

Microbial Transglutaminase Modifies Gel Properties of Porcine Collagen**

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ABSTRACT : We studied the gel properties of porcine collagen with microbial transglutaminase (MTGase) as a catalyst. A creep meter was used to measure the mechanical properties of gel. The results showed samples with high concentration of MTGase gelled faster than those with a low concentration of MTGase. The gel strength increased with incubation time and the peaks of breaking strength for 0.1, 0.2 and 0.5% MTGase were obtained at 40, 20 and 10 min incubation time, respectively. According to SDS-PAGE, the MTGase was successfully created a collagen polymer with an increase in molecular weight, whereas no change in formation was shown without MTGase. The sample with 0.5% MTGase began to polymerize after 10 or 20 min incubation at 50°C, and complete polymerization occurred after 40-60 min incubation. Scanning electron microscopic analysis revealed that the gel of porcine collagen in the presence of MTGase produced an extremely well cross-linked network. The differential scanning calorimetric analysis showed the peak thermal transition of porcine collagen gel was at 36°C, and that with MTGase no peak was detected during heating from 20 to 120°C. The melting point of porcine collagen gel could be controlled by MTGase concentration, incubation temperature and protein concentration. Knowledge of the structural and physicochemical properties of porcine collagen gel catalyzed with MTGase could facilitate their use in food products. (*Asian-Aust. J. Anim. Sci.* 2003, Vol 16, No. 2 : 269-276)

Key Words : Porcine Collagen Gel, Microbial Transglutaminase, Structural and Physicochemical Properties, Melting Point

INTRODUCTION

Collagen is the major component of animal connective tissues. For example, collagen represents about 70% of the dry weight of skin (Fujisaki and Hattori, 1999). Type I collagen from shark and land animals is a source of the potential protein in the production of some foods, cosmetics, microorganism culture materials, and medical materials, because of the unique structure and biological functions (Nomura et al., 2001). In addition, its use has recently expanded to new applications, such as health foods (Yoshimura et al., 2000). Collagen has also been studied for use in edible meat casing by collagen-elastin matrix reconstruction (Takahashi and Hattori, 1993). Gelatin is prepared by the thermal denaturation of collagen. Gelation is an important functional property of proteins, and could be a definite factor in the potential use of protein-rich material in food (Chronakis, 2001). The heat transformation of collagen into gelatin is interpreted as a disintegration of the

collagen triple helical structure into random coils (Watanabe et al., 1997).

Retaining the functional properties of gelatin is necessary in food industrial application. However, their physical and qualitative properties require some improvements, which can be made via enzymatic combination with others proteins (Nomura et al., 2000), protein mixing and enzymatic modification (Muguruma et al., 1999), structure modification (Alting et al., 2000), and other modifications. These enhancements can contribute firmness and texture of products such as jelly deserts, collagen film, casings for sausages, restructured meat, and ice cream. Cross-links are important factors in the gelation process, and increasing the cross-links content in gelatin can improve gel properties (Sakamoto et al., 1994; Takahashi et al., 1999; Lim et al., 1999).

One of the cross-linking agents widely used on production of protein polymer is microbial transglutaminase (MTGase), which is produced by *Streptoverticillium mobaraense* (Ando et al., 1989), and *Streptoverticillium ladakanum* (Tsai et al., 1996), and which is usually called as Ca²⁺-independent MTGase. Recent research on this MTGase has been focused on its use as a catalysis agent in the food industry. MTGase has previously been used to improve the functional properties of food proteins such as surimi and turkey breast gel (Ashie and Lanier, 1999), skim milk powder gel (Imm et al., 2000), and the barrier and tensile properties of gelatin film (Lim et al., 1999). We have used MTGase for improving the texture and properties of chicken sausage (Muguruma et al., 2003).

