RESEARCH ARTICLE

Effect of Recombinant Transglutaminase on the Quality Characteristics of Cooked Beef Meatballs

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Abstract

Transglutaminase (TGase) is an enzyme widely used in the food industry. In this study, the effect of transglutaminase enzyme on the chemical and physical characteristics of cooked beef meatballs was evaluated. For this aim, beef meatballs were prepared by using recombinant microbial transglutaminase (MTGase) and commercial TGase enzymes, after that physical and chemical tests were applied to meatball samples. The addition of MTGase enzyme improves the quality parameters of the beef meatballs. The myofibrillar proteins of cooked meatball samples were also analyzed with SDS-PAGE analysis. It was observed that, actin and myosin proteins bind covalently to form a new high molecular weight protein by the help of MTGase addition. These results indicated that recombinant MTGase enzyme can be used to obtain high quality restructured beef meat products.

Keywords: *Beef, Meatball, Recombinant enzyme, Transglutaminase, Pichia pastoris*

Rekombinant Transglutaminazın Pişmiş Sığır Köftelerinin Kalite Özelliklerine Etkisi

Öz

Transglutaminaz (TGase), gıda endüstrisinde yaygın olarak kullanılan bir enzimdir. Bu çalışmada, transglutaminaz enziminin pişmiş sığır köftelerinin kimyasal ve fiziksel özellikleri üzerine etkisi değerlendirilmiştir. Bu amaçla, sığır köfteleri rekombinant mikrobiyal transglutaminaz (MTGase) ve ticari TGase enzimleri kullanılarak hazırlanmış, ardından köfte örneklerine fiziksel ve kimyasal analizler uygulanmıştır. MTGase enziminin ilavesi, sığır köftelerinin kalite parametrelerini iyileştirmiştir. Pişirilmiş köfte örneklerinin miyofibriler proteinleri de SDS-PAGE yöntemi ile analiz edilmiştir. MTGase ilavesi ile, aktin ve miyosin proteinlerinin kovalent bağlanarak yeni bir yüksek moleküler ağırlıklı protein oluşturduğu görülmüştür. Bu sonuçlar, yüksek kaliteli yeniden yapılandırılmış sığır eti ürünleri elde etmek için rekombinant MTGase enziminin kullanılabileceğini göstermiştir.

Anahtar sözcükler: *Sığır eti, Köfte, Rekombinant enzim, Transglutaminaz, Pichia pastoris*

INTRODUCTION

Meatballs, an important ready-to-eat meat product, are the most common among hot pot materials and are well-liked by Turkish consumers. The cooking process contributes a special texture to meatballs due to the gelation of myofibrillar proteins [1,2]. However, high temperature cooking adversely affects the water holding capacity and the textural properties of the meatballs due to the poor gelation capacity of these proteins. The gel properties of heat-induced myofibrillar proteins are enhanced by modification of their structure. For this purpose, the transglutaminase enzyme is widely used in the food industry [3]. Transglutaminase enzyme (TGase, protein-glutamine g-glutamyltransferase, EC 2.3.2.13) is a binder agent that induces protein aggregation in muscle foods through isopeptide covalent cross-linking between glutamine residues (acting as acyl donor) and lysine residues (acting as acyl acceptor) [4,5]. TGase has been employed to improve the gel properties, water holding capacity and emulsion stability of food protein [6,7], and the quality characteristics of meat products [4,8,9], and during recent years, it has been used in the production of

restructured meat products [3,10,11]. Therefore, it increases the economic value of meat products and decreases waste. Besides, crosslinking proteins catalyzed by TGase containing various essential amino acids improve the nutritional value of meat products [12]. Studies have shown that TGase alone enhanced the functional and textural properties of meat products.

