flocculation الحادث بالمنفحة مقارنة باللبن غير المعامل بالحرارة. وقد ظهر تأثير التسخين على تثبيط التجبن الانزيمى من زيادة الوقت الذى بدأ فيه عمل الانزيم حيث زاد من ٤ق فى اللبن غير المعامل بالحرارة الى ١١، ٨، ١٢ ق فى اللبن المعامل بحرارة ٦٣°م /٣٠ ق، ٧٢°م/١٥ث، ٨٥ °م/١٥ث على التوالى . وقد ادت المعاملة الحرارية للبن الى دنترة denaturation بروتينات الشرش وتكوين خليط معقد من تجمعات بروتين الشرش وجسيمات الكازين المغطى ببروتينات الشرش، هذا بالاضافة الى أن معاملة اللبن حرارياً قد ينتج عنها تغيير فى التركيب البنائى وترابط الوحدات المكونة لجسيمات الكازين ودنترة بروتينات الشرش وأختلاف تركيز الكالسيوم المتأين مما جعل الانزيم يأخذ وقتا اطول فى ايجاد الرابطة الحساسة له ليقوم بعمله. وقد ادى التسخين الى ٧٢°م /١٥ث الى نقص طفيف فى ضغط القص (٧٠٠٩ داين/سم٢) مقارنة بـــاللبن غير المسخن (٧٤،٦ داين/سم٢).

اما الخواص الريولوجية: Intensity,Sphericity, Compactness, Surface Roughness, Matrix area, Pores area فقد تأثرت معنويا بالمعاملات الحرارية، فقد حصل جل اللبن الخام على اقل قيم لكل من : Intensity,Sphericity, Compactness, Roughness, Matrix area (٤٢،٦٪ و ٠،٥٧ و ٠،٤٤ و٠،٥٨ و٤٤،٥٪ على التوالى) والتى قد ترجع الى وجود بروتينات الشرش والكازين فى صورها الطبيعية أما فى حالة تسخين اللبن على ٦٣°م/٣٠ ق، ٧٢°م/١٥ث، ٨٥ °م/١٥ث فقد انخفضت بشكل معنوى مساحة مسامية الجيل Gel Pores (٣٠،٩ و٢٧،٤ و٠،٤٠،٧% على التوالى) مقارنة بتلك الخاصة بالجيل غير المسخن (٥٥،٤%). وقد بدى جيل اللبن الخام متجانسا وجزيئات شبكة البروتين ذات تركيب اكثر ارتباطا واكثر مسامية، ولم يظهر تأثير واضح على تركيب جسيمات كازين الجيل المتكون من جراء تسخين اللبن على ٧٢°م /١٥ث.