Influence of enzymatic treatment on stabilization of traditional Iranian yoghurt drink, Doogh

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Abstract— **Doogh is an Iranian fermented milk drink prepared through adding water and salt to yoghurt. In this study the effect of enzyme transglutaminase on Doogh characteristics was investigated. Also, the influence of two preparation methods i.e. water addition to yoghurt as a common method and fermentation of diluted milk as an alternative was studied. The enzyme was applied into the skim milk either before or after pasteurization followed by its thermal inactivation. Doogh preparation directly from milk resulted in more stable products with less phase separation. Enzymatic treatment after pasteurization of milk followed by water addition and fermentation yielded the least phase separation during storage. Improvement of the stability of Doogh samples was associated with increase in the apparent viscosity and uniformity of particles accompanied by finer meshed network and smaller pores in their microscopic structure.**

Keywords-component: Doogh; Transglutaminase, Stability, Viscosity, Microscopy.

I. INTRODUCTION

Drinking-type fermented milk products are prepared through adding water to yoghurt and can be considered as variations of stirred types with a rather lower viscosity [1]. The traditional Iranian yoghurt drink, Doogh, is a nutritious and refreshing drink. It is traditionally produced via adding water to full fat yoghurt and then churning it up in a special leather bag called "*Mashk*" followed by removing of fat along with the addition of salt and sweet-smelling herbs. In current commercial productions, the reduction of fat content is normally achieved using skim milk to prepare the yoghurt, and usually the final product is flavored with essential oils, such as those extracted from mint, oregano, thyme, etc. [2]. Similar products are available in other countries for instance *Ayran* in Turkey and *Laban* drink in most Arab countries [3]. These drinks differ from Doogh in, for example, the amount of added water, fat and salt content, rheological properties and taste [4]. Doogh is characterized by pH values less than 4.5 and non-fat-solid content more than 3.2% (w/w). The maximum salt content must be 1% (w/w) [5].

Enzymatic cross-linking of proteins to improve their functional properties has gained an increasing attendance as an alternative method to process the traditional foods [6]. The enzyme transglutaminase (TGase, EC 2.3.2.13) is a transferase which catalyzes an acyl transfer between γ-carboxyamide groups of peptide-bound glutamine residue (acyl donors) and ε-amino groups of lysine residues (acyl acceptor), leading to the formation of covalent cross-links in food proteins, including milk proteins [7]. The most significant application of transglutaminase in the dairy industry is in fermented milk gels [8] where the enzyme has been used to increase the gel strength and apparent viscosity, decrease the syneresis and produce a smooth surface in set and stirred type yoghurts [6, 9-11].

Serum separation is the main textural defect in drinking type fermented milk products during storage, which is industrially called "*Wheying off*". It is the separation of product into a casein-rich lower layer and a clear upper layer of serum [2]. Afonso & Maya (1999) proposed that acidification to pH values lower than the isoelectric point of protein particles results in the buildup of primary gel aggregates which are subsequently organized as self supportive super-aggregates structures. Stirring of yoghurt breaks down the gel network and produces the aggregate structures. In the preparation process of Doogh, these super-aggregates still exist after adding water to yoghurt; however, they are more separated and free to sediment under gravity, causing massive loss of stability. Furthermore, the presence of salt in this type of beverage intensifies the serum separation [12]. Hydrocolloids are very commonly used to prevent serum separation in fermented milk products by increasing the viscosity, electrostatic or/and steric repulsion [2, 13-15].

It has been reported that intramicellar cross-linking of caseins by transglutaminase can probably preserve the integrity of casein micelle when are acidified [16-18]. It was hypothesized at the start of the present study that cross-linking action of enzyme may also stabilize the super-aggregates in water-added yoghurt, as well as, in Doogh prepared directly from milk resulting in less phase separation.

To the best of authors' knowledge, there is no report in the literature on the influence of transglutaminase in fermented (such as Doogh and Ayran) and acidified milk drinks. The aim of this study was thus to decrease the phase separation of Doogh via treating with transglutaminase as a novel method to produce a high quality product.

II. MATERIALS AND METHODS

A. Materials

Purified lyophilized powder transglutaminase (TGase, EC 2.3.2.13) was purchased from Sigma-Aldrich® (Saint Louis, MO, USA). Each batch of the enzyme contained 500 mg with declared activity of 3100 units. The yoghurt culture DELVO®-YOG was obtained from DSM Food Specialties (Delft, Netherland) in lyophilized form. It was comprised by *Streptococcus thermophilus* and *Lactobacillus delbrueki ssp bulgaricus*. The milk was inoculated with 0.03% starter culture. Skim milk powder with 32.5% protein and 1.0% fat content was obtained from Ramak Dairy (Shiraz, Iran).