In general, the TGase enzyme is commercially obtained by extracting and purifying from the tissues or body fluids of plants and animals [13-15]. But low yield, time-consuming, high cost and complex purification procedures are the main problems in the extraction of this enzyme. Therefore, researchers have developed effective bacterial TGase expression systems like *Streptomyces lividans*, *Escherichia coli*, and *Corynebacterium glutamicum* to obtain high amount of TGase enzyme [16,17]. Because of rapid growth, bacterial expression systems are frequently used to express MTGase enzyme but there are some restrictions on the use of this system like inclusion body formation, complex refolding processes and incompatible with post-translational modifications. To overcome these problems yeast expression systems have become a good alternative. For recombinant protein production, *Saccharomyces cerevisiae, Pichia pastoris, Hansenula polymorpha, Yarrowia lipolytica,* and *Kluyveromyces lactis* are commonly used. *P. pastoris* is a methylotrophic yeast that is capable of high heterologous extracellular protein production. Additionally, this yeast can grow high cell densities in basic media (containing methanol, ethanol, glucose, and glycerol) and is suitable for genetic manipulations. Yang and Zhang [12] reported that *Streptomyces fradiae* pro-MTGase enzyme was expressed under the control of methanol inducible *AOX1* promoter in *P. pastoris* and the effect of the recombinant MTGase enzyme on the quality of restructured pork meat was evaluated. The results showed that the hardness and chewiness of the restructured meat were increased, and the adhesiveness decreased after the MTGase treatment.

To our current knowledge, there is no data available concerning the effect of recombinant MTGase enzyme on the quality of cooked beef meat in Turkey. Therefore, this study was designed to investigate the effect of the recombinant MTGase on the quality of cooked beef meatballs. For this aim, recombinant *Streptomyces mobaraensis* pro-MTGase enzyme was expressed in *P. pastoris* under the constitutive *GAP* promoter in a 5-L bioreactor [18] and then, beef meatballs were prepared by using the recombinant MTGase enzyme to evaluate chemical and physical characteristics of cooked beef meatballs.

MATERIALS AND METHODS

Chemical and Reagents

The protein marker used in this study was obtained from Thermo Fisher Scientific (ABD). Cultivation media constituents were purchased from Becton Dickinson and Company (BD) (Franklin Lakes, NJ, USA). Other chemicals and reagents were analytical grade and acquired from Sigma-Aldrich Co. (MO, USA), Merck (Deutschland). The recombinant MTGase enzyme produced in *P. pastoris* was used [18]. The *P. pastoris* X33 strain used in this study was obtained from Life Technologies (Carlsbad, CA. USA). Commercial transglutaminase enzyme was obtained from Ajinomoto Foods Europa SAS (Paris, France). The composition of the commercial enzyme consisted of 99% maltodextrin and 1% transglutaminase and its enzyme activity was reported as 100 Units (U)/g by the manufacturer. Ground beef with approximately 20% beef fat and 1% salt was obtained from a well-known butcher (Veli Cengiz Meat Products Ltd.) in Antalya. The purchased ground beef was a homogeneous mixture of lean beef cuts, beef fat and salt. Considering the tendencies to reduce food salt [4], this salt concentration would be sufficient to ensure the eating salinity (≈2%) of cooked meatballs.

The Production of Recombinant MTGase

The recombinant MTGase enzyme was produced under the control of constitutive *GAP* promoter in *P. pastoris* X33 strain as previously described [18]. In bioreactor level production of the MTGase, fermentation was conducted two-step fed-batch process. For this purpose, a vial of frozen culture was used to inoculate 100 mL BMGY and cultivated for 12 h. 100 mL (10 OD_{600nm}) pre-culture were inoculated to 2 L pH 5 citric acid media (2.0 g/L citric acid monohydrate, 45.6g/L glycerol (86%), 12.6g/L $(NH_4)_2HPO_4$, 0.5 g/L $MgSO_4*7H_2O$, 0.9 g/L KCl, 0.022 g/L $CaCl_2*2H_2O$) at the first step of the fermentation, the batch phase, and continued until sudden rise in dissolved oxygen (DO) level (about 16-20 h) at 28°C, pH: 5 and 900 rpm stirring conditions. After this carbon-exhaustion signal the second step, the fed-batch phase, was started with 50% glucose feed. At the beginning of the fed-batch phase, the pH and temperature values were adjusted to optimum pH and temperature values (pH 7 and 20°C) to obtain maximum enzyme production in the culture supernatants. During the 70 h fed-batch phase the feed rate of glucose solution was exponentially increased; it started with 3 mL/L/h and finished with 18 mL/L/h flow rate. DO level (20% saturation) of this phase was controlled by agitation speed, adding 1.5vvm airflow and supplying pure oxygen as necessary. At the end of enzyme production phase cells were separated by centrifugation and supernatants were collected. The harvested supernatant samples were analyzed with the BCA Protein Assay Kit (Thermo Fisher Scientific [USA]) to determine the amount of total protein. The enzyme was produced in an inactive pro-MTGase form and activated with Dispasel, considering the amount of protein. After activation of the enzyme MTGase activity was calculated as previously described [19] and used in the production of meatballs.

