Cheese Analogue-Making Process Using Microbial Transglutaminase: Effects on Texture and Syneresis

Ana M. Cadavid and Laia Bohigas BDF Natural Ingredients, Girona, Spain Email: acadavid@bdfingredients.com, laia@bdfingredients.com

M ónica Toldr à Dolors Par és, Carmen Carretero, and Elena Saguer University of Girona, Institute of Agro-Food Technology, Girona, Spain Email: {monica.toldra, dolors.pares, carmen.carretero, elena.saguer}@udg.edu

Abstract—The aim of the present paper was to determine the effects of adding microbial transglutaminse (MTGase) during the process of manufacturing fresh cheese analogues from a mixture of milk protein concentrate and milk cream, on their textural properties and syneresis. For this purpose, different MTGase concentrations (0, 1, 3 and 5 U g⁻¹ protein) were applied during the analogue-making process using two different coagulating agents, a chymosin produced by fermentation (CHY-MAX[®]) and a fungal aspartic protease (Fromase[®]). The results indicate that MTGase can be effectively used to enhance texture and water holding capacity of cheese analogues made with dairy ingredients, obtaining similar characteristics with either of the two tested coagulating agents. In any case, the best results were obtained with 3 U g⁻¹ protein-MTGase concentration.

Index Terms—cheese analogues, microbial transglutaminase, coagulating agents, dairy ingredients, texture, syneresis

I. INTRODUCTION

Since the end of the last century, several researchers [1], [2] were interested in the manufacture of cheese analogues by blending the individual components. According to them, it was a matter of time before this type of product was introduced into the cheese retail market. Currently, cheese analogues have a new business niche within the dairy industry with an annual production in the United States, the world's largest producing region, of around 300 000 t. Analogues are mainly applied as an ingredient in frozen pizzas and hamburgers as well as in salads, sandwiches and prepared foods [3], [4].

In cheese analogues manufacture, milk fat, milk protein or both can be partially or totally replaced by nondairy constituents, mainly of plant origin [1]. Cheese analogues containing milk proteins and fat, have an important advantage due to their similarity to the physical and functional characteristics of natural dairy constituents. In contrast, non-dairy cheese analogues commonly made by the substitution of casein for vegetable proteins, could

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exhibit deteriorated texture and off-flavors, having a negative effect on the consumers' acceptance [2], [4], [5], [6]. In consequence, organoleptic properties provided by dairy ingredients to the cheese analogues are a key aspect for the consumer approval. Moreover, dairy cheese analogues represent an interesting vehicle for the valorization of protein-based ingredients, facilitating the use of by-products and surpluses from the dairy industry [7]. With whey as one of the most problematic derivatives in cheese-making - 70-90% of the original volume of milk [8] -, the food industry has been working intensely to exploit its technological, functional and nutritional properties [8], [9].

Milk protein concentrates (MPCs), containing both caseins and whey proteins at concentrations very similar to those found in milk [10], [11], are a good example of innovative application that could be favored by the combination with modern processing techniques, including the treatment of milk with microbial transglutaminase (*MTGase*) [12]. In addition, the composition of the MPC makes possible the application of coagulants for the production of cheese analogues, providing them with a more similar character to enzymatic coagulation traditional cheeses [13], [14], [15].

Previous studies about the elaboration of enzymatic coagulation cheeses account for the positive effects of applying *MTGase* during their manufacturing process, on the physicochemical composition and techno-functional properties of the final product [16], [17], [18], [19], [20], [21].

Considering all the above mentioned, this work aimed to develop an analogue of enzymatic coagulation fresh cheese, from a mixture of MPC and milk cream, using two different coagulating agents (a chymosin produced by fermentation –FPC – and a fungal aspartic protease), and evaluate the effects of treating with different *MTGase* levels (0, 1, 3 and 5 U g⁻¹ protein) during the manufacturing process on the characteristics of the final product. Specifically, the present paper focused on determining the effects on texture and water retention capacity of cheese analogues. Also, the protein profile of casein fractions was analysed by SDS-PAGE under reducing conditions.