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Our previous research indicated that pig skin collagen was successfully polymerized by MTGase. Cross-links among single strands of collagen resulted in formation of new structure of biopolymer collagen (Erwanto et al., 2002). The structure of collagen cross-links through ϵ -(γ -glutamyl) lysine bonds may produce a biopolymer with new functional properties. The improvement of the functional properties of porcine collagen by MTGase may affect the qualities of many products, since porcine collagen gel is important as a food ingredient. However, the functional properties of collagen gel modified by MTGase and its thermal behavior have not yet been studied. Thus, the objectives of this research were to improve the gel properties of porcine collagen, and to investigate the effects of MTGase on polymerization of porcine collagen, and on the thermal behavior of cross-linked products.

MATERIALS AND METHODS

Materials

Porcine collagen powder (PK-100) was purchased from Nitta Gelatin Co., Inc. (Tokyo, Japan). Transglutaminase (E.C. 2.3.2.13) from the culture broth of *Streptovorticillium mobaraense* was obtained from Ajinomoto, Co.Ltd. (Tokyo, Japan). MTGase was dissolved in 20 mM NaCl and filtered through filter paper No. 5A (Advantec Toyo, Tokyo, Japan) after centrifugation at 5,800 g for 20 min at 4°C.

Breaking strength measurement

Porcine collagen powder was dissolved in 50 mM imidazole-HCl (pH 6.0) to give a concentration of 5% (w/v). MTGase was added to this solution in various concentrations (% weight per collagen weight). Aliquots of collagen solution (1.5 ml) were transferred into a micro well plate (48 holes, each with a diameter 11 mm) and incubated at 50°C for various times (0, 10, 20, 40 and 60 min). The quality of the gels was analyzed by measuring breaking strength according to the method of Nonaka et al. (1992). Breaking strength was measured with a creep meter (Rheoner, RE2-33005s, Yamaden Co., Tokyo, Japan) at 25°C. The spherical plunger (diameter 5 mm) was pressed against the center of the gel in the well at a speed of 60 mm/min. The breaking strength was measured and read on the force vs. deformation curve by the value of the first force peak (Newton). The measurement was repeated 4 times for each gel, and the means and standard deviations were calculated.

Confirmation of molecular weight by SDS-PAGE analysis

To confirm that the intermolecular covalent cross-link was formed by the enzyme treatment, the samples were analyzed by SDS-polyacrylamide gel electrophoresis (SDS-

PAGE). SDS-PAGE under reducing conditions was carried out on slab gels (4.5% acrylamide for stacking gel and 5-20% for separating gel) using an SDS-Tris-glycine discontinuous buffer system according to the method of Laemmli (1970). Prior to electrophoresis, protein samples were heated at 95°C for 5 min in the presence of 25 mM Tris-HCl (pH 6.8), 1% SDS, 20% glycerol, 0.01% bromo phenol blue, and 1% 2-mercaptoethanol. An aliquot of the sample solution was applied to SDS-PAGE gel and electrophoresis was performed at constant current (20 mA/slab gel). After running, the gel was stained with 0.25% Coomassie Brilliant Blue R-250 in 50% methanol and 10% acetic acid, and it was destained with the same solvent system without dye.

Scanning electron microscopy

A three-dimensional impression of the gel structure was obtained using a scanning electron microscope (SEM). The gel samples were washed with distilled water followed by centrifugation at 8,000 g for 20 min, then sliced into small pieces. The gel slices were fixed with 3% glutaraldehyde in 0.12 M cacodylic acid buffer (pH 7.2) at 4°C, and the medium was replaced 4 times every 2 h. After a washing with 0.12 M cacodylic acid buffer, the samples were fixed in 1.33% osmium acid (pH 7.2) at 4°C for 2 h, were dehydrated by immersion in a series of ethanol mixtures (50, 70, 80, 90, 95 and 100%), and finally immersed in isoamyl acetate. After dehydration, critical point drying was performed in liquid CO₂ in a pressurized chamber. The dried samples were mounted on an aluminum stub and coated with gold by an ion sputterer (E-1030, Hitachi Co., Tokyo, Japan). All samples were then observed and photographed using an SEM (S-4100M, Hitachi Co., Tokyo, Japan).

