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A Novel Bone Gelatin Prepared by Enzymatic Catalysis with High Crosslinking Activity of MTGase for Gelatinization Properties of Minced Pork

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Abstract: A novel gelatin prepared by enzymatic catalysis (type-E bone gelatin) was developed in our group. In this study, the high crosslinking activity of type-E bone gelatin with microbial transglutaminase (MTGase) was found and further used for the gelatinization properties of minced pork. The results showed that the contents of lysine and glutamine in type-E bone gelatin were higher than that of traditional gelatin prepared by acid (type-A gelatin) and alkali (type-B gelatin) methods, which are as action sites for MTGase. The crosslinking degree (79%) of type-E was approximately 4.9 times that of type-A and 5.6 times that of type-B at 1.44 U/g MTGase. Moreover, the type-E gel showed thermal irreversibility when the MTGase concentration was higher than 0.90 U/g due to high crosslinking activity. For minced pork gel, the water-holding capacity and texture properties of minced pork modified with type-E bone gelatin crosslinked by MTGase were improved and cooking loss was significantly reduced.

Keywords: bone gelatin; microbial transglutaminase; crosslink; minced pork; thermo-reversibility

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1. Introduction

As a natural product, gelatin contains plenty of excellent physical and chemical properties, such as hydrophilicity, gelatinization, surface activity, etc., so it is widely used in food, medicine, cosmetics, and photosensitive materials [1]. Bone gelatin, obtained from the hydrolysis of collagen, is generally derived from animal bones, including pigs, cattle, poultry, and fish [2]. According to the traditional gelatin production process, gelatin is classified into type-A and type-B gelatin. Type-A gelatin is extracted from raw material treated with acid, and type-B gelatin is obtained from raw material using alkali [3–5].

In the food industry, gelatin is a key component in modern cuisine for its gelling capabilities [6]. It can be applied in meat products such as meatballs, meatloaf, dried meats, and hams to retain water and provide the skeleton and heat-transfer medium in food processing [7]. However, due to the thermo-reversibility of gelatin, the formation of gelatin gel is easily influenced by temperature. The three-dimensional (3D) physical crosslinking network could disintegrate, and the gel state will turn into a melted colloidal state when the temperature rises [8]. Therefore, gelatin is greatly affected by temperature during food product processing, especially heat processing. Microbial transglutaminase (MTGase) is a biocatalytic cross-linking agent, which is widely recognized for its high efficiency and safety. In recent years, some studies have shown that the use of MTGase may be a source of dangerous allergens [9]. At present, MTGase is still a commonly used processing aid in the food industry, such as protein food processing, and the addition of MTGase helps to provide a stronger chemical cross-linking structure [10]. MTGase can catalyze the acyl-transfer reaction between γ -carboxyamide groups of glutamine residues as an "acyl donor" and ε-amino groups of lysine residues as an "acyl acceptor", which

leads to the formation of intra- and intermolecular crosslinks of ε-(γ-glutamyl) lysine of the protein [11,12]. The traditional alkaline gelatin (type-B gelatin) and acid gelatin (type-A gelatin) can be catalyzed by MTGase [13–15], but some researchers believe that the traditional gelatin, especially type-B gelatin, has weak cross-linking activity or even cannot be catalyzed by MTGase [16]. Alkali leaching for a long duration during the processing of type-B gelatin destroys the covalent crosslinking of ossein and promotes the conversion of asparagine and glutamine to aspartic acid and glutamic acid [17]. In general, there are differences in amino acid compositions and MTGase action sites due to the different production processes of gelatin [18]. Based on this, we developed a one-step biocatalytic type-E bone gelatin without the long-term liming process. The process conditions were relatively mild and environmentally friendly [19]. The amount of glutamate produced by glutamine deamination is lower than that of alkaline bone gelatin, so it has more potential to interact with MTGase.

This study aimed to investigate the crosslinking and rheological behavior of type-A and type-B bone gelatin and novel type-E bone gelatin under the action of MTGase. The high crosslinking activity of type-E bone gelatin with MTGase was found, which was further used for the gelatinization properties of minced pork.

2. Materials and Methods

2.1. Materials

Defatted bone particles and type-B bone gelatin (obtained from alkali process, ~180 Bloomg) were kindly provided by Tian Zheng Medicine Co., Ltd. (Changchun, China), while type-A bone gelatin (obtained from acid process, ~180 Bloomg) was purchased from Henan E-King Gelatin Co., Ltd. (Henan, China). Pepsin (20,000 U/g) was purchased from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). Microbial transglutaminase (MTGase, 120 U/g) was obtained from Jiangsu Yi Ming Biological Co., Ltd. (Taizhou, China). Pork (Posterior gluteal) was purchased from a local market (Wuxi, Jiangsu, China) and cut into approximately 5 cm × 5 cm × 1 cm pieces. All chemicals were of analytical grade.