II. MATERIALS AND METHODS

A. Materials

Milk protein concentrate (MPC 65, DairyTec, Fayrefield Foods GmbH, Wasserburg, Germany), with a chemical composition of 2.4% water, 66% protein, and 7.7% ashes. Cream (Central Lechera Asturiana, Asturias, Spain), containing 38% fat, 56% water, 2.3% protein and 0.4% ashes. Coagulant agents: CHY-MAX Powder Extra NB (Chr. Hansen Holding A/S, Hoersholm, Denmark), 100% chymosin produced by fermentation (FPC) by Aspergillus niger var. awamori with a coagulant activity of 2235 IMCU g⁻¹; and Fromase[®] 220 TL BF (Royal DSM, Heerlen, The Netherlands), a thermosensitive aspartic protease from Rhizomucor miehei with a coagulant activity ≥ 220 IMCU L⁻¹. Commercial preparation of MTGase Probind CH 2.0 (BDF Natural Ingredients SL, Cassà de la Selva, Spain), with an enzymatic activity of 125 U g⁻¹ protein.

B. Experimental Design

Experimental days (3) were considered as blocks in a randomized complete block design in order to study the effect of treating a mixture of MPC and milk cream with different *MTGase* levels (0, 1, 3 and 5 U g⁻¹ of protein) to produce enzymatic coagulation cheese analogues by using two different coagulating agents (CHY-MAX and Fromase). Specifically, the measured parameters included chemical composition as a process control parameter, texture from the Strength-Time curve, and water retention capacity measured under forced conditions. The protein profiles of casein fraction corresponding to the different treatments were analysed by SDS-PAGE under reducing conditions.

C. Manufacturing enzymatic coagulation cheese analogues

To manufacture cheese analogues (1 kg per treatment for each experiment), 450 g of water and 390 g of cream were firstly mixed in a kitchen robot (Thermomix TM5, Vorwerk, Wuppertal, Germany) for 30 s at speed 3. After that, 160 g of MPC 65 were added and the blend was mixed again for 90 s under the same conditions. To prevent the fat separation, it was homogenized in the same robot at a speed 5 and 50 $\,$ $^{\circ}$ C for 5 min. The homogenized mixture was pasteurized at 72 °C for 15 s at speed 2 and, after cooling to 38 °C in a water:ice bath, 10 g of NaCl were added along with the amount of MTGase commercial product (Probind CH 2.0) needed to obtain de desired final concentration (0, 1, 3 and 5 U g^{-1} protein). Then, the resulting mixture was newly mixed in the kitchen robot for 1 min at a speed 2 and put in a bottle before adding the coagulating agent (50 IMCU L^{-1}), hand shaking for 1 min, distributing the content in containers (50 g in each one) and incubating at 38 °C for 30 min to coagulate the samples. After coagulation, cheese analogues were kept at 4 °C for 20 h, before carrying out the compositional, textural and syneresis analyses.

D. Proximate Composition Analysis

Moisture and protein contents of cheese analogues were determined according to the Association of Official Analytical Chemists methods (AOAC, 2000).

E. Texture Analysis

Texture analyses were carried out using a texturometer AEMETEK CT3-10K (Brookfield Laboratories, Middleboro, MA, USA), with a 15 mm- cylindrical stainless-steel penetration probe. The Strength-Time curve was recorded during the probe penetration up to 5.0 mm from the analogue's surface at 1 mm s⁻¹ for 6 different analogues per treatment, right after taking samples from the refrigerator, without sample extraction and taking only one measure per analogue. The measured parameters were: firmness (peak force in N during the downstream stage of the penetration test); final distance strength; consistency (work done from the beginning of the test to final load); and stickiness (work necessary to overcome the attractive forces between the surface of the product and the surface of the probe at the end of the penetration test, expressed in absolute value in this paper).

F. Forced Syneresis

A technique combining filtration and centrifugation based on that proposed by [22] and slightly modified was used. Gel cylinders (20 mm length and 8 mm dia) were placed into cylinders of PVC with polyester mesh (100 mm aperture; Henry Simon, Cheshire, UK) in the bottom, which were suspended inside 50 mL-centrifuge tubes and then centrifuged at 2000 x g for 10 min at 15–20 °C. The results are reported as percentage (w/w) of water released after centrifugation. Three replicates were measured for each sample.

