

INFLUENCE OF MICROBIAL TRANSGLUTAMINASE ON SOME PHYSICAL AND MICROSTRUCTURAL PROPERTIES OF COW MILK COAGULUM SUPPLEMENTED WITH WHEY PROTEINS

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ABSTRACT

Recently, a focus on the utilization of whey proteins (WP) has been extensively investigated due to their nutritional value and their potential to enhance functional characteristics of the dairy product. The effect of microbial transglutaminase (TG) on the characteristics of skim-milk prepared with the addition of WP products (α -lactalbumin, β -lactoglobulin and whey protein isolate (WPI)) was studied. For comparison, full-fat and skim-milk gels without addition of TG were also examined. The results show that TG significantly ($P < 0.05$) increased the yield values and decreased curd firmness obtained by formagraph, compared to skim-milk gel without TG. Furthermore, addition of WP enhanced the reactivity of TG as indicated by the extended rennet coagulation time and the further less firmness of the curd. This result was confirmed by the appearance of new high molecular weight protein polymers bands in SDS-PAGE, particularly, within WPI and β -lactoglobulin treatments. The highest curd yield was obtained when WPI was added (an increase of ~50 %, compared with the TG-treated skim-milk), while the α -lactalbumin addition exhibited the least reactivity with TG on coagulation properties. Also, scanning electron microscope (SEM) analysis approved the same trend of the results represented in the more dense structure accompanied by finely dispersed clusters of WP strands attached to protein matrices with particular intense in WPI and β -lactoglobulin treatments. In conclusion, the cross-linking by TG with the addition of whey proteins intensely altered the functional properties of the resulting milk coagulum. This novel functionality of the milk coagulation network offers interesting possibilities for enhancing dairy products by adding whey proteins before treating milk with TG.

Keywords : Transglutaminase, cross-linking, Whey proteins, microstructure, formagraph.

INTRODUCTION

Transglutaminase (TG) is an enzyme (EC 2.3.2.13) that capable of forming both inter- and intra-molecular isopeptide bonds in and between many proteins by cross-linking of the γ -carboxamide group of peptide bound glutamine residue (acyl donor) and primary amino group of lysine residue (acyle receptor), forming $\epsilon(\gamma$ -glutamyl)lysine linkages (Wróblewska *et al.*, 2008; Jaros *et al.*, 2010).

TG has been used by the food industries over the recent decades to improve the texture and to generally modify the functional properties of the prepared foods, i.e. seafood, surimi products, noodles and dairy products (Kuraishi *et al.*, 2001). Various researches demonstrate the potential of TG performance with different food proteins such as caseins (Kruif *et al.*, 2002; Mugurama *et al.*, 2003), gluten (Bauer *et al.*, 2003), oat globulin (Siu *et al.*, 2002), myosin (Tseng *et al.*, 2002) and soy proteins (Mugurama *et al.*, 2003).

Milk protein gels being made traditionally are considered stabilized mainly by weak non-covalent interactions. Introduction of new covalent bonds leads to gels which are, therefore, different in structure and functional properties. The use of TG offers such opportunity to improve the rheological and other quality properties of dairy products, which in-turne, has attracted considerable attention in dairy research (Nielsen *et al.*, 1995; Schorsch *et al.*, 2000).

Whey proteins (WP) compose about 20% of cow milk and are generally produced as a co-product of the cheese industry. In addition to the high potential nutritional value of the whey proteins and its easily digested feature, it possess cluster of functional properties including the hydration ability, emulsification, gelation, stabilization, whipping/foaming and thickening properties, that in turne excessively alter and modify the product quality attributes (Eissa and Khan, 2006).