2.2. Preparation of Type-E Bone Gelatin

The extraction of type-E bone gelatin referred to our previous method: One-step biocatalysis [19]. Before extraction, defatted bone particles were crushed and passed through a 30-mesh sample sieve. First, the processing of demineralization and enzymatic hydrolysis was carried out simultaneously: Bone powder was immersed in 1mol/L hydrochloric acid (the ratio of the solid solution was 1:9 (*w*/*v*)) with constant stirring at 30 °C for 3 h, of which the pepsin concentration was 40 U/g bone powder. Then, the mixture was centrifuged at 7000 rpm for 8 min. The resulting precipitate was named ossein, which was twice washed with deionized water at a ratio of 1:2 (*w*/*v*). The rinsed ossein was added to a double volume of deionized water, and the gelatin was extracted at pH 5.2–5.6 for 3 h at 60 °C with constant stirring. Then, the mixture was centrifuged at 8000 rpm for 10 min. Next, the supernatant fluid was dried at 40 °C for 24 h. Eventually, the obtained dried gelatin was classified as type-E bone gelatin.

2.3. Preparation of MTGase Modified Bone Gelatin

Different concentrations of the MTGase solution were prepared and added to the type-A bone gelatin, type-B bone gelatin, and type-E bone gelatin (6.67% (*w*/*v*)). The concentrations of MTGase were 0, 0.09, 0.36, 0.90, and 1.44 U/g bone gelatin. After sufficient mixing, the mixed solution was placed in a water bath at 50 °C for 1 h, followed by transfer into a water bath at 75 °C with 15 min for inactivation.

2.4. Glutamine Analysis

We referred to the method of Kuhn [20] to determine the content of glutamine with modifications. The mixture containing 1 mL of the 0.1 g/mL gelatin solution, 1 mL of the 0.4 g/mL BTI-dimethylformamide solution, and 0.5 mL of the 100 μmol/mL pyridine solu-

tion was placed in a water bath at 50 °C for 4 h. Then, the water and dimethylformamide were dried with nitrogen at room temperature. Hydrochloric acid (8 mL, 6 mol/L) was added to the sample and hydrolyzed at 120 °C for 24 h, then it was neutralized (4.8 mL, 10 mol/L) with sodium hydroxide solution. The neutralization solution was fixed to 25 mL, filtered with double-layer filter paper, and centrifuged at 10,000 rpm for 10 min, while the supernatant was used for glutamic acid measured at 338 nm by high-performance liquid chromatography (HPLC, Agilent 1100, Agilent Technologies Inc., Santa Clara, CA, USA). Finally, the glutamine content of the sample was the difference value between the glutamate content of the control sample and the BTI-derived sample.

2.5. Crosslinking Degree

The detection of the cross-linking degree was based on the change in the number of free amino groups in the gelatin solution before and after MTgase modification [21]. The measurement was carried out with a slight modification according to Nielsen's method [22]. We dissolved 7.620 g sodium tetraborate and 200 mg sodium dodecyl sulfate in 150 mL of deionized water. Then, we accurately weighed 160 mg o-phthalaldehyde (OPA) (97%) and dissolved it in 4 mL ethanol. Next, 176 mg DTT (99%) was added to the mixture of the above two solutions. Finally, the mixed solution was diluted to 200 mL, which was the OPA test reagent [23]. The diluted gelatin solution sample (2 mg/mL, 400 μL) was added to the 3 mL OPA test reagent and then the mixed samples were incubated at room temperature in the dark for 2 min. The absorbance of the reaction solution was measured at 340 nm by an ultraviolet-visible spectrophotometer (UV-1800, AOE Instrument Co., Ltd., Shanghai, China) and the crosslinking degree was calculated, as follows:

$$\text{Crosslinking degree\%} = (1 - A_m/A_0) \times 100\% \quad (1)$$

where A_m is the absorbance of the modified sample and A_0 is the absorbance of the unmodified sample.

2.6. Rheological Analysis

The temperature sweep program of gelatin gel samples was operated by a HAAKE MARS Rheometer (DISCOVERYHR-3, TA, New Castle, DE, USA) with a probe of a 40 mm plate. Moreover, the parameters of the temperature sweep were as follows: Temperature range, 5–50 °C and 50–5 °C; heating/cooling rate, 5 °C/min; strain, 1%; frequency, 1 Hz. The storage modulus (G') and loss modulus (G") were recorded as functions of temperature. The intersection of heating/cooling procedures G' and G" is considered to be the melting temperature and gelation temperature of the gelatin gel, respectively. The apparent viscosity of the sample was measured at 50 °C, and the shear rate ranged from 0.1 to 100 s^{-1}.

2.7. Preparation of Minced Pork Gels

The pork was trimmed of visible fat and connective tissue and repeatedly ground 3 times in an HX-J330 food processor (AUX, Ningbo, China) through a plate with 5 mm diameter orifices until the meat was minced. First, 2% salt was added to minced pork alongside kneading for 15 min. Then, the salted minced pork was divided into 8 groups, and 1.5% different types of bone gelatin and 0.8% MTGase were added according to the experimental design. The detailed ingredients of each group were as follows: Control (minced pork), MTG (minced pork with 0.8% MTGase), A (minced pork with 1.5% type-A bone gelatin), MTG+A (minced pork with 1.5% type-A bone gelatin and 0.8% MTGase), B (minced pork with 1.5% type-B bone gelatin), MTG+B (minced pork with 1.5% type-B bone gelatin and 0.8% MTGase), E (minced pork with 1.5% type-E bone gelatin), and MTG+E (minced pork with 1.5% type-E bone gelatin and 0.8% MTGase). Gelatin and MTGase were added in sequence via kneading for 10 min, and water was added to adjust the moisture content of each component to be consistent during the kneading process. The mixture was stuffed into a glass casing (inner diameter, 3.5 cm; length, 2 cm) and stored at 4 °C for 15 h. Then, they were split into two parts. One part was non-cooked, while the rest was heated

in a water bath at 80 °C for 25 min, followed by rapid cooling in ice water (0 °C) for 10 min, and then the gels were stored at 4 °C overnight.