Determination of thermal transition by differential scanning calorimetry

A differential scanning calorimeter (DSC; Perkin-Elmer, Norwalk, USA) was used to measure the thermal stability of porcine collagen gel with and without MTGase. The collagen solution 10% (w/v) was mixed with 0.2% MTGase and was incubated at 50°C for 30 min. The reaction was stopped with heating at 75°C for 30 min, and the solution was stored at 4°C until DSC analysis. About 20 mg of gel was charged and sealed in an aluminum sample pan (Perkin-Elmer sample pan kit No. 0219-0062). The temperature of the scan was from about 20 to 120°C, and the average heating rate was 5°C/min.

Melting point measurement

Porcine collagen solution (5%, w/v) was diluted to various concentrations in the presence of 50 mM sodium-phosphate buffer (pH 6.5) and 100 mM NaCl. Diluted protein solution (2 ml) was mixed with various

concentrations of MTGase (0, 0.1, 0.2, 0.5 and 1%) and then aliquots of these mixtures were transferred to a 100 μ l micro pipette (Drummond Scientific Co., Pennsylvania, USA). Aliquots were incubated at 4°C for 16 h or at 50°C for 1 h followed by storage at 4°C for 12 h. The melting point was measured using a Taitec aluminum heating bath (DTU-1C, Taitec Co. Saitama, Japan). Silicon oil was used as the heat transfer medium, and the average of heating rate was 3°C/min. Melting point temperature was defined as the moment at which the gel began to melt. When the sample solution was not gelled, the first melting point was not observed, and melting point temperature was referred to as zero. Data measurement was repeated 5 times for each sample, and the means and standard deviations were calculated.

Statistical analysis

The effects of treatments on breaking strength and melting point were analyzed using Super ANOVA computer software as a two-way ANOVA. When ANOVA revealed a significant effect, differences among treatment means were tested using Duncan's Multiple Range Test (Steel and Torrie, 1980).

RESULTS AND DISCUSSION

Rheological properties of porcine collagen gel

Changes in rheological properties of the porcine collagen gel with and without MTGase, respectively, are shown in Figure 1. The breaking strength of collagen gel at 0.1% MTGase showed linear increases ($p < 0.01$) up to 40 min, after which it decreased; the peak breaking strength was 1.87 N (Figure 1A). At the level of 0.2% MTGase, the gel strength increased ($p < 0.01$) and the peak breaking strength (1.74 N) was reached at 20 min incubation, after which it decreased (Figure 1B). When the MTGase concentration increased to 0.5%, the peak of breaking strength (1.82 N) was shown at 10 min (Figure 1C). The samples without MTGase (control) showed no significant change ($p > 0.05$) in breaking strength up to 60 min incubation. Previous research on casein gelation (Nonaka et al., 1992) showed a similar phenomenon. Nonaka et al. reported that the caseinate gel made with MTGase could be divided into two types: one difficult to break by axial compression {incubated at an enzyme concentration of 10 units/g protein (low concentration)}; the other was more fragile under axial compression. {incubated at an enzyme concentration of 50 or 100 units/g protein (high concentration)}. MTGase introduces a number of cross-linkages to collagen, and the resulting cross-linked collagen molecules self-assemble more quickly into fibrils than do native collagen molecules (Nomura et al., 2001).