B. Doogh preparation

Doogh samples were either prepared from yoghurt, (coded as Y) or milk (coded as M) adopting different methods. For preparation of Y-coded samples, water was added to yoghurt while M-coded samples were produced directly through the fermentation of diluted milk, samples Y1 and M1were prepared with no enzyme treatment. Skim milk powder was used for the preparation of milk, and 1 unit transglutaminase was applied per g of milk protein before (samplesY2 and M2) and after (samples Y3 and M3) batch pasteurization at 90°C for 15 min. When the enzyme was added after milk pasteurization, a rapid and direct post-incubation heat treatment at 90°C for 1 min was applied to inactivate the enzyme. This additional heating was approximately equalized for other samples through a 1 min longer pasteurization i.e. 90°C for 16 min. The dry matter content of Doogh samples and pH values were adjusted to 6 ± 0.1 %, and 4 ± 0.02, respectively, followed by refrigeration storage at 4°C to prevent further acidification by starter culture.

C. Flow behaviour

Flow behaviour characteristics of samples were determined at 4°C by Paar Physica MCR 301 rheometer (Anton-Paar, GmbH, Graz, Austria) using a double gap concentric cylinder geometry covered by a cap to prevent evaporation during measurements. With the purpose of structure recovery and temperature equilibration, each sample was left standing for 5 min. Viscosity and shear stress were measured at shear rates of 2-100 s^{-1}. The data from the measurement were submitted to the Power Law model using non-linear regressions.

D. Light scattering measurement

Size distribution of particles in Doogh samples was studied by a static light scattering laser-based particle size analyzer (Mastersizer Hydro 2000S, Malvern Instruments Ltd., Malvern, Worcestershire, UK). Analyses were performed 24 h after preparation of samples. Particle characteristics are reported by surface area moment or Sauter mean diameter, $D_{[3,2]}$, volume moment or De Brouckere mean diameter, $D_{[4,3]}$, specific surface area, and uniformity. Absolute deviation from the median which is indicative of polydispersity is reported as uniformity (U). $D_{[3,2]}$ and $D_{[4,3]}$ are calculated as below:

$$D_{[3,2]} = \frac{\sum_i^n D_i^3 n_i}{\sum_i^n D_i^2 n_i} \quad (1)$$

$$D_{[4,3]} = \frac{\sum_i^n D_i^4 n_i}{\sum_i^n D_i^3 n_i} \quad (2)$$

where n_i is the number of particles of D_i diameter [19]. Uniformity is expressed by the following equation:

$$U = \frac{1}{d(0.5)} \frac{\sum_i v_i |d(0.5) - d_i|}{\sum_i v_i} \quad (3)$$

where v_i is the volume of the number of particles present between the two consecutive diameters [14].

E. Microscopic observation

Microstructure of Doogh samples was observed by divert phase contrast inverted microscope (Leitz, Wetzlar, Germany). The images were taken by the mounted digital camera (Euromex CMEX-5000, Arnhem, Netherland) connected to a computer. There was no need to dilute the samples and protein staining.

F. Serum separation

Doogh samples were placed in 15 mL gauged test tubes and stored at 4°C. The separation of serum and sedimentation was measured during 15 days of storage. Separated phases were clear to detect.

G. Statistics analysis

The analysis of variance was done using PASW Statistics 18 (SPSS Inc., Chicago, IL., USA). Significant differences were defined at $P < 0.05$. Sensory evaluation data means were compared by Duncan's multiple comparison test. All measurements were repeated at least 2 and generally 3 times.