2.8. Texture Analysis

The general texture Analyzer (TA-XT plus, Stable Micro Systems, Surrey, UK) was used for the texture analysis in this study. For non-cooked minced pork gel, it is necessary to equilibrate at room temperature for 2 h before use, and the relevant experimental parameters were as follows: Probe type, P/5; test speed, 1 mm/s; penetration depth, 1 cm. Gel strength was calculated according to the Penetration Hardness (maximum penetration force, g) and the Penetration Depth:

$$\text{Gel strength (g·cm)} = \text{Penetration Hardness} \times \text{Penetration Depth} \quad (2)$$

The texture profile analysis (TPA) of cooked minced pork gel was carried out using the texture Analyzer, and the test parameters were as follows: Probe type, P/36R; pre-test speed, 1.5 mm/s; test speed, 1 mm/s; post-test speed, 1 mm/s; strain, 30%; trigger force, 5 g; time interval between two compressions, 5 s. The hardness, springiness, chewiness, gumminess, cohesiveness, and resilience of each sample were calculated according to the TPA curve.

2.9. Water Holding Capacity

The water-holding capacity (WHC) of gel samples was measured according to the method of Pietrasik et al. [24]. In short, the cooked minced pork gels were cut into small pieces (2 g, m_1), which were wrapped with double-layer filter paper (Whatman No. 1), placed between two glass sheets, and pressed for 5 min by standard weight (5 kg). The samples were then removed from the papers and weighed again (m_2). The WHC calculation formula was as follows:

$$\text{WHC\%} = (m_1 - m_2)/m_1 \times 100 \quad (3)$$

2.10. Cooking Loss

The cooking loss of minced pork gels in each treatment was determined by weighing samples before and after cooking. For the cooked minced pork gels, the residual mucus on the surface was gently wiped off before weighing. The cooking loss was calculated as follows:

$$\text{Cooking loss (\%)} = (M_{\text{non-cooked}} - M_{\text{cooked}})/M_{\text{non-cooked}} \times 100 \quad (4)$$

2.11. Statistical Analysis

In this study, all treatment groups were set to three parallels. Data were analyzed by one-way analysis of variance (ANOVA) of each experiment using the SPSS 25.0 statistic program (SPSS Inc., Chicago, IL, USA). The statistical significance among the treatments was performed by Duncan's multiple comparisons at a significant level of $p < 0.05$.

3. Results and Discussion

3.1. The Action of Glutamine and Lysine on Crosslinking Degree of Gelatin with MTGase

The presence of glutamine and lysine is a prerequisite for bone gelatin to be modified by MTGase [25]. The contents of glutamine and lysine of type-E bone gelatin were higher than those of type-A and type-B bone gelatin ($p < 0.05$) (Figure 1). Type-A and B bone gelatin possess similar amounts of lysine but different amounts of glutamine. According to previous studies [16], processing methods had a certain influence on the amino acid composition of gelatin. Long-term alkali leaching of bone gelatin induced the hydrolysis of amide groups and promoted the conversion of some glutamine to glutamic acid [26]. In contrast, a long acid-leaching treatment in the production of type-A bone gelatin avoided the conversion of glutamine to glutamic acid [2]. It was notable that the type-E bone gelatin prepared by enzyme catalysis has higher contents of glutamine and lysine, providing more

action sites for MTGase. The results suggested that type-E gelatin has more potential to be modified by MTGase.

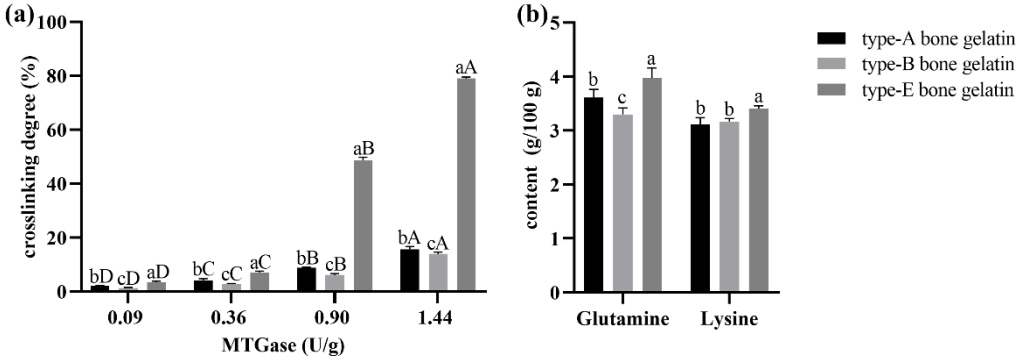

Figure 1. Crosslinking degree (%) (**a**) of type-A, type-B, and type-E bone gelatin modified with different MTGase levels. Bars represent the standard deviations (n = 3). Different lowercase letters (a, b, c) on the column indicate that the cross-linking degree of different types of bone gelatin was significantly different ($p < 0.05$) under the same MTGase concentration. Different uppercase letters (A, B, C, D) on the column indicate that the cross-linking degree of the same type of bone gelatin was significantly different ($p < 0.05$) under different MTGase concentrations. Glutamine and lysine content (g/100 g) (**b**) in type-A bone gelatin, type-B bone gelatin, and type-E bone gelatin without MTGase modification. Bars represent the standard deviations (n = 3). Different lowercase letters (a, b, c) on the column within the content of Glutamine or lysine denote significant differences ($p < 0.05$).