Various studies have approved the capability of TG to work on whey proteins (Sharma *et al.*, 2002; Rodriguez-Nogales, 2006; Gauche *et al.*, 2008). The SDS-PAGE analysis indicated the reactivity of TG with the milk-WP (α -lactalbumin and β -lactoglobulin) without the need for any prior treatment (Sharma *et al.*, 2002 and Gauche *et al.*, 2008). It is worthwhile to note that the researches generally deal with the isolated or concentrated milk-WP mainly through its natural form or with other ingredients in aqueous solution forms. Instead, milk-WP polymerization by TG in the milk matrix (phase) has not been much reviewed. Accordingly, in the study presented herein, the influence of crosslinking between different whey protein products (α -lactalbumin, β -lactoglobulin and WPI) and casein *via* TG in milk was investigated. These effects were studied under chosen conditions that of which resembling production scheme of many cheese varieties with respect to soft cheese types. Therefore, glucono- δ -lactone was added to slightly acidify the milk before rennet addition. Hence, acidic-rennet induced coagulation of fresh cow milk was performed. The functional impact of WPP addition and TG treatment on milk-gels were investigated by physical and microstructure means.

MATERIALS AND METHODS

Fresh cow milk was obtained from the Norwegian University of Life Science farm. A quantity of whole milk was kept as control ,and the remaining amount of the milk was subjected to separation giving skim milk of 0.1% fat. The microbial rennet (CHY-MAX™ Plus, Christian Hansen, Hørsholm, Denmark) was used.

The whey protein products (WPPs) used were BiPRO® ,which is whey protein isolate (WPI), Davisco's Alpha-lactalbumin (Alpha) and Davisco's Beta-lactoglobulin (Beta) (Davisco Food International, INC., Minnesota, USA). The dispersion of WPPs in milk was made according to the manufacturer's recommendations. The WPPs were initially dispersed in a portion of the raw skim milk from their respective vats in a proportion of 0.2% (w/w) for all of them using a high speed blender for 2–5 min.

The used microbial TG preparation was Activa® YG (E.C. 2.3.2.13) (Ajinomoto Foods Europe S.A.S., Hamburg, Germany), which had a declared specific activity of 100 U g⁻¹ powder. TG was added at a concentration of 2 U g⁻¹ milk proteins to the respective treatments.

Acid-rennet induced gelation was carried-out in six treatments. The experimental design was performed to compare full-fat milk and skim-milk gels as controls (without addition of TG and coded as F and S treatments, respectively) with four different skim-milk gels consisted of the following treatments: skim-milk with addition of TG, skim-milk with addition of TG + Alpha, skim-milk with addition of TG + Beta and skim-milk with addition of TG + WPI; where are represented in codes STG, STG-Alpha, STG-Beta and STG-WPI, respectively. The factorial design were made with two factors: Replicate block (3 levels), and milk treatment (6 levels).

All milks were heat treated at 78°C/2min in double-walled stainless-steel vats followed by cooling to 32°C, glucono-δ-lactone (GDL from Sigma, Italy) was added to all milk treatments as a powder (0.5% w/v) 30 minutes prior to rennet addition (0.015%, v/v). TG was added simultaneously with the GDL addition and prior to rennet addition for an incubation time of 30 min.

The total nitrogen content (TN %) was measured by the Kjeldahl method (International Dairy Federation (IDF), 1993). Total protein content was calculated by multiplying the TN % by 6.38. Milk fat content was determined by the Gerber method according to (Ling, 1963). Milk total solids (TS %) content was determined according to (AOAC, 1990). pH was measured in the fresh milk samples and 30 min after the addition of GDL using a digital pH-meter PHM92 (MeterLab™, Radiometer Analytical S.A., France). All samples were analyzed in triplicate.

The yield was determined by a centrifugation method according to Bönisch *et al.* (2008). The yield after centrifugation in % (w/w) was defined as the weight of the gel attained after centrifugation in relation to the initial weight of milk sample All measurements were carried out in triplicate.

The gelation kinetics of the viscoelastic milk gels of all treatments were performed by formagraph (Lattodinamografo, FOSS ITALIA S.p.A., Via Belgio, 35127 Padova, Italy) according to the method of McMahon and Brown (1982).