III. RESULTS AND DISCUSSION

A. Flow behaviour

The shear rate vs. shear stress and viscosity profiles of Doogh treatments are shown in Fig. 1. It is clear that samples prepared directly from milk (M-coded) were significantly of higher viscosities than those of prepared from yoghurt (Y-coded). It is argued that adding of water to milk before acidification (M-coded) resulted in more immobilization of water by proteins during the fermentation; whilst, the water added to yoghurt base could not well interact with protein super-aggregates. All samples of Doogh showed non-Newtonian shear thinning behaviour, according to tabulated parameters in Table I. Similar flow behaviours have been reported for Doogh and Ayran [2, 12, 14]

The data reported in Table I indicate that the application of transglutaminase in Doogh preparation resulted in a

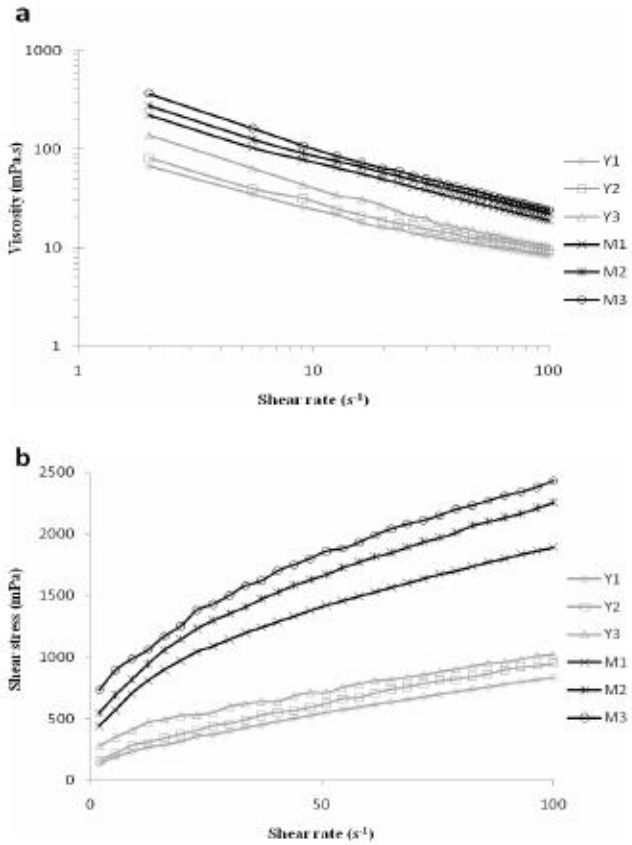

Figure1. Effect of transglutaminase treatment on a) apparent viscosity and b) shear rate-shear stress profile of Doogh samples.

Table I. Viscosity parameters of Doogh samples.

Sample	Determination coefficient (r^2)	n^a	K^b (mPa.s^n)	$\eta_{app}{}^c$ (mPa.s)	Standard Error
Y1	0.994	0.569±0.022	59.69±5.50	10.54±1.91	0.126
Y2	0.993	0.558±0.024	71.70±7.27	12.20±1.77	0.146
Y3	0.982	0.394±0.026	160.50±9.20	13.9 ± 0.93	0.176
M1	0.999	0.405±0.007	290.2 ± 4.80	26.73±1.07	0.093
M2	0.998	0.411±0.009	343.17±6.20	31.68±2.32	0.103
M3	0.992	0.370±0.015	434.18±7.33	34.6 ± 2.01	0.098

[a] Flow behaviour index
[b] Consistency coefficient
[c] Apparent viscosity at shear rate of 55 s^{-1}

significant ($P < 0.05$) increase in consistency coefficient (K) and apparent viscosity of product. Furthermore, transglutaminase caused a decrease in flow index (n) of Doogh samples prepared either directly from milk or yoghurt base. The farther the value of n depart from 1.0 to 0.0, the greater is the deviation from Newtonian flow [20]. Hence, enzyme treatment resulted in an increase in the shear-thinning (pseudoplastic) property of Doogh samples. The consistency coefficient and apparent viscosity of Y3 and M3 samples were significantly ($p < 0.05$) higher than those of their counterpart treatments (Table I). This indicates that treating the milk by enzyme after a severe heat treatment (90°C, 15 min), which made the whey proteins more susceptible to enzyme action resulted in a significant structural enhancement of Doogh. Similar results have been reported in the production of microbial transglutaminase treated set type yoghurt by Şanli, et al. (2011). Casein micelles owing to their highly accessible and flexible open chain structure are excellent substrates for transglutaminase [21]. Whey proteins in their native globular structure are, by comparison, less prone to the cross-linking reaction, mainly due to the stabilization of globular conformation by disulphide bonds limiting the accessibility of cross-linking sites [22]. However, denaturation of whey proteins either by heat treatment or addition of reducing agents can improve their enzymatic susceptibility [23, 24].