G. SDS-PAGE Analysis

Prior to the analysis of the casein fraction from cheese analogues by reducing SDS-PAGE, caseins were separated from whey proteins. For this purpose, 30 g of each cheese analogue were blended with 30 g of Milli-Q water using mixer MSM6500/03 (BSH а Electrodom ésticos España SA, Zaragoza, Spain). After homogenization and pH adjustment to 4.6 with 2 M HCl, the mixture was cooled to 10 °C and centrifuged (Allegra[®] X-22 Series - Beckman Coulter, Inc, L'Hospitalet de Llobregat, Spain) at 4500 rpm and 10 °C during 20 min. Caseins (insoluble fraction) were recovered after washing with 0.5 M Na-acetate buffer (pH 4.6) and centrifuged at 7000 rpm and room temperature for 15 min, three times. The clean casein fraction was frozen and lyophilized using a freeze-dryer CRYODOS-80 (Telstar, Terrassa, Spain).

50 mM phosphate buffer and Laemmli 4X buffer (Bio-Rad Laboratories Inc. Hercules, CA, USA) containing 200 mM DTT were added to thawed samples to obtain a final protein concentration of 0.75 μ g· μ L⁻¹. Sample solutions were heated at 100 °C for 5 min and, then, cooled to 5 °C. After that, 20 μ L of each sample were loaded to the polyacrylamide gel (stacking gel 5%; resolving gel, 15%) prepared using a solution of acrylamide/bis 29:1 (Bio-Rad Laboratories Inc). Precision Plus Protein TM Dual Color Standards, (Bio-Rad Laboratories Inc.) was used for molecular weight estimation in the range from 10 to 250 kDa. Electrophoresis was performed at 90 V for 10 min, and followed for a second step at 200 V for 30 min. The resulting gel was stained with Coomassie Brilliant Blue R-250 (Bio-Rad Laboratories Inc.) for 10 min and destained in 7% acetic acid solution for 24 h. All gels were analyzed using the image analyzer Molecular Imager Gel Doc XR System equipped with the software Quantity One 1-D Analysis (Bio-Rad Laboratories Inc.).

H. Statistical Analysis

The statistical analyses were carried out using IBM SPSS Statistics version 23.0 for Windows (IBM SPSS Statistical software Inc., Chicago, IL, USA). In all cases, data were submitted to ANOVA using the general linear model procedure (Proc GLM) and considering *MTGase* concentration and kind of coagulating agent as fixed effects, and experimental day as random effect. When significant effects were obtained, the Tukey's test was used for the *post hoc* analysis to compare means. The significance level for all tests was $\alpha = 0.05$.

III. RESULTS AND DISCUSSION

A. Proximate Composition

Results from chemical composition of cheese analogues produced using two different coagulating agents (CHY-MAX and Fromase) and applying different *MTGase* concentrations (0, 1, 3 and 5 U g⁻¹ protein) were used only as process control parameters because in this type of dairy products there are no material losses during their processing. As expected, statistical analysis did not show any significant effect of the studied factors (p>0.05). All cheese analogues contained around 66% water and 11% protein.

B. Texture

Globally, the statistical analyses of the results corresponding to the different textural parameters (firmness, final distance strength, consistency, and stickiness) of cheese analogues showed that the applied coagulant agent had only significant effects (p<0.05) in the case of the stickiness. That is why this textural attribute results have been separately analysed for each of the two coagulating agents used. By contrast, the applied *MTGase* concentration significantly (p<0.05) affected all the analysed parameters.

Regarding firmness, and according to the Tukey's mean separation test, this textural attribute was only significantly increased (p<0.05) when the prepared mixture for making cheese analogue was treated with 3 U g⁻¹ *MTGase*. Intermediate firmness values were obtained for the two others tested *MTGase* levels (1 and 5 U g⁻¹ protein) but being not significantly different (p>0.05) neither between them nor from those obtained with the aforementioned treatments, i.e. 0 and 5 U g⁻¹ protein (Fig. 1).