Fig. 1 shows the output results of the Formagraph. Rennet clotting time (RCT) which is referred to coagulation time, required to achieve a curd firmness of 20 mm (K20) reflecting the firming rate of the curd and curd firmness (A30) ,which is measured 30 min after the addition of rennet were recorded at 32°C. The overall time of every Formagraph run was 60 min. Measurements were carried out in at least triplicate in the same milk sample.

For scanning electron microscopy (SEM) of the fresh prepared milk gels, small cylindrical pieces from the center of the gel blocks (approximately 3mm in diameter and height) were prepared and examined according to Sandoval-Castilla *et al.* (2004) using a high vacuum Zeiss Scanning Electron Microscope EVO-50-EP (Carl Zeiss SMT Ltd., Cambridge CB1 3JS, UK) to view the samples at 5 KV and magnification of 7500x.

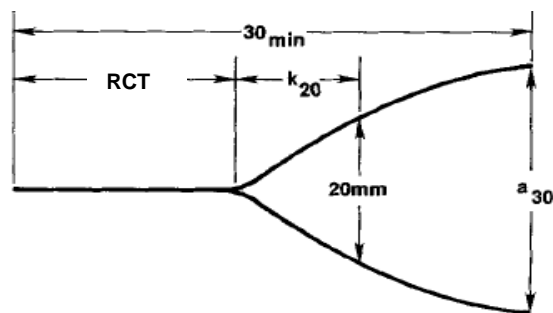


Fig. 1. A typical diagram of coagulation and curd firmness as a function of time obtained by the Formagraph (McMahon and Brown, 1982)

Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE) was carried by using a BIO-RAD Mini PROTEAN II unit. SDS-PAGE under reducing conditions has been used to estimate the relative molecular weight to evaluate the polymerization extent of the protein chains (Laemmli, 1970).

Data were statistically analyzed by ANOVA using general linear model of SPSS (ver. 11.0.1, SPSS Inc., USA, 2001), and significant differences ($P \leq 0.05$) between treatments were determined.

RESULTS AND DISCUSSION

The average composition and some of the physicochemical properties of fresh cow milk used in the present study are given in Table 1. The total solids of skim milk were reduced as a result of skimming. On the other hand, pre-incubation of milk for 30 minutes after addition of GDL resulted in rather acidified milk (pH ~5.9) prior to renneting step.

Table (1): Composition and physicochemical properties of full-fat and skim-milks used in manufacture of milk-gels.

	Full-fat milk	Skim-milk
Fat %	3.35 ± 0.17	0.12 ± 0.04
Protein %	3.31 ± 0.05	3.44 ± 0.08
Total solids %	11.87 ± 0.24	9.07 ± 0.15
pH	6.68 ± 0.02	6.65 ± 0.01
pH-30min*	5.94 ± 0.06	5.92 ± 0.05

Values are means ± standard deviation.

* pH values of milks after 30 min from GDL addition.

By the Formagraph technique, the progress of the curd formation can be detected. Table (2) shows milk-clotting parameters derived from formagraph at 32°C. RCT values have increased significantly ($P < 0.05$) as a result of adding TG, and even rather higher within treatments of whey protein products addition, compared to the control treatment (F and S). Increased

coagulation times as a result of the pre-incubation of milk by TG reflecting the impact of the TG cross-linking on the primary enzymatic phase. This result was also described by O'Sullivan *et al.* (2002) and Bönisch *et al.* (2008). The *k*-casein molecule, which is located at the surface of the casein micelle, possesses four potential glutamine residues that are available as cross-linking sites rendering *k*-casein susceptible to TG, which in turn retard the lag phase of rennet-induced coagulation (Sharma *et al.*, 2001; Tolkach & Kulozik, 2005).

Table (2): Impact of TG treatment and addition of whey protein fractions on milk-clotting parameters derived from formagraph at 32°C and yield%, as indicated by the analysis of variance.