Incubation time affected the increase in breaking

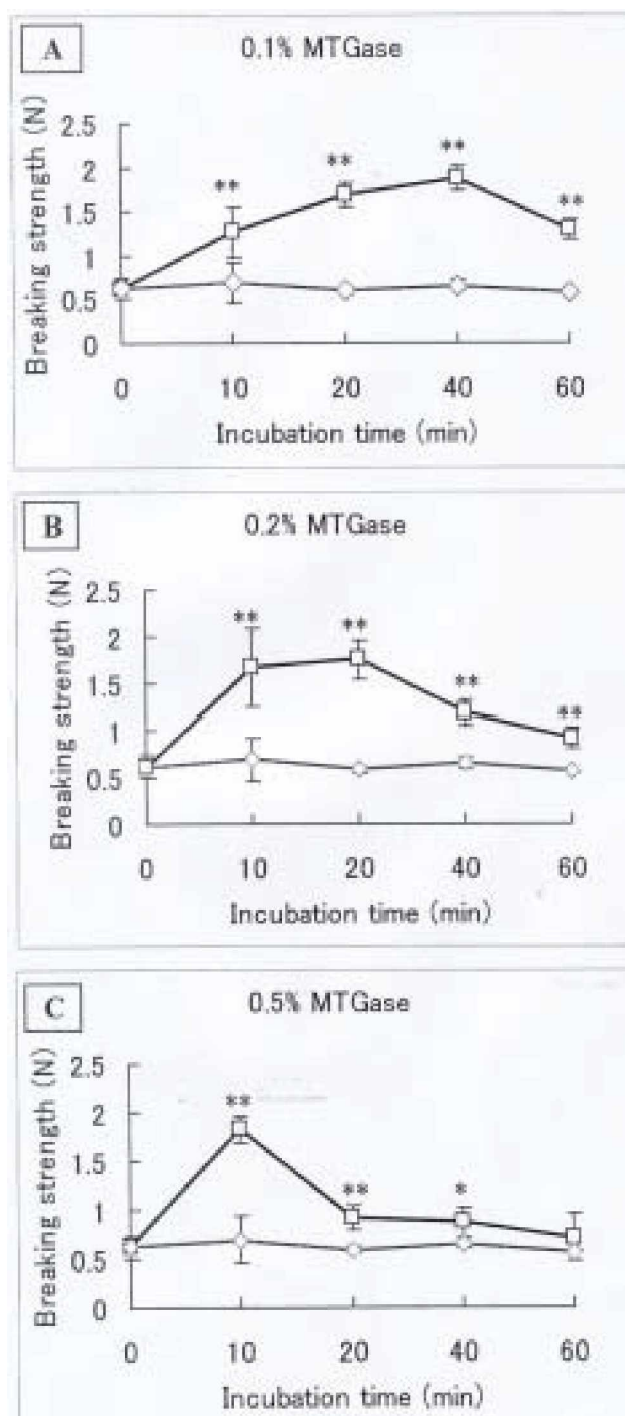


Figure 1. Change in breaking strength of porcine collagen gel during transglutaminase reaction. Samples were incubated at 50°C in various time incubations. \diamond , control; \square , with MTGase. Values represent means and standard deviations for four measurements. * Values are significantly different ($p < 0.05$). ** Values are very significantly different ($p < 0.01$).

strength (Figure 1A). Increasing breaking strength was observed in ϵ -(γ -glutamyl) lysine cross-links in protein gels as a result of MTGase (Nonaka et al., 1992; Sakamoto et al., 1995; Jiang et al., 1998). The breaking strength was

estimated to increase with increasing of the ϵ -(γ -glutamyl) lysine cross-links content (Sakamoto et al., 1995). Tseng et al. (2002) noticed that curd strength increased due to the high content of the ϵ -(γ -glutamyl) lysine cross-links.

When gels were prepared with heating during the last stage of gelation, the interaction between proteins included more than covalent cross-linkage through ϵ -(γ -glutamyl) lysine cross-links (Sakamoto et al., 1995). The interactions between protein molecules led to aggregation, coagulation, precipitation, and gelation in protein dispersion. The forces responsible for the molecular interaction include hydrogen bonds, Van der Waals forces, ionic bonds, covalent bonds, and hydrophobic interactions (Lee and Rha, 1979). Thus, the decreasing of breaking strength may be caused by the weakening of the intermolecular interactions of protein under thermal conditions.