Different concentration levels of MTGase were used for crosslinking. The crosslinking degree of the three types of bone gelatin increased significantly with the increase in MTGase concentration ($p < 0.05$) (Figure 1). Under the same concentration of MTGase, the crosslinking degree of type-E bone gelatin was significantly higher than that of type-A and type-B bone gelatin ($p < 0.05$). When the MTGase concentration was 1.44 U/g, the crosslinking degree of type-E reached 79%, whereas those of type-A and type-B were 16% and 14%, respectively. The higher glutamine and lysine contents of type-E bone gelatin provided more action sites for MTGase.

3.2. The Crosslinking Activity of Gelatin with MTGase

Temperature is one of the most important factors affecting the application of gelatin in food manufacturing [6]. During the heating/cooling program, the temperature corresponding to the intersection of the storage modulus (G′) and storage loss (G″) was considered to be the melting temperature (T_m) and gelation temperature (T_g) of gelatin gels, respectively [1,27]. Figure 2. shows the T_m and T_g of three types of gelatin gel modified with different concentrations of MTGase. Without MTGase modification, the three types of bone gelatin showed similar thermal transition trends in the course of cooling and heating, which indicated that the thermal stability of the three types of bone gelatin was similar. The T_m and T_g among different kinds of bone gelatin are in the order of $T_{mA0} < T_{mB0} < T_{mE0}$ and $T_{gA0} < T_{gB0} < T_{gE0}$ (Figure 2), which may be related to the differences in pretreatment methods during the production of bone gelatin and the types and contents of amino acids that make up gelatin [28].