Preparation of the Meatballs

The meatballs used in the research were prepared in

three different compositions: control, commercial and recombinant. Differences in the formulation were sourced from the TGase enzyme. Except for salt, no ingredients such as black pepper, paprika or cumin were used in the formulation of the meatball. The meatballs that included no TGase enzyme were used as a control. Both commercial and recombinant MTGase enzymes were added 400 U to 1 kg ground beef. The enzyme amount and concentration were determined as 0.4 U/g, which is the concentration used by a local company conducting restructured meat products experiment in Antalya. Ground beef was randomly divided into 3 groups for different compositions. All groups consisted of 1 kg ground beef (about 10 meatballs), and a total of 3 kg ground beef were used for one replication. Two replications were carried out for all analyses, so a total of 6 kg ground beef was used.

After the TGase enzyme was added to the ground beef, the mixture was kneaded by hand for five minutes, and homogeneous meatball dough was obtained. After the meatball dough was spread in about 2 cm thickness, meat-balls were shaped using petri dishes of 9 cm in diameter. Then, the samples were spread in one layer on cooking paper and kept in an incubator at 40°C for 2 h to catalyze the enzymatic reaction before cooking. A maximum period of 2 h was applied for enzyme activation at 40°C to avoid beef spoilage. The meatballs were cooked in a preheated oven (Siemens HB86K575, South Africa) for about 20 min using the meatball cooking program (the cooking temperature set to 180°C) until the temperature at the geometric centre reached 72°C. At the end of cooking, all samples were allowed to cool at room temperature (25°C), packed in seal plastic bags and stored at refrigerator temperature (4°C) overnight before determining their quality properties.

Chemical and Physical Analysis

The cooking loss of samples was calculated by the difference in the weight of meatballs before and immediately after cooking. The dimension change of the samples was calculated by the difference of the diameter and thickness of the meatballs, measured with a caliper, before and after they were cooked.

The thiobarbituric acid reactive substances (TBARS) were determined according to the method of Lemon [20] and expressed as μmol malondialdehyde (MDA)/kg of the sample.

The water holding capacity (WHC) of meatballs was determined based on the method detailed by Wang et al.[2] Meatballs were cut into cubes (approximately 30×30×20 mm³). Each cube was placed between the filter papers and then pressed with a 5 kg mass for 2 min. Values of WHC were calculated by the ratio between weight before pressing and weight after pressing.

Color parameters of the samples were measured by using a CR-400 Chromameter (Konica Minolta Inc., Osaka, Japan) and expressed as $L*$ (lightness), $a*$ (redness), and $b*$ (yellowness) values. The color device was calibrated by using its white ceramic plate before actual use. Color values were measured using 3 cubes per group and 3 measurements per cube. Accordingly, the results were reported as the mean value of nine replicates for each group.

The texture profile of the meatball samples was determined using a TA.XTplus Texture Analysis Device (Stable Micro-systems, UK). The meatballs removed from the refrigerator were kept at 25°C for 1 h and then cut into cubes to be subjected to texture profile analysis (TPA). The hardness, springiness, cohesiveness and chewiness properties of the meatball cubes were determined. For the analysis, a 100 mm cylinder probe (P/100) and Heavy Duty Platform (HDP/90) accessories were used. Before and after the TPA test, the probe speed was set to 2 mm/sec, the test speed was set to 5 mm/sec, the waiting time was set to 2 sec, the trigger strength was set to 5 g, the load cell was set to be 50 kg and the distance was set to the distance that would provide 40% deformation.

Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

The meatball samples were analyzed with the SDS-polyacrylamide gel electrophoresis method to show the formation of a covalent cross-link between intermolecular protein. The raw minced beef meat and, the commercial and recombinant MTGase enzyme treated cooked beef meatballs were analyzed to observe the changes in protein patterns. Proteins were extracted according to method described by Sorapukdee and Tangwatcharin [21]. In order to solubilize the samples, 27 mL of 5% SDS was added to 3 g samples and homogenized with ultra-turrax (IKA-T18, Staufen, Germany) and incubated at 85°C for 1 h and centrifuged at 3000 g for 20 min. After centrifugation, undissolved debris was removed and supernatant samples were collected. The amount of total protein was determined by using BCA Bradford Assay kit and about 15 μg protein was treated with dithiothreitol (DTT) and incubated 70°C for 10 min. Then samples were loaded to 10% SDS-PAGE gels and subjected to electrophoresis for 1 hour at 100V to determine myofibrillar protein bands. After separation, the gel was stained with Coomassie Blue (G250) and scanned with the Odyssey Infrared Imaging System (LI-COR, Lincoln, NE, USA). The protein bands aligned to Page Ruler unstained protein ladder (Thermo Fisher Scientific, MA, USA) and actin, myosin and the other protein bands were identified. The relative quantification of the target protein bands was determined with ImageJ program.

Statistical Analysis

The meatball production was made in two replicates, and the analyses of the samples were held in parallel. Variance analysis (ANOVA) was made for the data and a Duncan Multiple Comparison Test was applied to the important

factors. All statistical calculations were done using SAS Statistics Software (v.7.00, SAS Institute Inc., Cary, NC, USA), and the values were given as mean±standard error.

RESULTS

Table 1 shows the cooking loss for the meatballs formulated with different TGase enzymes. The cooking loss values of recombinant and commercial meatballs were slightly lower than that of the control, but not significantly (P>0.05). The increase in thickness (22.73%) and the decrease in diameter (34.75%) of control meatballs were higher than those of other meatballs *(Table 1)*, indicating that both TGase enzymes were useful in retaining moisture in the product during cooking and maintaining the shape of the meatballs. The results of the WHC were also presented in *Table 1*. There is no significant difference in WHC of meatballs after it is treated with TGase (P>0.05).

The TBARS values of meatball samples were significantly affected (*P*<0.01) from the use of the TGase enzyme *(Table 1)*. The highest TBARS value was detected in control meatballs. Both TGase enzymes slowed down lipid oxidation but did not inhibit it. The *L** value of commercial and recombinant meatballs was higher than that of the control *(Table 2)*. The other color parameters *a** and *b** were not significantly affected by the formulations. The textural properties of the meatballs are given in *Table 3*. Except for chewiness (P<0.05), the textural properties were not significantly (P>0.05) affected by the addition of TGase.

Hardness, springiness and, cohesiveness showed a mean value of 0.18 kg, 0.91 and 0.74, respectively.

The result of SDS-PAGE analysis of raw minced meat and restructured cooked meatballs was presented in *Fig. 1*. The raw minced meat without MTGase addition was used as a control, and actin and myosin bands were detected on the gel. The densitometric profiles of actin, myosin and newly formed protein band were shown in *Fig. 2*. The relative quantities of actin, myosin and newly formed protein bands were analyzed and the calculated peak areas were shown above the related peaks. When compared to the control group, actin and myosin peak areas were decreased and newly formed protein peak areas were increased in both commercial and recombinant MTGase treatments *(Fig. 2)*.