According to the above results, the collagen gelation with MTGase required optimum temperature, MTGase level and incubation time to obtain good gel strength. Optimal setting times still need to be determined for collagen gelation involving bovine collagen, porcine collagen, and other proteins. Previous research (Sakamoto et al., 1994) on the effect of incubation time on breaking strength during MTGase reaction reported that about 120-250 min at 37°C was optimal for caseinate and commercial gelatin gel to obtain maximum breaking strength. They reported that an incubation temperature of 50°C was required to obtain maximum breaking strength. With regard to the combination of MTGase concentrations and incubation time, the maximum breaking strengths for 0.1, 0.2 and 0.5% MTGase were obtained at 40, 20 and 10 min incubation time, respectively.

SDS-PAGE of modified porcine collagen by MTGase

To confirm that an intermolecular covalent cross-link was formed by the enzyme treatment, samples were analyzed by SDS-PAGE. The effect of MTGase concentration on porcine collagen polymerization is shown in Figure 2. The sample without MTGase (Figure 2, lane 1) showed similar patterns with pig skin collagen bands in our previous research (Erwanto et al., 2002) and there were no intermolecular collagen cross-links, although the incubation time was extended to 60 min (data not shown). The polymerization was noted at the high concentration of MTGase (Figure 2, lane 3). The number of collagen molecules with low molecular weight apparently decreased with increased enzyme concentration. Since no bands appeared in the L region of the SDS-PAGE gels (Figure 2), such a decrease in porcine collagen bands may be ascribable to cross-linking of inter molecular collagen through ϵ -(γ -glutamyl) lysine bonds.

The appearance of high molecular-weight products would be more difficult to dissociate in a sample buffer for

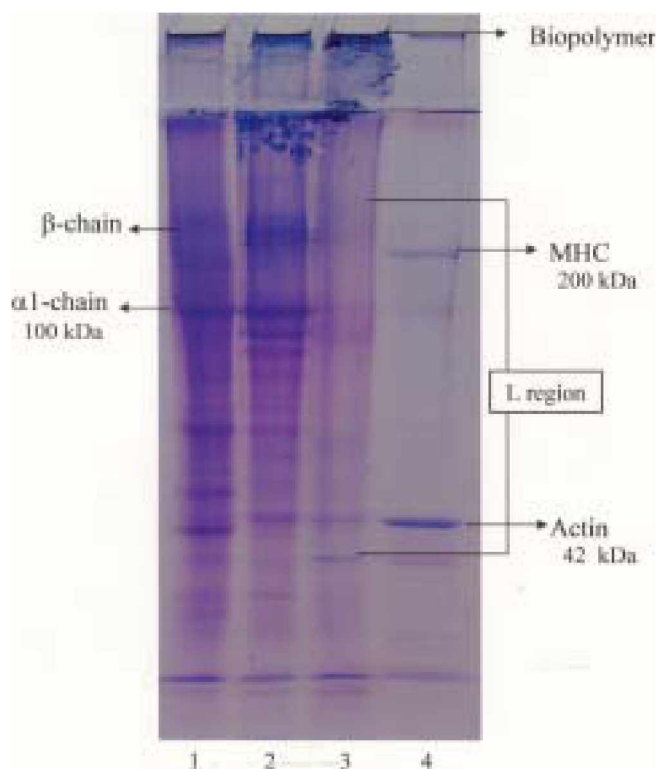


Figure 2. SDS-PAGE patterns of porcine collagen. 1) control (without MTGase), 2) 0.2% MTGase, 3) 0.5% MTGase, and 4) Myosin B (marker). Samples were incubated with MTGase at 50 for 60 min.

SDS-PAGE analysis, and finally it could not enter the stacking gel. The coincided results were previously reported in surimi gel cross-linking with MTGase (Jiang et al., 2000), and in gelatin film prepared using MTGase (Lim et al., 1999). They found 0.1% MTGase had a high ability to increase the formation of intermolecular cross-links. Ashie and Lanier (1999) reported that the MTGase (1.5 units/g protein) was able to polymerize turkey breast muscle and surimi at 50°C. In this study, through the result of SDS PAGE analysis, we confirmed that porcine collagen could be used as a substrate for MTGase.