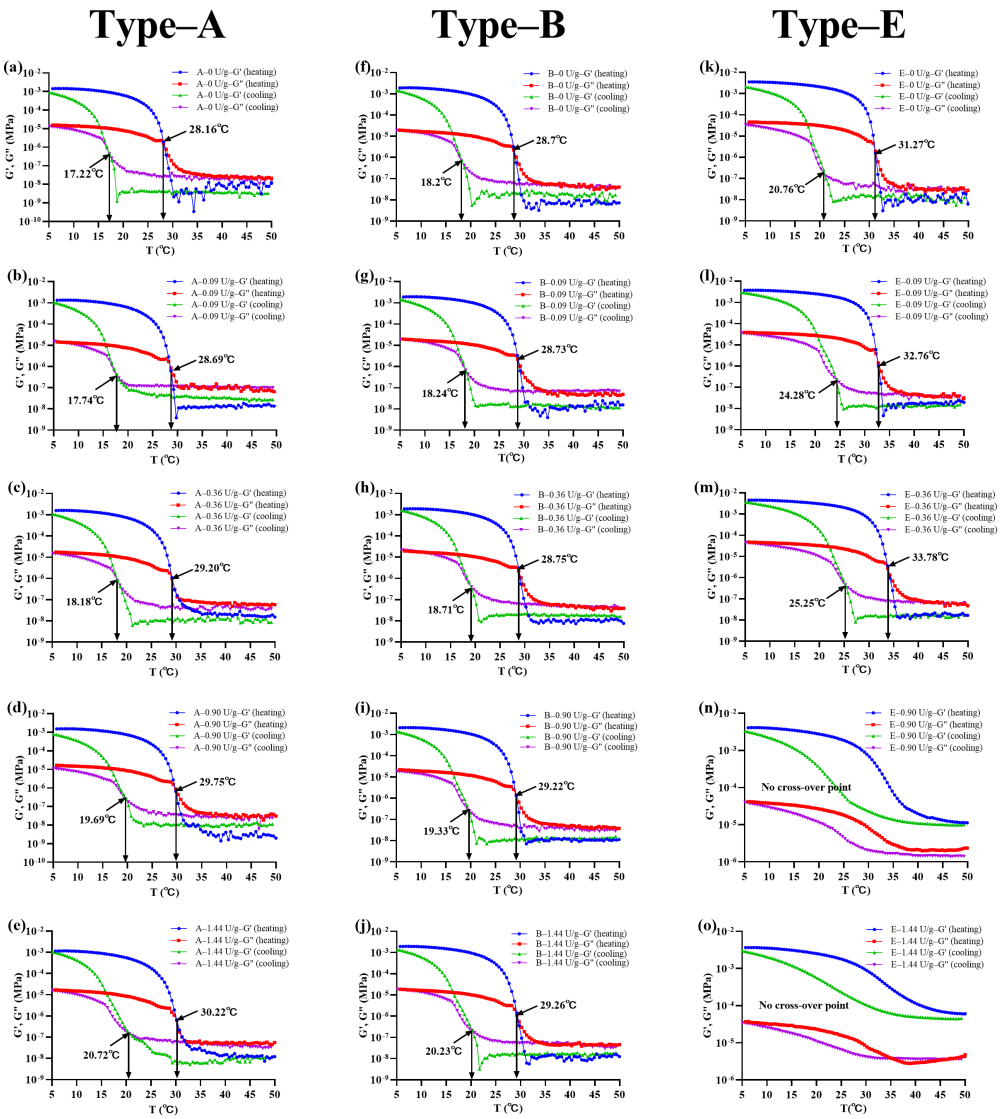

Figure 2. Evolution of storage modulus (G′) and loss modulus (G″) of native gelatin ((**a**,**f**,**k**)) and MTGase-treated gelatin (**b**–**e**,**g**–**j**,**l**–**o**) upon heating from 5 °C to 50 °C and cooling from 50 °C to 5 °C. A: Type-A bone gelatin; B: Type-B bone gelatin; E: Type-E bone gelatin. Gelatin was treated with MTGase using different enzyme levels for 0, 0.09, 0.36, 0.90, and 1.44 U/g gelatin, respectively.

As shown in Figure 2, type-A and type-B bone gelatin gels with different treatment groups had a crossover point between G′ and G″, which indicated thermoreversible behavior [29]. At the same time, the T_m and T_g of gelatin gel increased slightly with the increase in MTGase concentration, which suggested that the addition of MTGase promoted the crosslinking degree of gelatin molecules and improved the thermal stability of gelatin gels. For type-E bone gelatin, the T_m and T_g of gelatin gel were increased at the addition amount of MTGase of 0.09 and 0.36 U/g, respectively (Figure 2). However, the values of G′ and G″ in the heating/cooling program decreased with the increase in temperature, and the crossover point between G′ and G″ was not observed when the concentration of MTGase was 0.90 U/g and 1.44 U/g, which implied that the over-modified type-E bone gelatin gels remained in the gel state with thermal irreversibility [2]. Thermal reversibility is a unique property of the gelatin solution, where the single strands form a triple helix structure via the action of hydrogen bonds, ionic bonds, van der Waals forces, etc., which contribute to the formation of a three-dimensional (3D) gel network dominated by physical crosslinking [30]. The thermal properties of gelatin gel and MTGase-modified gelatin gels are shown in Figure 3. The triple helix structure disintegrated, and the gelation state of the

gelatin gel returned to the flow state with the increase in temperature (Figure 3a.). According to previous research, the γ-carboxyamide groups of glutamine residues and ε-amino groups of lysine residues of gelatin formed ε-(γ-glutaminyl)-lysine isopeptide bonds of chemical crosslinking in gelatin gel under the catalysis of MTGase [25]. The gelatin gel still showed thermal reversibility under modification with MTGase (Figure 3b). During cooling, a stronger chemical-physical 3D network was formed for the triple helix structure and ε-(γ-glutaminyl)-lysine isopeptide bonds. When the temperature increased, the triple helix and the chemical-physical 3D network disintegrated, and the gelation state was transformed into a flow state. However, when the gelatin gel was over-modified with MTGase, the main body of the gel with a chemical 3D network will hinder the disintegration of the triple helix structure, and at this time, the gelatin gel showed thermal irreversibility (Figure 3c). These results demonstrated that gelatin modified with MTGase can increase the T_m and T_g of gelatin gel. In this study, the loss of thermal reversibility of modified type-E bone gelatin is significant, which may be due to the abundant glutamine and lysine of type-E bone gelatin providing more action sites for MTGase to form more covalent crosslinks within and among the gelatin chains.

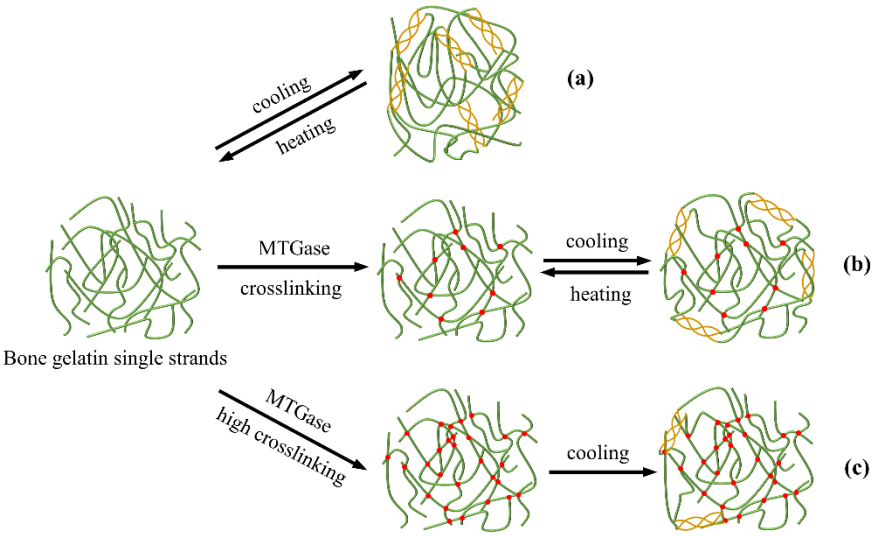

Figure 3. Schematic diagram illustrating the mechanism of MTGase catalyzing bone gelatin. (**a**) no crosslinking, (**b**) moderately crosslinking, (**c**) high crosslinking.