Figure 1. Firmness of cheese analogues as a function of *MTGase* concentration applied during the analogue-making process. Mean \pm SD considering together the results obtained with both coagulating agents (n=6). Different minor letters mean significant differences between *MTGase* levels, according to Tukey's mean separation test (α =0.05).

As a whole, these results may indicate that the behavior of analogue's firmness as a function of the *MTGase* concentration adopts an upside-down U-shaped parabola, showing an optimum at a concentration of 3 U g⁻¹ protein and with a setback in the improvements being obtained when it is exceeded.

MTGase (U g ⁻¹)	Final Distance Strength (N)	Consistency (N s)
0	$0.90 \pm 0.08 \text{ a}$	3.02 ± 0.25
1	1.01 ±0.11 ab	3.33 ± 0.32
3	1.15 ±0.12 b	3.38 ± 0.30
5	1.05 ± 0.19 b	3.08 ±0.37

 TABLE I. TEXTURAL PROPERTIES OF CHEESE ANALOGUES PRODUCED

 USING DIFFERENT MTGASE CONCENTRATIONS

Table I shows the values corresponding to final distance strength. As can be observed, its behavior is very similar to that one of firmness although the values tend to be slightly lower in some treatments.

From Strength-Time curves obtained through the penetration test for both coagulating agents (Fig. 2a and 2b), it can be verified that, indeed, not in all the curves the firmness value is reached when reaching the maximum penetration distance. Despite in many food products force peak matches the final distance, it does not always happen in fresh cheeses.

Also from Fig. 2, it seems that, under the processing conditions established in the present study, the maximum firmness that can be reached is approximately 1.20 N, and from this value, the force required to continue with the probe displacement until the established final distance is kept practically constant.

Although the statistical analysis did not show a significant interaction (p>0.05) between the two tested factors (*MTGase* concentration and kind of coagulating agent), slight differences in the behavior pattern relative to the effect of the applied *MTGase* level on the Strength –Time curve can be observed between the two tested coagulating agents by comparing Fig. 2a and Fig. 2b.

^{*} Mean \pm SD considering together the results obtained with both coagulating agents (n=6). Different minor letters in the same column mean significant differences between *MTGase* levels, according to Tukey's mean separation test (α =0.05).



Figure 2. Strength-Time curves corresponding to enzymatic coagulation cheese analogues treated with different *MTGase* concentrations, using two coagulating agents: a) CHY-MAX, and b) Fromase. Each curve represents the average of 6 replicates.

In the case of CHY-MAX (Fig. 2a), the trend for the two lower *MTGase* concentrations (1 and 3 U g L⁻¹) is similar to that of the untreated one (0 U g L⁻¹), but achieving higher strength values as the enzyme concentration increases. However, a negative effect on the texture seems to happen when the enzyme concentration increases beyond 3 U g L⁻¹.

From Fig. 2b, it can be maintained that the combined action of *MTGase* and Fromase is more efficient than the one of the cross-linking enzyme activity and CHY-MAX, since a firmness value around 1.20 N is already achieved at the lowest tested *MTGase* concentration (1 U g⁻¹ protein) with Fromase. In addition, applying a *MTGase* concentration of 5 U g⁻¹ protein in cheese analogues does not contribute with practically any improvement when compared with the untreated ones.

Regarding analogue's consistency (Table I), a significant effect of the *MTGase* concentration was actually obtained for this parameter, although the probability value (p<0.043) was very close to the significance level. Possibly, that is the reason why Tukey's test was not able to separate the means corresponding to different treatments. However, from an accurate analysis of the data, it was possible to detect a behavior as a function of the *MTGase*-concentration similar to the one of firmness.

Stickiness of cheese analogues was significantly (p<0.05) affected not only by the applied *MTGase* concentration but also by the particular coagulating agent, without both factors interacting. As can be observed in Fig. 3, for both coagulating agents, the cheese analogues obtained after treating the ingredient's mixture with one of the two highest *MTGase* concentrations (3 and 5 U g⁻¹

protein) were significantly less sticky (p<0.05) than those treated at the lowest one (1 U g⁻¹ protein). Also, for *MTGase*-treated cheese analogues, the stickiness values were always significantly (p<0.05) lower to those from the untreated ones.