Fig. 1.a shows the obtained viscosity plots versus shear rate. A swift disruption of the structure occurs on the initial shearing followed by relatively slower changes at higher shear rates. Such behavior is observed at drinking yoghurt and stirred yoghurt [6, 25, 26]. A fully different picture of viscosity changes was observed by the influence of transglutaminase on Doogh samples. Fig. 1.b demonstrates that a higher shear stress was required to disrupt the super-aggregates into the primary gel particles in Doogh made from transglutaminase-treated milk. This led to a higher viscosity at low shear rates (Fig. 1.b). Similar results were reported by Jaros et al. (2007) in stirred yoghurt.

B. Particle size measurement

The mean diameter of particles in Doogh samples is reported in Table II. Particle size is a quality measurement index for the studying of acidified milk drinks [27]. Modification of protein particles through introducing new covalent bonds by transglutaminase affected the size and distribution of supper-aggregates in Doogh. In overall, enzymatic treatment led to the smaller and more uniform particles. Results indicate that using transglutaminase resulted in good polydispersity of treated Doogh samples. Samples that whey proteins were involved in enzymatic cross-linking i.e. Y3 and M3, had the smallest particles and the most uniform size distribution, as well as, the highest surface area values (Table II). It is hypothesized that whey protein probably are cross-linked with each other and surface of casein micelles. It seems that breaking of transglutaminase treated gel during preparation of Doogh resulted in homogeneous supper-aggregates dispersion. Hence, high values of uniformity were obtained.

Table II. Particle size parameters of Doogh samples.

Sample	$D_{[3,2]}$ µm	$D_{[4,3]}$ µm	Uniformity	Specific surface area $m^2 g^{-1}$
Y1	12.72[a]	34.92[a]	0.60[c]	0.472[f]
Y2	6.96[b]	27.83[b]	0.61[c]	0.862[e]
Y3	0.95[f]	17.87[e]	0.78[b]	6.31[a]
M1	1.39[d]	19.46[d]	0.68[d]	4.33[c]
M2	2.37[c]	25.58[c]	0.71[c]	2.53[d]
M3	1.14[e]	18.23[e]	0.88[a]	5.25[b]

Means with different superscripts within the same column differed significantly ($P < 0.05$).

C. Microscopic observations

In Fig. 2 the microscopic images of Dooghs are illustrated. In the untreated samples i.e. Y1 and M1 great clusters of proteins are clearly visible and super-aggregates are separated from each other by large water contained pores. Transglutaminase cross-linking of proteins yielded in an organized distribution of super-aggregates leading in a finer meshed network and smaller pores. Images of M3 and Y3 revealed that the application of transglutaminase after pasteurization improved the homogeneity of these two samples. [28] stated that interactions amongst casein micelles are altered due to changes in the surfaces of micelles through transglutaminase induced cross-linking resulting in changes in the microstructure of protein network.

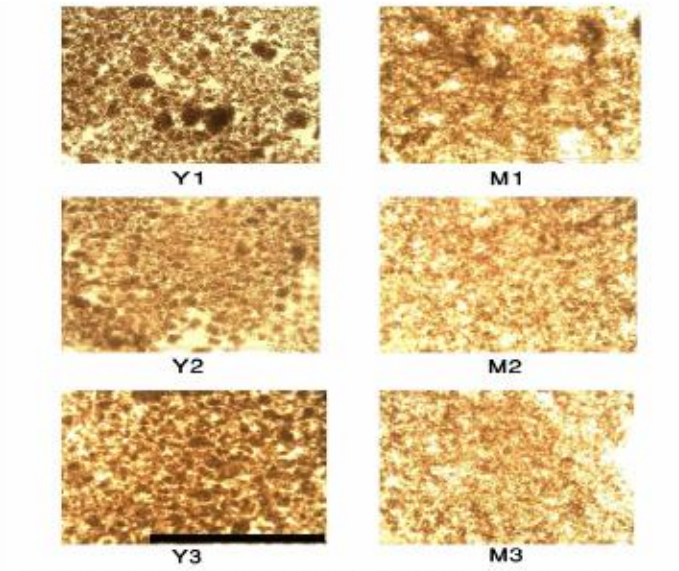

Figure 2. Microscopic images of Doogh samples. Y-coded are prepared from dilution of yoghurt and M-coded are produced through fermentation of diluted milk. Transglutaminase addition is done before pasteurization in Y2 and M2 and after pasteurization in Y3 and M3. The scale bar denotes 100 µm.