	RCT (min.)	K20 (min.)	A30 (mm)	Yield (%)
F	8.08±0.14 ^a	5.92±0.29 ^a	27.01±1.03 ^a	32.23±0.75 ^a
S	8.67±0.14 ^a	6.67±0.14 ^a	32.75±0.68 ^b	23.24±0.33 ^b
STG	12.08±0.38 ^b	10.00±1.09 ^b	28.28±2.02 ^a	35.09±0.73 ^c
STG-Alpha	14.25±0.50 ^c	11.92±0.52 ^b	23.35±0.50 ^c	43.48±1.00 ^d
STG-Beta	15.33±0.52 ^d	14.58±1.01 ^c	19.53±1.20 ^d	48.83±0.19 ^e
STG-WPI	14.58±0.38 ^c	14.33±0.58 ^c	21.56±0.69 ^d	52.31±0.58 ^f

Values are means of triplicate analyses of three individual milk samples ($n = 9$), ± standard deviation.

Means with different superscripts in the same column are significantly differ ($P < 0.05$).

Furthermore, the significant increased ($P < 0.05$) RCT values within treatments of whey protein products (STG-Alpha, STG-Beta and STG-WPI), compared to that of TG treated skim-milk without addition of whey proteins indicate that an induced and excessive cross-linked caseins performed through the pre-incubation time (30 min.) with TG, that resulted in an extended clotting time. This trend of result confirms that whey proteins are favorable substances for its performance.

The Formagraph data presented in Table 2 obviously show that there was no significant difference between full-fat and skim milk treatments with respect to clotting time and curd firming rate values, whereas skim-milk gel exhibited a significant ($P < 0.05$) higher curd firmness, compared to the full-fat milk gel (A30-values increased by 21%). This observation was expected due to the bereft of milk-fat in S treatment. In this context, treating skim-milk with TG resulted in a significant reduction ($P < 0.05$) in A30-values and reaching no significance with full-fat treatment (F). As previously concluded by Bönisch *et al.* (2008), the present obtained results indicate that not only the primary phase (lag phase) of rennet coagulation, but in particular the secondary phase and the formation of a three dimensional gel structure appears to be affected significantly by TG.

Regarding the impact of whey proteins addition, it is noticed that STG-Beta treatment obtained the highest K20-values and the lowest A30-values. It can be clearly indicated from A30-values that all whey protein treatments were significantly ($P < 0.05$) softer than the TG-treated skim milk (Table 2). This reduction in curd firmness was of 17%, 31% and 24% for STG-Alpha, STG-Beta and STG-WPI treatments, respectively, compared to that of the TG-treated skim milk. Surprisingly, whey protein treatments were

significantly ($P < 0.05$) softer than the full-fat treatment (F), reflecting a great enhanced reactivity of the protein cross-linking, and hence altering the functional properties of milk-gels.

The curd yield (%) after centrifugation are shown in Table 2. It appeared that the yield of full fat milk gels is more than the skim milk gels which, is of normal finding due to the presence of milk fat. A significant increase ($P < 0.05$) in curd yield (~ 51%) was observed in skim milk when treated with TG (Table 2). Bönisch *et al.* (2008) elucidated that the increased curd yield is rather explained by the enhanced serum binding of the gel network stabilized by additional covalent bonds than by the incorporation of native WP into the gel network.

Furthermore, addition of whey protein products significantly ($P < 0.05$) increased the curd yield compared to STG treatment. This increase was of 23%, 39% and 49% for STG-Alpha, STG-Beta and STG-WPI treatments, respectively (Table 2). Bönisch *et al.* (2008) elucidated that the increased curd yield is rather explained by the enhanced serum binding of the gel network stabilized by additional covalent bonds than by the incorporation of native WP into the gel network. Additionally, the highest yield value obtained by STG-WPI might be explained not only through its enhancement of TG reactivity, but also to its well-known higher water holding capacity as stated by Truong *et al.* (2004) and Li *et al.* (2005). The more hydrated texture and more excessive cross-linking action may together contribute to a softer and less firm texture obtained in STG-Beta and STG-WPI treatments compared to both control treatments (F and S) and STG treatment.