Scanning electron microscopy

It has been accepted that there is a close relationship between the physical properties of a gel and its network structure (Kitabatake et al., 1989) and also between gelling properties and protein structure (Handa et al., 2001). In this study, SEM was used to analyze the network structure of porcine collagen gel formed by MTGase reaction (Figure 3). In the case of native porcine collagen, the microstructure of collagen showed a fibril structure and did not have an intermolecular cross-linked network. SEM revealed that the gel of porcine collagen in the absence of MTGase had a poor network structure, showing fewer clumps of aggregated protein and no intermolecular cross-links or a sheet-like structure (Figure 3A). As shown in Figure 3A, in

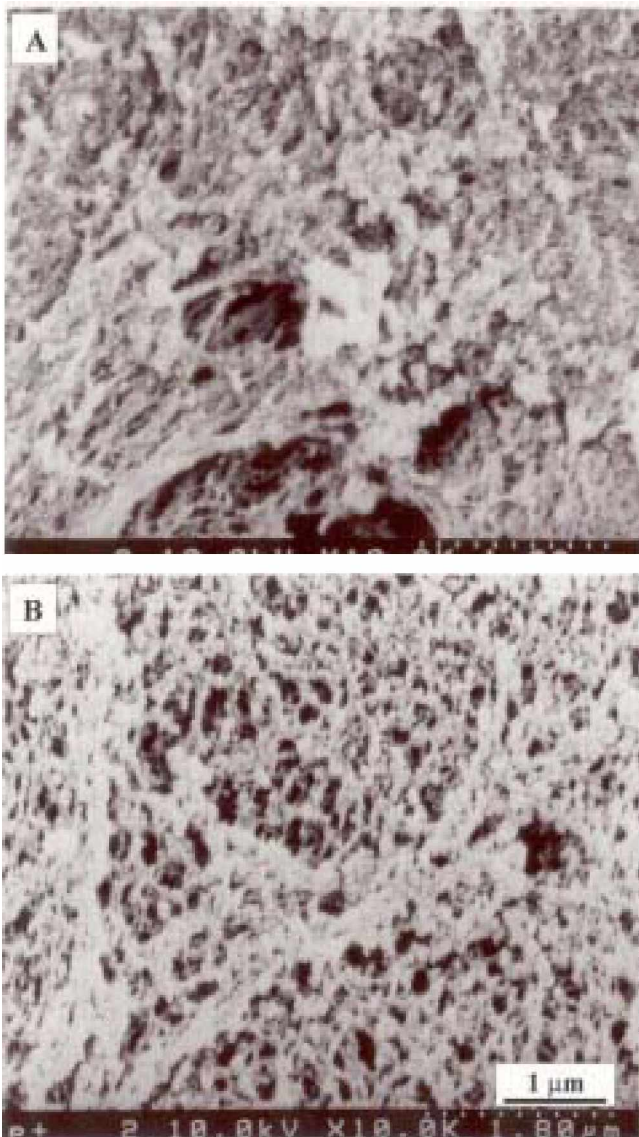


Figure 3. SEM photographs showing the gel network formed by MTGase reaction. (A) Porcine collagen without MTGase; (B) with MTGase (0.5% w/w). Samples were prepared by incubation with MTGase at 50°C for 1 h. Magnification is 10,000 \times and the calibration bar represents 1 μ m.

the porcine collagen gel without MTGase there is poor aggregation of protein and, consequently, its breaking strength was low (Figure 1). The gel of porcine collagen in the presence of MTGase produced an extremely well cross-linked network, in which intermolecular bonds and a sheet-like protein structure are shown (Figure 3B). This microstructure agrees with the mechanical properties of the gels formed by MTGase (Figure 1), which showed high values of breaking force.