It is observed in Fig. 2 that M-coded samples prepared from the fermentation of diluted milk showed a finer and well-ordered distribution of super-aggregates in comparison to Y-coded treatment. It can be concluded that addition of water before fermentation results in the arranged aggregation of caseins micelles during acidification. As will be discussed in next section, the finer is the meshed network, the smaller are the pores and the thinner are the particle strands, the less water is squeezed out [16].

D. Phase separation

The influence of treatment with Transglutaminase on the phase separation of Doogh samples in 15 day storage is shown in Fig. 3. As expected, the application of enzyme reduced the serum separation of Doogh. This is probably caused by the influence of transglutaminase on the flow behavior properties and the pore size of protein network, as discussed in previous sections. [29] Confirmed that water holding capacity of gel networks is improved via treatment by transglutaminase. The greatest portion of sedimentation for all samples is observed in the first 3 days of storage. The most stable sample with ≅ 13% serum separation was M3, the Doogh prepared from pasteurized milk treated with the enzyme followed by fermentation of the diluted milk. Results suggested that samples prepared directly from milk were less prone to supper-aggregates sedimentation than those prepared from yoghurt.

IV. Conclusion

Employment of transglutaminase was found efficacious to prevent serum separation of Doogh. The enzyme application after heat treatment resulted in more stabilization, better uniformity of supper-aggregates dispersion, smaller particle size and higher viscosity. The method of preparation is another factor to improve the physico-chemical characteristics of Doogh.

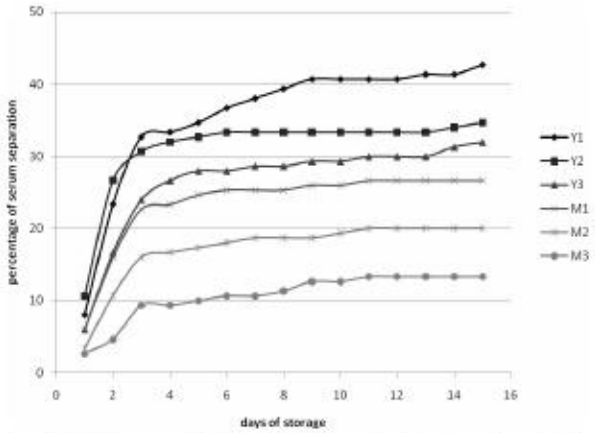

Figure 3. Influence of transglutaminase treatment and methods of preparation on phase separation of Doogh samples during storage at 4°C.

When Doogh was prepared directly from milk a faster preparation with less energy consumption, especially at the cooling step is achieved.

V. REFRENCES

[1] A.Y. Tamime, A. Hassan, E. Farnworth, T. Toba, "Structure of fermented milks," in: A. Tamime (Ed.) Structure of dairy products, Society of Dairy Technology-Blackwell Publishing Ltd, UK., 2007, pp. 134-170.

[2] H. Kiani, M. Mousavi, H. Razavi, E. Morris, "Effect of gellan, alone and in combination with high-methoxy pectin, on the structure and stability of doogh, a yogurt-based Iranian drink," Food Hydrocolloids, 24 (2010) 744-754.

[3] L.-E. Nilsson, S. Lyck, A.Y. Tamime, "Production of Drinking Products," in: A. Tamime (Ed.) Fermented Milks, Society of Dairy Tehnology-Blackwell Science Ltd, UK, 2006, pp. 95-128.

[4] H. Kiani, S.M.A. Mousavi, Z. Emam-Djomeh, "Rheological properties of Iranian yoghurt drink, Doogh," International Journal of Dairy Science, 3 (2008) 71-78.

[5] ISIRI, Iranian National Standard 2453, in: Doogh - Specifications and test methods, Karaj, Iran, 2008.

[6] D. Jaros, C. Heidig, H. Rohm, "Enzymatic modification through microbial transglutaminase enhances the viscosity of stirred yogurt," Journal of texture studies, 38 (2007) 179-198.

[7] S.-J. Jiang, X.-H. Zhao, "Transglutaminase-induced cross-linking and glucosamine conjugation of casein and some functional properties of the modified product," International Dairy Journal, 21 (2011) 198-205.

[8] D. Jaros, H. Rohm, "Enzymes Exogenous to Milk in Dairy Technology | Transglutaminase," in: W.F. Editor-in-Chief: John (Ed.) Encyclopedia of Dairy Sciences (Second Edition), Academic Press, San Diego, 2011, pp. 297-300.