The electrophoresis profile in SDS-PAGE was carried out in order to confirm the polymerization reaction of whey protein fractions with milk proteins by TG (Fig. 2). The extent of cross-linking was demonstrated by disappearance of protein bands and/or appearance of new high molecular weight protein polymers bands. It could be noticed from lanes 2 and 3 that without addition of TG, no cross-linking was observed. The appearance of high molecular weight bands in lanes 4, 5 and 6 shows different rates of cross-linking between whey protein fractions added and milk proteins. Moreover, it could be noticed that the addition of α -lactalbumin and β -lactoglobulin fractions resulted in an increase in the band sizes of these fractions, while the addition of WPI resulted in the increase in the size of bands of both α -lactalbumin and β -lactoglobulin (Fig. 2).

As indicated in the SDS-PAGE patterns (Fig. 2), the intensity of the protein polymer formed due to cross-linking catalyzed by TG was high when β -lactoglobulin and WPI were added, while it was low when α -lactalbumin was added. This suggests that the rate of cross-linking was bigger in case of β -lactoglobulin and WPI treatments than that in the case of α -lactalbumin treatment. In fact, the rate of crosslinking by TG is dependent on the macromolecular structure of each protein substrate and reactive glutamine residues available.

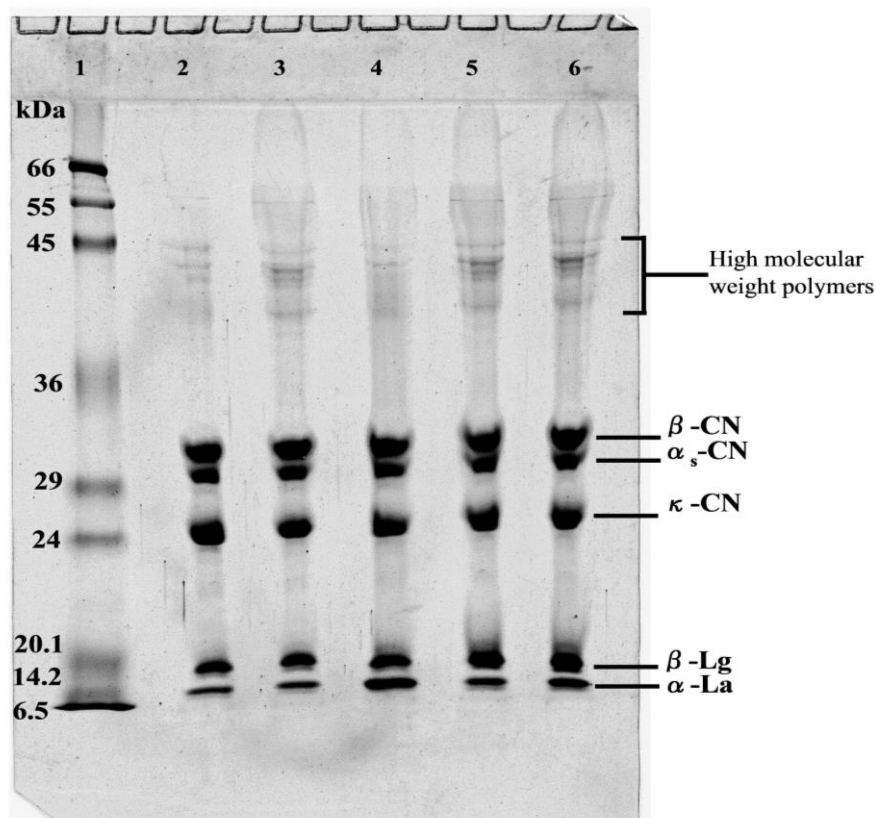


Fig (2): SDS-PAGE gel. Lane 1 is the Molecular weight marker. Lane 2 is untreated milk sample, lane 3 milk sample treated with transglutaminase. Lane 4, 5 and 6 are milk mixed with α -lactalbumin, β -lactoglobulin and whey protein isolate, respectively, and treated with TG.