As shown in Figure 4, the apparent viscosity of all samples decreased with the increase in shear rate, which is the typical shear thinning effect for pseudoplastic materials [31]. Under high-speed shear, the equilibrium state of the sample was destroyed, and the flow resistance was decreased with the expansion of the molecular chains, resulting in a decrease in the apparent viscosity [32]. At the shear rate of 10 s^{-1}, the apparent viscosity of all three types of gelatin was increased with increasing contents of MTGase. It is worth noting that when the MTGase concentration was 0.90 and 1.44 U/g, the apparent viscosity of type-E bone gelatin was higher than in other treatment groups. The catalysis of MTGase on intra- and intermolecular crosslinking of gelatin changed the structure of gelatin and formed a high-molecular-weight polymer with the increase in enzyme concentration, which thus increased the apparent viscosity of gelatin. A large number of molecules inside the gelatin will aggregate to form a strong covalent bond at a high MTGase concentration, resulting in a significant increase in apparent viscosity.

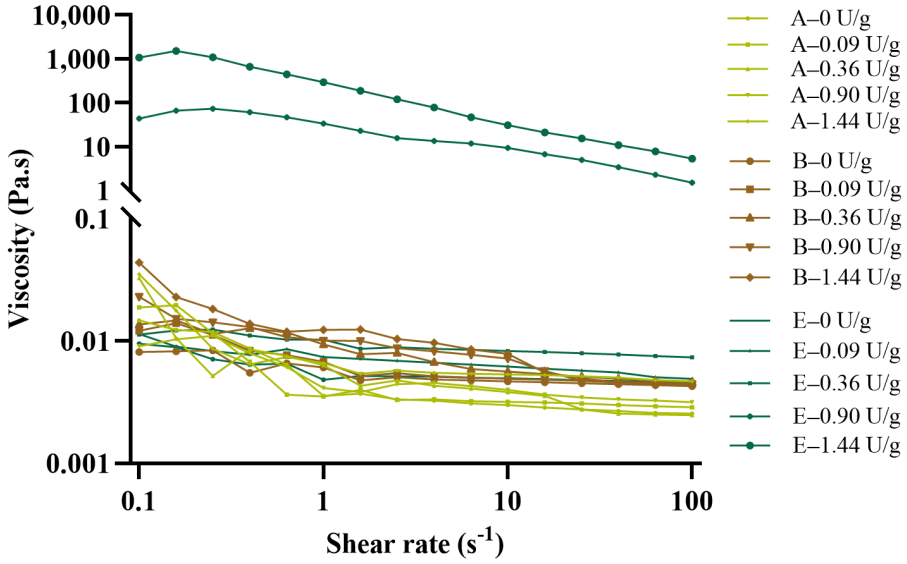

Figure 4. Apparent viscosity of native gelatin and MTGase-treated gelatin solution upon shear rate from 0.1 to 100 s^{-1}.

3.3. The Use of Gelatin and MTGase for Gelatinization Properties of Minced Pork

Affected by thermal properties, meat products such as meatballs, steaks, and sausages find it difficult to meet the processing demand by relying only on the gelling ability of meat protein itself during processing. Figure 5 shows the gel strength of the non-cooked meat gels mixed with three types of bone gelatin and MTGase. Compared with the control, all experimental groups had a significantly ($p < 0.05$) beneficial effect on the texture properties of the non-cooked meat gels. Because of the excellent viscosity, type-A, B, and E bone gelatin can all increase the cohesiveness of the mixture through physical bonding. Meanwhile, the gelatin solution will change from fluid to semi-solid to form a thermal reversibility gel at low temperatures, which provides suitable gel conditions for non-cooked meat gel so as to improve the gel strength. Enacting a single physical modification of pork gel unable to combat its temperature changes in thermal processing due to the thermo-reversibility of gelatin. The combined addition of MTGase and bone gelatin further increased the gel strength of the non-cooked meat gel, especially the combination of type-E bone gelatin and MTGgase, whereby the meat gel treated with this combination has the highest gel strength (Figure 5). This consequence indicated that the addition of type-E gelatin modified with MTGgase endowed the minced pork with the most crosslink formations.

The texture profile analysis (TPA) results are summarized in Table 1. In comparison with the control group, the hardness of the mixture was significantly increased when bone gelatin and MTGase were used alone or together ($p < 0.05$). Under the same gelatin concentration, the hardness of the mixture was improved by adding three types of bone gelatin ($p > 0.05$), and the hardness was further increased when adding the same amount of MTGase to the mixture containing type-A or type-E bone gelatin ($p < 0.05$). For springiness, gumminess, and chewiness parameters, the combination of type-E bone gelatin and MTGase also showed a significant beneficial effect on the texture properties of the mixture compared with other treatments ($p < 0.05$). For the cohesiveness parameter, the combined use of MTGase and type-E bone gelatin increased the cohesiveness, and the use of type-B bone gelatin only lowered the cohesiveness of the mixture ($p < 0.05$), while other treatment groups had no effect on the cohesiveness compared with the control ($p > 0.05$). For the resilience parameter, its variation trend is similar to that of the cohesiveness parameter. The combined use of MTGase and type-E bone gelatin still showed a positive effect compared with the control ($p < 0.05$), and there was no significant difference between the Control, MTG, MTG+A, MTG+B, and E groups ($p > 0.05$), while the use of type-A or type-B bone gelatin alone produced a negative result compared with the control group ($p < 0.05$).