Figure 3. Stickiness (in absolute values) of enzymatic coagulation cheese analogues as a function of *MTGase* concentration and type of coagulating agent (CHY-MAX or Fromase). Means ±SD. Different minor letters mean significant differences between *MTGase* concentrations for each coagulating agent, according to the Tukey's mean separation test (α = 0.05, n= 3).

Regardless of the effect of *MTGase* concentration, cheese analogues manufactured using CHY-MAX showed significantly (p<0.05) higher stickiness values (in absolute value) than those produced with Fromase (0.22 ± 0.11 N s and 0.18 ± 0.08 N s, respectively).

C. Forced Syneresis

Statistical analysis of forced syneresis data indicated a significant interaction (p<0.05) between the main factors included in the model. Consequently, it was mandatory to analyze the effects of the cross-linking enzyme for each coagulation agent separately. As can be seen in Fig. 4, only in the case of CHY-MAX the amount of water released after centrifugation significantly (p<0.05) varied with the applied *MTGase* concentration. Specifically, water losses tended to be reduced as the *MTGase* concentration increased. By contrast, no significant effects (p>0.05) on this parameter were observed when Fromase was used to induce milk coagulation.



Figure 4. Forced syneresis (expressed as a percentage of water released after centrifugation) of enzymatic coagulation cheese analogues as a function of *MTGase* concentration and kind of coagulating agent (CHY-MAX or Fromase). Means \pm SD. Different minor letters mean significant differences between *MTGase* concentrations, according to the Tukey's mean separation test (α = 0.05, n= 3).

This discrepancy in the behavior between the two coagulating agents may be due to the lower water retention capacity of analogues produced with CHY-MAX in the absence of the polymerizing enzyme.

D. SDS PAGE Analysis

MTGase-induced cross-linking of milk proteins during the cheese analogue manufacture was clearly evidenced by the formation of new high molecular weight protein polymers, when comparing the protein profiles corresponding to the casein fraction of the untreated analogues (0 U g⁻¹ protein) with the *MTGase*-treated ones, for both coagulating agents (Fig. 5). The new-formed polymers were visible at the top of the electrophoresis gel - between 75 and 250 kDa - for the different MTGasetreated analogues; and, even, a dark band not able to penetrate the stacking gel can be observed in these samples. Untreated samples, by contrast, did not exhibit any of these bands. By contrast, in all the profiles a band corresponding to para- κ -CN - next to the α -lactoalbumin and formed as a consequence of the treatment with the coagulating agents - is evident.



Figure 5. SDS-PAGE profiles of casein fraction from cheese analogues elaborated with two different coagulating agents (Fromase and CHY-MAX) and treated with different *MTGase* concentrations (0, 1, 3 and 5 U g⁻¹ protein). Std: Molecular weight standards.

SDS-PAGE profiles also show a decrease in the size of α_s -CN and β -CN bands in *MTGase*-treated cheese analogues in comparison to the untreated ones, concomitant to the appearance of new-formed polymers. κ -CN band was also markedly diminished, as expected, not only due to its high reactivity with MTGase and its affinity for the formation of bonds with serum proteins and other caseins. Additionally, a slight decrease in the intensity of low molecular weight products was observed as the concentration of MTGase increased, indicating the possible inclusion of them into the new-formed polymers by the action of this enzyme. In this sense, and as reported by other authors, the cross-linking degree of the different caseins is related to the respective location of each type of casein within the casein micelle [23], [24] and their particular reactivity with MTGase.

The fact that the electrophoretic profiles were obtained under reducing conditions with no more differences in the manufacture process for both *MTGase*-treated and untreated cheese analogues than the addition of this enzyme, confirmed that the changes observed in the band pattern were due to the cross-linking of milk proteins by the catalysis of the *MTGase*.