Therefore, the low rate of cross-linking with α -lactalbumin might be due to its lower content of reactive residues (8 glutamine residues and 12 lysine residues) than β -lactoglobulin (16 glutamine residues and 15 lysine residues) in its protein chain (Gauche *et al.*, 2008). These data support the obtained results of formagraph and yield (Table 2). In fact, the full fat milk sample was not used in this test as the previous results showed that the presence of fat in milk did not affect the extent of cross-linking of milk proteins by TG (Hinz *et al.*, 2007).

The SEM micrographs of cow milk gels as influenced by addition of protein cross-linking and whey protein fractions are shown in Fig. 3. The protein matrix (gray area) formed a continuous phase permeated by an amorphous system of voids filled with serum, which in turn revealed the spatial dimensions of these images.

As it might be seen from these micrographs, there is an obvious and large variation in the microstructural properties of full-fat and skim-milk gels

(F and S) as control treatments without addition of TG (Fig. 3A and 3B). A spongy-like structure was obtained in skim-milk gel, whereas a continuous phase of smooth protein aggregate network characterized by a more compacted and dense structure accompanied by less voids revealed in the full-fat milk gel, where the spherical fat globules (pointed with white arrows) were obviously dispersed and scattered uniformly throughout the protein matrices. This result is in parallel with that of Lobato-Calleros, *et al.* (2002 and 2007) and Liu *et al.* (2008).

The manifested microstructure in Fig. 3C clearly revealed that addition of TG to skim-milk (STG treatment) promoted regularly aggregated protein matrices accompanied by homogeneous systematic pores that obviously much finer and less in size compared to that of control treatments (F and S; Fig. 3A and 3B, respectively). This expressed manner of protein matrix might attribute to the influence of TG addition that is capable of forming both inter- and intra-molecular isopeptide protein bonds (Kuraishi *et al.*, 2001). Moreover, remarkable clusters of cross-linked strands and partially fused protein aggregates (pointed with black arrows) were noticed in this micrograph which most probably resulted from the cross-linking action between whey protein particles and casein micelles as an impact of TG addition. These observations in accordance to those reported by Kruif *et al.* (2002) and Bönisch *et al.* (2008).

As it might be seen in Fig. 3D, 3E and 3F, the addition of whey protein products promoted the overlapping of cross-linking protein aggregates (appeared in the images as whiter gray areas and pointed with black arrows) that distributed irregularly through the gel matrix giving much finely dispersion and dense vision of the protein matrices, compared to that of skim milk treated with TG and without addition of whey protein products (Fig. 3C). These results are conformable with the gelation kinetics obtained by formagraph (Table. 2), and are in agreement with those of Lobato-Calleros, *et al.* (2007) and Supavititpatana, *et al.* (2009) in white fresh cheese and corn-milk yogurt, respectively.

Despite that, a uniform addition of whey protein products (0.2% w/w) to the skim milks was considered throughout preparation of those treatments (STG-Alpha, STG-Beta and STG-WPI), a noticeable changes in microstructure properties was evident among them.

Obviously, addition of α -lactalbumin fraction tended to cause the least influence on the milk-gel network structure, whereas a more inclusion of finely cross-linked strands and protein aggregates (pointed with black arrows) resulted in within treatments of β -lactoglobulin and WPI addition as it could be seen in Fig. 3D, 3E and 3F, respectively. This finding goes in parallel with the results trend obtained by SDS-PAGE electropherograms, as the addition of β -lactoglobulin and WPI tended to cause inclusion of a high cross-linked protein aggregates. This in turn resulted in more homogenous dense structure, which rather resembled, to some extent that of full-fat structure regardless the presence of milk-fat globules. However, the addition of α -lactalbumin showed minimal changes in the microstructure (Fig. 3D), which is most probably attributed to the weak reactivity with the TG, as earlier

explained. This finding consistent with the observations of Puventhiran *et al.* (2002) and Supavititpatana *et al.* (2009).