From the SEM studies, the presence of MTGase could be expected to induce a compact and more aggregated gel structure. This was found to be the case when porcine collagen gels were made with and without MTGase, respectively (Figures 3A and B). Findings indicated that the addition of MTGase to the gelation process of porcine

collagen gel affected the structure of the gels. If a collagen gel has a well developed, intermolecular cross-linked, and fully aggregated protein, it should be stronger and more compact. If the gel has a poorly developed structure, especially that with an aggregation-type network, it is softer. It was demonstrated in the breaking strength analysis that a gel with MTGase has greater strength than that without MTGase. A similar result was reported for the gelation of soy bean glycinin (Kang et al., 1994), and Handa et al. (2001) found the strong correlations between the average of molecular weights and physical properties. Their results indicated an increase of breaking strength with increases in molecular weight. Comprehensive study of collagen gels treated with MTGase may allow controlled modification of gel structure, enabling creation of gels with specific properties.

Thermal stability of porcine collagen modified MTGase

A DSC was used to investigate the influence of MTGase on the endothermic transitions of porcine collagen gel. Figure 4 shows the DSC curves for porcine collagen gels formed with and without the MTGase (0.2% w/w). DSC curve of the gel without MTGase showed a sharp thermal

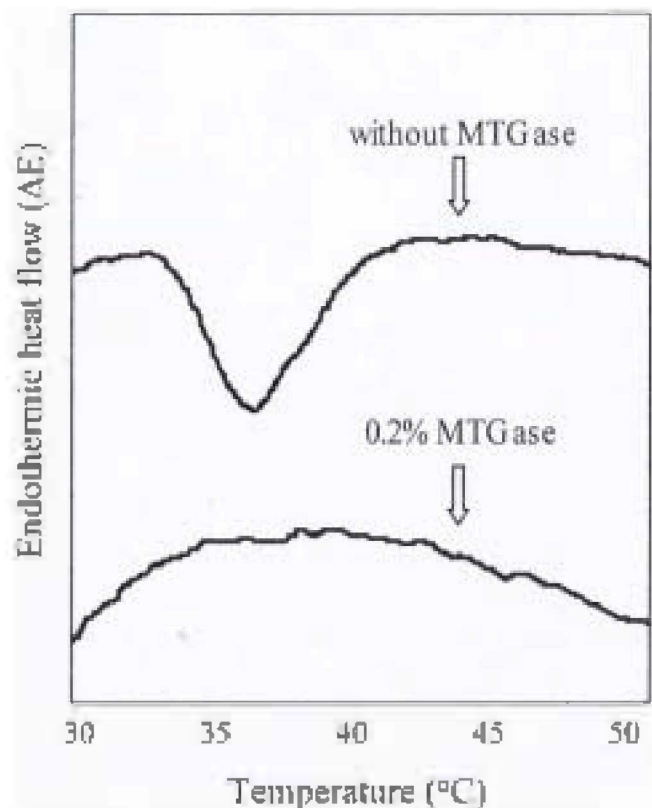


Figure 4. Differential scanning calorimetry curves of porcine collagen gels (10% w/v) with and without 0.2% MTGase. Heating rate was 5°C/min.

transition peak at 36°C, whereas the gel with 0.2% MTGase had no thermal transition peak over the range of about 20 to

120°C. This suggests that fibrils of the collagen molecule cross-linked by MTGase have a high thermal stability with additional crosslinkage. Through MTGase-induced intermolecular crosslinking, the thermal transition of porcine collagen is probably elevated in the transition to the polymer. This result is in accord with previous research by Mizuno et al. (1999), who found that the glass transition of casein was elevated by the cross-links caused by MTGase treatment.

Generally, the glass transition of synthetic polymers increases with increasing of molecular weight (Chartoff, 1997). The thermal transition of porcine collagen polymer that has a high molecular weight is considered to occur over a wide range of temperatures, and the thermal transition of those polymers cannot be reliably detected within the limited sensitivity range of an instrument. MTGase gave rise to the thermal resistance in the collagen gel, which therefore has the potential to expand the collagen applications in food materials (Nomura et al., 2001). The effect of MTGase treatment on the thermal transition of other proteins requires more study prior to wider applications of MTGase in the food industry.