DISCUSSION

Lower cooking loss value may have been due to the TGase enzyme promoted strong protein interactions, enhancing the water holding capacity and consequently, decreasing the cooking loss. Tseng et al.[4] reported that the cooking yield of low-salt chicken meatballs containing the TGase enzyme was significantly higher than the control group. Monteiro et al.[10] reported that the levels of TGase enzyme from 0% to 0.8% led to a significant increase in cooking yield of restructured tilapia steaks. It was reported that the cooking loss of pork sausages decreased with the addition of a combination of TGase, hydrocolloids, acorn powder, and mung bean powder, due to improved water

Table 1. *The physico-chemical properties of meatballs*

Meatballs	Cooking Loss (%)	Increase in Thickness (%)	Decrease in Diameter (%)	WHC (%)	TBARS (µmol MDA/kg)
Control	51.97±0.39[a]	22.73±0.19[a]	34.75±1.37[a]	99.68±0.05[a]	43.09±1.62[a]
Recombinant	46.45±3.17[a]	17.87±0.06[b]	27.20±1.55[ab]	99.72±0.03[a]	32.73±0.07[b]
Commercial	45.77±0.92[a]	16.22±0.11[c]	26.59±2.09[b]	99.71±0.02[a]	32.03±0.36[b]

[a,b,c] *Means with different letters within the column indicate differences*

Table 2. *Color values of meatballs*

Meatballs	L*	a*	b*
Control	50.26±0.07[b]	7.69±0.22[a]	11.76±0.03[a]
Recombinant	53.36±0.40[a]	8.09±0.41[a]	11.81±0.20[a]
Commercial	52.92±0.38[a]	7.36±0.11[a]	11.92±0.07[a]

[a,b] *Means with different letters within the column indicate differences*

Table 3. *Textural properties of meatballs*

Meatballs	Hardness (kg)	Springiness	Cohesiveness	Chewiness (kg)
Control	0.16±0.01[a]	0.91±0.02[a]	0.75±0.01[a]	0.10±0.00[c]
Recombinant	0.19±0.04[a]	0.91±0.01[a]	0.73±0.02[a]	0.15±0.01[a]
Commercial	0.19±0.02[a]	0.92±0.00[a]	0.75±0.01[a]	0.13±0.01[b]