IV. CONCLUSIONS

MTGase-treatment of the dairy ingredient's mixture used in the elaboration of cheese analogues had a concentration-dependent positive effect on their textural properties thanks to its protein cross-linking activity. Taking together all the texture results, it can be affirmed that 3 U g⁻¹ protein is the optimal *MTGase* concentration to improve the texture of cheese analogues prepared under the conditions established in this work, regardless of the type of coagulating agent. By contrast, forced syneresis was only improved by protein cross-linking when fermentation produced chymosin (CHY-MAX) was used, with the above-mentioned *MTGase* concentration being also the recommended one.

Although the experimental conditions used in the elaboration of cheese analogues did not enable to obtain a cheese-like product actually comparable to a natural enzymatic coagulation fresh cheese, the *MTGase* cross-linking activity could contribute with better results after modifying some process conditions, for example during the homogenization step.

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Ana M. Cadavid was born in Medell ń, Colombia on November 18, 1986. She holds a BSc degree in Biological Engineering from the National University of Colombia (2010) and a MSc degree in Food Biotechnology from the University of Girona (2013). Currently a Ph. D. candidate at University of Girona in association with the company BDF Natural Ingredients, where she is developing her doctoral thesis related with the application of Microbial Transglutaminase (*MTGase*) in dairy products. She has an important background in the implementation and application of the hydroxamate-based method for *MTGase* quantification and experience in the application of the enzyme in different protein-based food products.



Laia Bohigas was born in Girona on August 08, 1980. She holds a degree in Food engineering and she has been working in the Food sector for more than 15 years. After finishing her degree, she worked in a Bakery company as R+D and Quality Control technician for 3 years and a half. After this period, she worked in a Meat company as Quality Manager for 1 year. From 11 years ago, she has been working in BDF Natural Ingredients, she started as food technician in

the Technical department. Together with the growth of the company, she had been specializing in Microbial Transglutaminase (*MTGase*) application area, which includes different sectors: meat, fish, dairy and bakery. Nowadays she is the Transglutaminase Product Manager and responsible for the projects coordination of the Technical Department. She is also part of the innovation committee of BDF Natural Ingredients.



Mònica Toldr à was born in Barcelona, Spain, on September 21, 1968. She holds a degree in Veterinary Medicine from the Autonomous University of Barcelona (UAB) and a PhD in Biology from the University of Girona (UdG), in Spain. She is an Associate Professor of Food Technology in the Department of Agricultural, Chemical Engineering and Agrifood Technology and researcher of the Institute of Agrifood Technology of the

University of Girona. Her research focuses on the field of the use of coproducts from the meat industry and the development of technofunctional food ingredients from low commercial value meat coproducts. She has participated in several research projects and research and technology transfer agreements with food companies.



Dolors Par és was born in Girona, Spain on January 14, 1964. She completed Doctorate in the University of Girona (UdG) in 1998. Her career as a teacher and researcher has developed at the Polytechnic School of the University of Girona in Spain. She is a member of the research group "Food Technology" of the Agrifood Technology Institute (INTEA). Her research interests have been focused on the use of meat industry byproducts aimed at obtaining functional and/or

nutritional added-value ingredients for food and feed purposes. She is senior lecturer in Food Technology specialized in industrial microbiology and biotechnology, food safety and meat technology.



Carmen Carretero was born in Zaragoza, Spain on June 18, 1959. She completed her doctorate in Food Technology in the Department of Food Technology of the Autonomous University of Barcelona. She has projects and publications about dairy technologies, and upgrading technologies of porcine blood and meat by-products. She has been working as Professor in Food Technology in University of Girona since 1993.



Elena Saguer was born in Girona, Spain on August 28, 1965. She holds a degree in Biological Sciences from the Autonomous University of Barcelona (UAB) and a PhD in Biology from the University of Girona (UdG). She is an Associate Professor of Food Technology in the Department of Agricultural, Chemical Engineering, who teaches in subjects related to food analysis and food biotechnology. Her research mostly focuses on meat by-product valorization, including the use of microbial transglutaminase to improve technofunctional properties of proteins. At present, she is also interested in applying this enzyme on food proteins from other sources.