[9] T. Şanlı, E. Sezgin, O. Deveci, E. Şenel, M. Benli, "Effect of using transglutaminase on physical, chemical and sensory properties of set-type yoghurt," Food Hydrocolloids, 25 (2011) 1477-1481.

[10] M.P. Bönisch, M. Huss, K. Weitl, U. Kulozik, "Transglutaminase cross-linking of milk proteins and impact on yoghurt gel properties," International Dairy Journal, 17 (2007) 1360-1371.

[11] C. Guyot, U. Kulozik, "Effect of transglutaminase-treated milk powders on the properties of skim milk yoghurt," International Dairy Journal, 21 (2011) 628-635.

[12] A. Kõksoy, M. Kılıç, "Effects of water and salt level on rheological properties of ayran, a Turkish yoghurt drink," International Dairy Journal, 13 (2003) 835-839.

[13] F. Azarikia, S. Abbasi, "On the stabilization mechanism of Doogh (Iranian yoghurt drink) by gum tragacanth," Food Hydrocolloids, 24 (2010) 358-363.

[14] E.G. Gorji, M.A. Mohammadifar, H. Ezzatpanah, "Influence of gum tragacanth, Astragalus gossypinus, addition on stability of nonfat Doogh, an Iranian fermented milk drink," International Journal of Dairy Technology, 64 (2011) 262-268.

[15] A. Koksoy, M. Kilic, "Use of hydrocolloids in textural stabilization of a yoghurt drink, ayran," Food Hydrocolloids, 18 (2004) 593-600.

[16] C. Schorsch, H. Carrie, I.T. Norton, "Cross-linking casein micelles by a microbial transglutaminase: influence of cross-links in acid-induced gelation," International Dairy Journal, 10 (2000) 529-539.

[17] M.A. Smiddy, J.E.G.H. Martin, A.L. Kelly, C.G. de Kruif, T. Huppertz, "Stability of casein micelles cross-linked by transglutaminase," Journal of Dairy Science, 89 (2006) 1906-1914.

[18] T. Huppertz, C.G. de Kruif, "Structure and stability of nanogel particles prepared by internal cross-linking of casein micelles," International Dairy Journal, 18 (2008) 556-565.

[19] M. Alderliesten, "Mean particle diameters. From statistical definition to physical understanding," Journal of Biopharmaceutical Statistics, 15 (2005) 295-325.

[20] M.C. Bourne, "Food texture and viscosity: Concept and measurement.," Academic Press, New York, 2002.

[21] R. Sharma, P.C. Lorenzen, K.B. Qvist, "Influence of transglutaminase treatment of skim milk on the formation of ε-(γ-glutamyl) lysine and the susceptibility of individual proteins towards crosslinking," International Dairy Journal, 11 (2001) 785-793.

[22] M. Faergemand, J. Otte, K. Qvist, "Enzymatic cross-linking of whey proteins by a Ca^{2+}-independent microbial transglutaminase from *Streptomyces lydicus*," Food Hydrocolloids, 11 (1997) 19-25.

[23] R. Sharma, M. Zakora, K.B. Qvist, "Susceptibility of an industrial α-lactalbumin concentrate to cross-linking by microbial transglutaminase," International Dairy Journal, 12 (2002) 1005-1012.

[24] J.M. Rodriguez-Nogales, "Effect of preheat treatment on the transglutaminase-catalyzed cross-linking of goat milk proteins," Process Biochemistry, 41 (2006) 430-437.

[25] G. Patocka, R. Cervenkova, S. Narine, P. Jelen, "Rheological behaviour of dairy products as affected by soluble whey protein isolate," International Dairy Journal, 16 (2006) 399-405.

[26] I.M. Afonso, J.M. Maia, "Rheological monitoring of structure evolution and development in stirred yoghurt," Journal of Food Engineering, 42 (1999) 183-190.

[27] T. Salomonsen, M.T. Sejersen, N. Viereck, R. Ipsen, S.B. Engelsen, "Water mobility in acidified milk drinks studied by low-field ^{1}H NMR," International Dairy Journal, 17 (2007) 294-301.

[28] M. Færgemand, K.B. Qvist, "Transglutaminase: effect on rheological properties, microstructure and permeability of set style acid skim milk gel," Food Hydrocolloids, 11 (1997) 287-292.

[29] M. Motoki, K. Seguro, "Transglutaminase and its use for food processing.," Trends in Food Science and Technology, 9 (1998) 204-210.