[a,b,c] *Means with different letters within the column indicate differences*
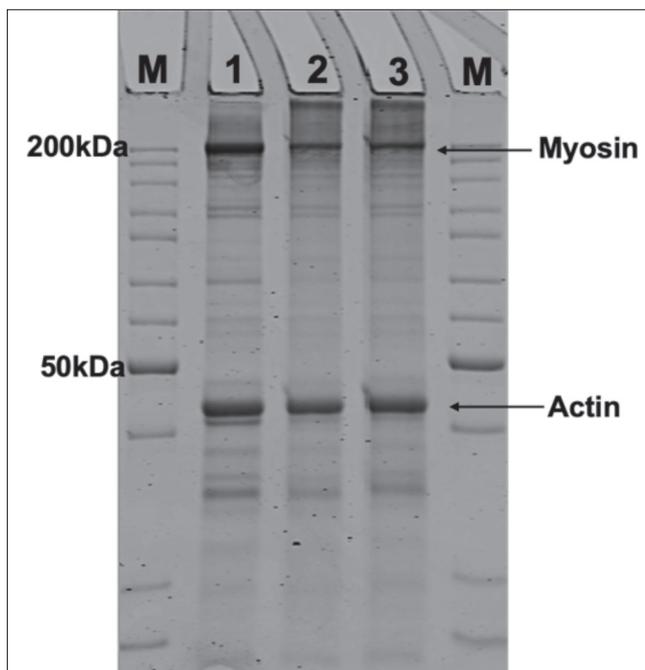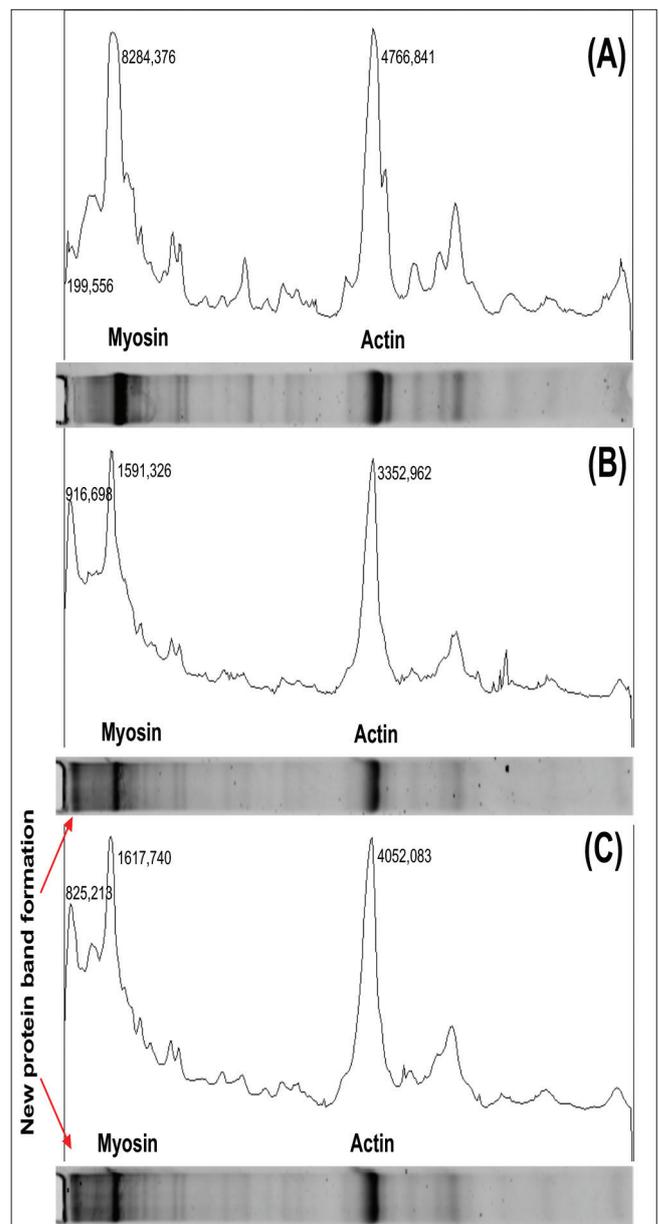
Fig 1. SDS-PAGE analysis of myofibrillar proteins of raw minced meat (Lane 1) and commercial (Lane 2) and recombinant MTGase (Lane 3) treated cooked meatballs, respectively. M, Page ruler unstained protein ladder

Fig 2. Densitometry plots of SDS-PAGE gels of myofibrillar protein. A: Control group, B: Cooked meatballs with the addition of commercial MTGase, C: Cooked meatballs with the addition of recombinant MTGase

binding properties [22]. Similar results were also reported for reduced-salt frankfurters treated with sea mustard [23] and for restructured beef steaks with plant proteins [24].

Shrinking of control meatballs may be sourced from the volume of the diverging water and the mobility of the protein matrix during cooking. TGase enzymes used in meatballs provided the formation of covalent disulfide bonds and aggregation of the exposed hydrophobic amino acids via hydrophobic interactions, in turn leading to the formation of a regular gel network. Tseng et al.[4] also reported that low-salt chicken meatballs made with TGase formed firmer and more regular gel network structures than the control samples which has a looser gel network.

High WHC values indicated the retention of less moveable water and the maintenance of juiciness in meatballs. These results were probably due to the salt content of meatballs (salinity, 1%). It was reported that the use of microbial TGase (without salts) can result in meat products with poor water-binding properties [25]. Tseng et al.[4] reported that low-salt (1%) chicken meatballs with TGase had better emulsion stability and hydration properties. These results suggest that salts are therefore required to improve the protein-water interactions in cooked meat products, along with the TGase enzyme.