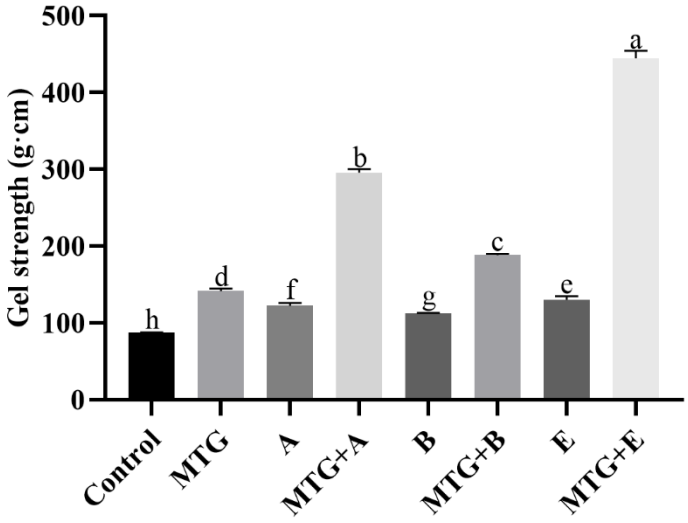

Figure 5. Gel strength analysis of the non-cooked minced pork gels with three types of bone gelatin and MTGase added. Bars represent the standard deviations (n = 3). Different lowercase letters (a, b, c, d, e, f, g, h) on the columns indicate significant differences ($p < 0.05$) in gel strength between experimental groups.

Table 1. Texture profile analysis of the cooked minced pork gels with three types of bone gelatin and MTGase added.

Samples	Hardness/kg	Springiness	Cohesiveness	Gumminess/kg	Chewiness/kg	Resilience
Control	5.27 ± 0.27 ^{c}	0.90 ± 0.01 ^{b,c}	0.82 ± 0.03 ^{a,b}	4.86 ± 0.10 ^{c}	4.65 ± 0.03 ^{c}	0.44 ± 0.04 ^{a,b}
MTG	6.51 ± 0.12 ^{b}	0.90 ± 0.02 ^{b,c}	0.83 ± 0.06 ^{a,b}	5.33 ± 0.19 ^{b}	5.08 ± 0.27 ^{b,c}	0.44 ± 0.03 ^{a,b}
A	6.45 ± 0.20 ^{b}	0.91 ± 0.01 ^{b}	0.80 ± 0.06 ^{a,b}	5.36 ± 0.35 ^{b}	4.90 ± 0.27 ^{b,c}	0.43 ± 0.02 ^{b}
MTG + A	6.83 ± 0.11 ^{a}	0.91 ± 0.01 ^{b}	0.85 ± 0.03 ^{a,b}	5.64 ± 0.40 ^{a,b}	5.14 ± 0.31 ^{b}	0.48 ± 0.02 ^{a,b}
B	6.46 ± 0.19 ^{b}	0.88 ± 0.02 ^{c}	0.78 ± 0.05 ^{b}	5.19 ± 0.30 ^{b,c}	4.86 ± 0.29 ^{b,c}	0.41 ± 0.03 ^{b}
MTG + B	6.52 ± 0.15 ^{b}	0.91 ± 0.01 ^{b}	0.82 ± 0.06 ^{a,b}	5.53 ± 0.20 ^{a,b}	5.03 ± 0.15 ^{b,c}	0.46 ± 0.05 ^{a,b}
E	6.34 ± 0.10 ^{b}	0.91 ± 0.02 ^{b,c}	0.81 ± 0.05 ^{a,b}	5.46 ± 0.24 ^{a,b}	4.95 ± 0.19 ^{b,c}	0.46 ± 0.04 ^{a,b}
MTG + E	7.05 ± 0.11 ^{a}	0.96 ± 0.01 ^{a}	0.88 ± 0.02 ^{a}	5.89 ± 0.15 ^{a}	5.60 ± 0.18 ^{a}	0.50 ± 0.04 ^{a}

Values are mean ± SD (n = 3). Different lowercase superscripts in the same column denote the significant difference ($p < 0.05$).

Overall, compared with the addition of MTGase or gelatin alone, the combined use of MTGase and bone gelatin was beneficial to the improvement of the textural properties of the mixture, especially the joint use of type-E bone gelatin and MTGase. The application of MTGase in meat products, catalyzing the formation of ε-(γ-glutamyl) lysine bonds to improve the quality and texture characteristics, had been widely accepted [24], but MTGase cannot work efficiently due to the limited amount of the protein matrix that can be modified in meat. The addition of bone gelatin can bond the meat matrix together to form compact aggregate macromolecules [33], but the gelatin solution exuded from the mixture due to the thermal reversibility of gelatin during the heating process, which impeded the application of gelatin. The combined use of MTGase and bone gelatin increased the content of crosslinking protein substrates for MTGase and promoted the formation of a crosslinking 3D network in the mixture; furthermore, the mixture of meat and gelatin modified by MTGase formed a stronger covalent bond to avoid the loss of gelatin during heating. Therefore, the rich lysine and glutamine content of type-E bone gelatin provide more sites for MTGase, which makes a stronger network structure at the same MTGase concentration and provides a supporting skeleton for the meat–gelatin mixture, thus affecting the texture properties of meat gels.