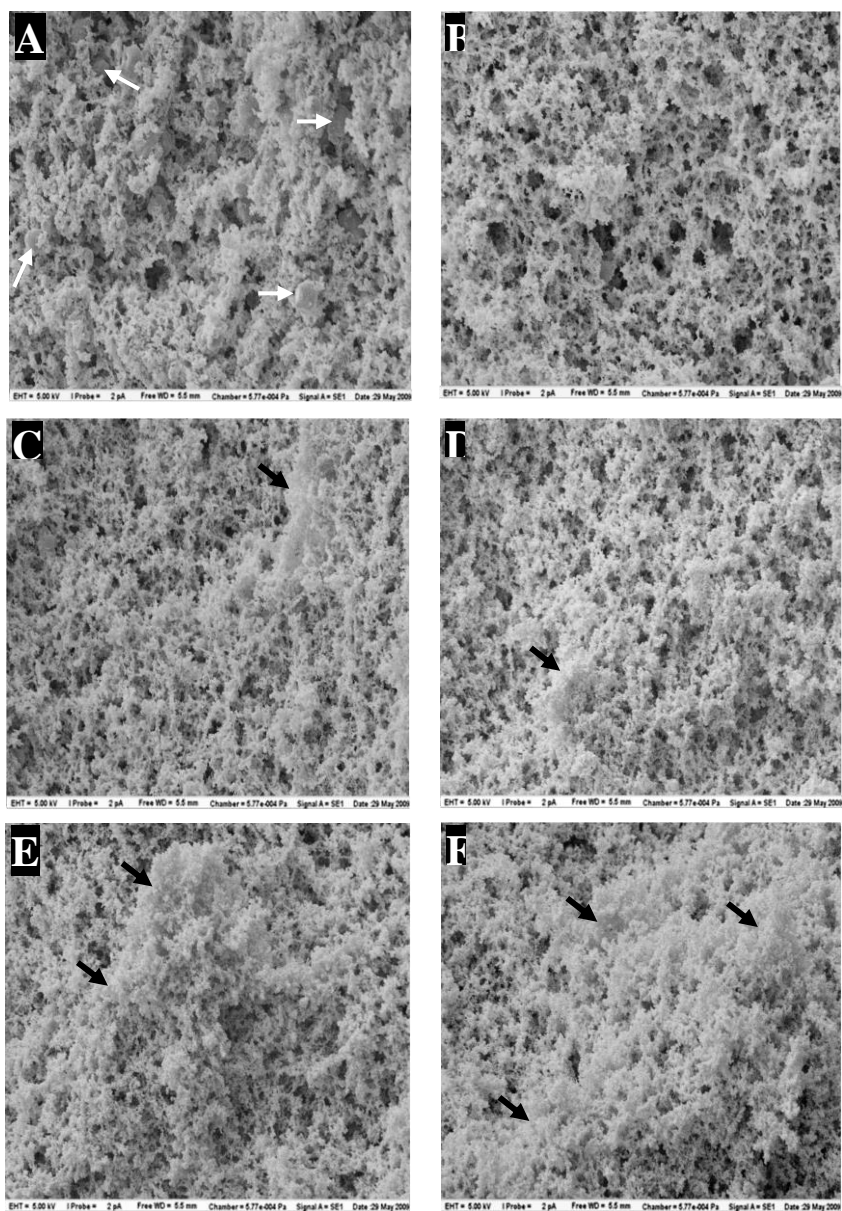


Fig (3): Scanning electron micrographs of rennet-induced gel of; (A) full-fat milk, (B) skim-milk, (C) Skim-milk treated with Tg, (D) skim-milk mixed with α -lactalbumin and treated with Tg, (E) skim milk mixed with β -lactoglobulin and treated with Tg and (F) skim milk mixed with WPI and treated with Tg.