Thiobarbituric acid reactive substances results showed that free radicals could be more stable in meatballs with TGase. These results are in line with Gharibzahedi et al.[26], where cross-linked TGase microcapsules with edible oils

had high effectiveness to delay the lipid oxidation process because the use of TGase could stabilize the polymeric structure of the microcapsule against the diffusion of prooxidants and digestive enzymes. Additionally, it was reported that emulsions stabilized by TGase treated protein isolate showed the inhibiting effects against lipid oxidation due to their larger particle size than the control emulsion [27]. However, it was reported that the addition of different percentages of binder admixture including TGase did not affect lipid oxidation in restructured meat [28]. Baugreet et al.[24] reported that meat alone was affected by lipid oxidation during processing.

Similar to color results, Martínez et al.[29] found that *L*, a**

and *b** values in beef patties with TGase were 50.60, 9.65 and 16.02, respectively. It was reported that doses of TGase from 0% to 0.5% led to a slight increase in the *L** value of chicken breast patties, from 41.81 to 43.10 [30]. Park et al.[31] reported that *a** and *b** values of cooked meat batters with or without TGase treatment showed no significant difference. Cofrades et al.[32] also reported that the addition of TGase did not affect any of the color parameters of the raw and cooked meat products. Additionally, color is one of the most important visual traits of the beef products perceived by consumers [33]. Therefore, the results indicate the application potential of this recombinant TGase enzyme in beef meatballs.

The hardness, springiness and cohesiveness values of control group were not different from those of recombinant and commercial enzyme added meatballs. However, the chewiness of meat products was related to these properties and reflected the acceptability of food by the consumer. The chewiness of recombinant meatballs was significantly higher than those of the others, possibly from an increase in the formation of cross-linking between glutamine and lysine residues. It was reported that increasing levels of TGase (from 0% to 2%) increased the chewiness of the reduced-salt frankfurters from 0.09 kg to 0.13 kg but did not have a significant effect on the springiness and cohesiveness properties [23]. Yang and Zhang [12] reported that the chewiness of the restructured pork catalyzed by recombinant TGase was higher than that of the control group which had no TGase and suggested that recombinant TGase can improve tenacity and the taste of mixed foods. This study suggests that the addition of recombinant TGase, followed by controlled heating at 40°C, could improve the textural properties of meatballs.

When MTGase was added to restructured meat, there was a decrease in the density of the actin and myosin bands, while a new extra band was formed at the top of the gel (Lane 2 and 3 in *Fig. 1)*. The formation of this new band was observed in both commercial and recombinant MTGase enzyme treatments but was not observed in the control group. Our findings were consistent with the other studies in the literature [21,34]. In addition, *Fig. 2* showed that myosin and actin covalently bond to form a new, cross-linked, high molecular weight protein on the top of the gels. The reduction of actin and myosin bands with the addition of MTGase has been shown in many studies [35,36]. Both commercial and recombinant MTGase enzyme induced cross-linking of polypeptide chains in the cooked beef meatball samples owing to the disulfide bonds. According to these findings, the recombinant MTGase enzyme [18] produced in *P. pastoris* can be used as meat glue like commercial TGases to obtain restructured meat products.

This study showed that the effects of MTGase enzyme on the cooking loss, water holding capacity (WHC), color parameters and texture profile of the meatball samples. The addition of the MTGase enzyme into the meatballs

improves the textural properties of the samples. In addition, the TBARS values of the meatballs decreased with MTGase treatment. All of these physical, chemical and SDS-PAGE analysis showed that the recombinant MTGase and the commercial MTGase enzymes had similar effects on the restructured beef meat. As a conclusion, the recombinantly produced MTGase can be a good alternative for cooked beef meatballs in the reconstituted meat industry.

CONFLICT OF INTEREST

The authors declared no potential conflicts of interest

STATEMENT OF AUTHOR CONTRIBUTIONS

Conception and design: M. İnan. Analysis and interpretation of data: F. Ersöz, E. Aykın-Dinçer. Drafting the article: F. Ersöz, E. Aykın-Dinçer. Revising it for intellectual content: A. Türkanoğlu Özçelik. Final approval of the completed article: F. Ersöz, E. Aykın-Dinçer, A. Türkanoğlu Özçelik, M. İnan.

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