In food processing and applications, the water-holding capacity (WHC) is very important to the stability of the products, and a higher WHC value indicates a higher ability to

combine and retain moisture in the heating process [34]. Figure 6a shows the WHC values of the minced pork gels mixed with three types of bone gelatin only and combined with MTGase separately. The use of type-A, B, and E bone gelatin without MTGase reduced the WHC of minced pork gels, in the order of Control > E > B > A ($p < 0.05$). Previous studies showed that adding collagen to meat products resulted in low WHC [33,35], which is consistent with the results of this experiment. The negative effect of bone gelatin on the WHC of minced pork gels may be related to the thermal reversibility of gelatin. In particular, the results showed that the lowest WHC value was obtained by adding type-A bone gelatin at the same gelatin concentration, which may be related to the viscosity, gel strength, pH, or other properties of gelatin. The addition of MTGase alone can improve the WHC of the mixture because the use of MTGase catalyzes the formation of the 3D network via cross-linking of the meat protein, which is conducive to the retention of moisture. When MTGase and bone gelatin were added to the mixture together, the WHC was higher than that of MTGase alone ($p < 0.05$), especially the WHC of the mixture treated with the combination of type-E bone gelatin and MTGase. Under the catalysis of MTGase, protein–protein, protein–gelatin, and gelatin–gelatin covalent bonds in meat supported each other to form a complex physical-chemistry 3D network, which has a good effect on moisture retention. For this reason, type-E bone gelatin showed a higher degree of cross-linking with MTGase compared to type-A and type-B bone gelatin, which resulted in higher water-holding capacity.

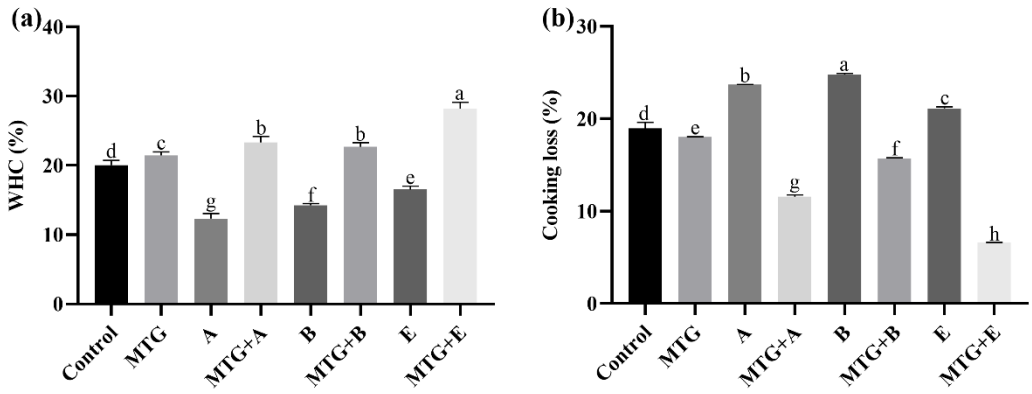

Figure 6. WHC (%) (**a**) and cooking loss (%) (**b**) of the cooked minced pork gel treated with three types of bone gelatin and MTGase or their combination. Bars represent the standard deviations ($n = 3$). Different lowercase letters (a, b, c, d, e, f, g, h) on the columns indicate significant differences ($p < 0.05$) in WHC or cooking loss between experimental groups.

The results of cooking loss are shown in Figure 6b, and there were significant differences in cooking loss among the treatment groups ($p < 0.05$). The cooking loss was increased using type-A, type-B, and type-E bone gelatin without MTGase, in which the cooking loss was the highest by adding type-B gelatin. Affected by the thermal reversibility, gelatin melted during heating, and a large amount of water was taken away from the mixture, which caused the high values of cooking loss [36,37]. Compared with the control group, all the treatments with MTGase reduced the cooking loss of the mixture in descending order: Control > MTG > MTG + B > MTG + A > MTG + E. Additionally, under the modification of MTGase, part of the meat protein and bone gelatin form strong intra- and intermolecular covalent bonds to build a complex physical-chemistry 3D network, which can effectively resist the damage to the gel structure during thermal processing, thereby reducing the moisture loss during cooking. The significant reduction of cooking loss via the combination of MTGase and type-E bone gelatin (about one-third of the control group) may be due to the high crosslinking degree of type-E bone gelatin, which reduced the amount of un-crosslinked gelatin and formed a complex gel network, thus reducing the cooking loss of gelatin due to melting.

4. Conclusions

Bone gelatin has great potential in the application of minced pork with the modification of MTGase, especially the novel type-E bone gelatin. The type-E bone gelatin had greater crosslinking potential than type-B due to rich glutamine and lysine contents. Rheological analysis showed that the gelation temperature and melting temperature of bone gelatin gel rose with the increase in the MTGase concentration. The type-E gel showed thermal irreversibility when the MTGase concentration was greater than 0.90 U/g due to high crosslinking activity. The combined use of type-E bone gelatin and MTGase significantly improved the gel strength, texture properties, and water-holding capacity of minced pork gel compared with the control. These results provide new ideas for the application of novel type-E bone gelatin in the food industry.

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