Conclusion

Addition of whey protein products enhanced the reactivity of TG that represented in the extended clotting time and the marked increased curd yield with much less curd firmness as well as the appearance of new high molecular weight protein polymers bands in SDS-PAGE, compared to the other treatments with/without TG. It should be noted that the excessive cross-linking was more pronounced within WPI and β -lactoglobulin addition than that of α -lactalbumin addition. The microstructure analysis confirms these findings through the presence of much closure and dense casein aggregates accompanied by clusters of WP strands attached to protein matrices with particular intense in STG-Beta and STG-WPI treatments.

In sum, the results of this study imply that TG as well as whey protein products greatly altered the functional properties of the protein gel network. Among the whey protein products, WPI was of more recommended due to the highest curd yield as well as its nutritional value, compared to the individual whey protein fractions. The modified functional properties of the gel network obtained in this study contribute to further understanding of how different ingredients interact and modify casein-based structure, allowing manifold applications in dairy products with particular respect to the enhancement and optimization of low-fat cheese varieties.

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تأثير إنزيم الترانسجلوتامينيز علي بعض الخواص الفيزيائية والتركيب البنائي
الدقيق لخرثة اللبن البقرى والمضاف لها بروتينات الشرش
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شهدت الأونة الأخيرة تعدد الدراسات التي تناولت زيادة استخدام بروتينات الشرش في منتجات الألبان وذلك لما لها من أهمية غذائية عالية ولمقدرتها على تحسين الصفات الوظيفية لتلك المنتجات. ولذا تناول هذا البحث دراسة تأثير المعاملة بإنزيم الترانسجلوتامينيز الميكروبي علي الخواص الوظيفية والتركيب البنائي الدقيق لخرثة اللبن البقرى الناتجة من التجبن الحمضى-الإنزيمى و المضاف له بروتينات الشرش (الألفالاكتالبيومين أو البييتالاكتوجلوبولين أو معزول بروتينات الشرش " WPI ") ، وتمت دراسة صفات الخثرة الناتجة من كل من اللبن الكامل الدسم واللبن الفرز بدون اضافة الإنزيم وعينة لبن فرز أخري اضيف اليها الإنزيم كعوامل مقارنة. وأشارت النتائج المتحصل عليها الي أن هذا الإنزيم قد أدى الي زيادة التصافي وانخفاض صلابة الخثرة الناتجة والتي تم قياسها بواسطة جهاز الفورماجراف بالمقارنة بالعينة التي لم يضاف اليها الإنزيم. أيضا لوحظ أن اضافة منتجات بروتينات الشرش قد أدت لتحسين نشاط و زيادة عمل الإنزيم و الذى انعكس فى الزيادة الكبيرة لطرارة الخثرة الناتجة. وأكد ذلك أيضا ظهور Bands جديدة بواسطة التفريد الكهربى نتيجة تكوين بوليمرات بروتينية عالية الوزن الجزيئى وبصفة خاصة عند اضافة WPI أو البييتالاكتوجلوبولين ، في حين كانت اضافة الألفالاكتالبيومين أقل تأثيرا. كما أدت اضافة منتجات بروتينات الشرش الي زيادة كبيرة فى تصافى الخثرة و خاصة تلك المضاف اليها WPI و التى حققت أعلى نسبة تصافى مقارنة بالمعاملات الاخرى . أيضا أكدت نتائج التركيب الدقيق بواسطة الميكروسكوب الإلكتروني الماسح النتائج السابقة فقد ظهر التركيب البنائي الدقيق للخثرة المتكونة أكثر تماسكا وظهرت بروتينات الشرش مندمجة في هذا التركيب البنائي وظهر ذلك أكثر وضوحا في حالة اضافة WPI والبييتالاكتوجلوبولين.

وبصفة عامة يمكن الاستنتاج أن استعمال انزيم الترانسجلوتامينيز مع اضافة بروتينات الشرش أدى الي تحسين الصفات الوظيفية للخثرة المتكونة وبناء عليه يمكن تحسين الصفات الوظيفية للمنتجات اللبنية بإضافة بروتينات الشرش قبل معاملة اللبن بانزيم الترانسجلوتامينيز .

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