Melting point

The changes in melting point as a result of MTGase reaction are shown in Figure 5. The melting points of collagen gel treated with MTGase were significantly higher than those of the gel without MTGase ($p < 0.01$). Increasing MTGase concentration increased melting point. Incubation temperature significantly affected melting point ($p < 0.01$). The melting points of gels incubated at high temperature were higher than those incubated at low temperature. No significant differences ($p > 0.05$) was noticed between high and low incubation temperature at 40 mg/ml of collagen with 1% MTGase (Figure 5C). The high temperature incubation (50°C) affected the ability of MTGase capability to polymerize porcine collagen; consequently, the melting point was increased by the optimal activity of MTGase. The optimum temperature for this enzyme was 50°C, and MTGase fully sustained its activity, for 10 min, even at 50°C. MTGase still expressed its activity at 10°C, and still retained some activity at temperature just above the freezing-point (Motoki and Seguro, 1998).

Increases in the melting point of collagen with MTGase may be a result of collagen cross-links. The mechanism by which these increases occurred was assumed to be similar to that by which increases in breaking strength were achieved. These are likely to be due to the increases in molecular weight or in the stability of collagen polymeric materials (DSC analysis) during polymerization by MTGase. Takahashi et al. (1999) also reported that production of cross-links between elastin and collagen with MTGase resulted in films showing increases in denaturation

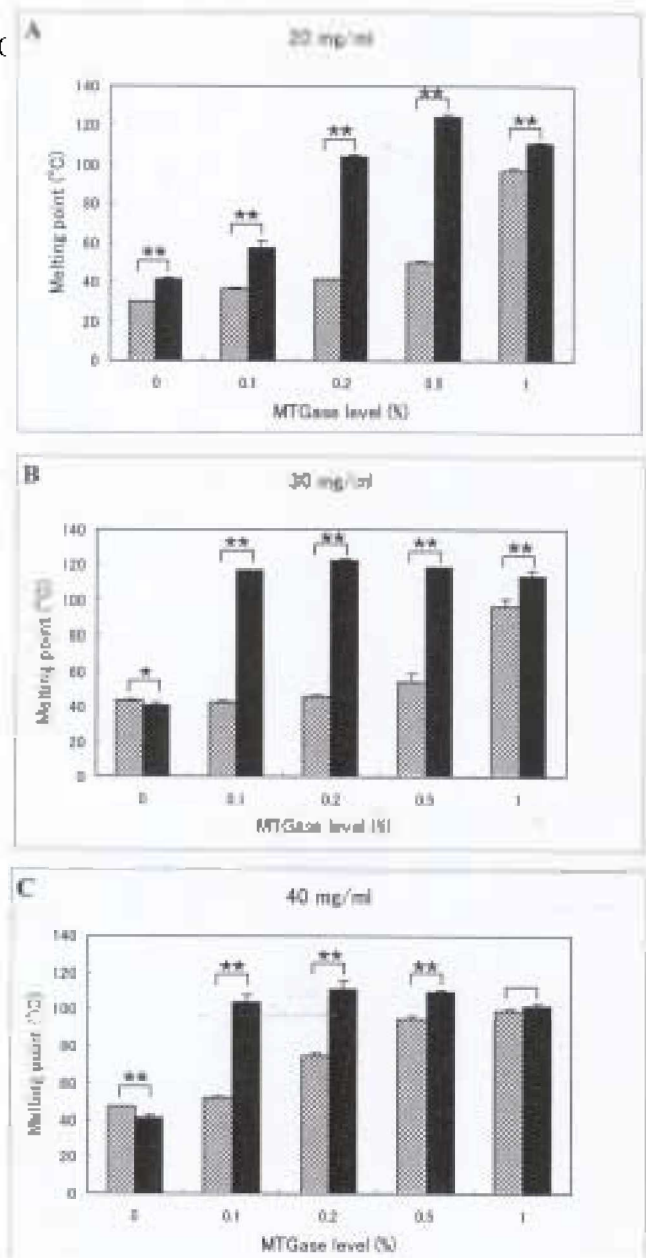


Figure 5. Changes in melting point temperature of collagen gels at various MTGase and protein concentrations. ▨ Incubated at 4°C; ■, Incubated at 50°C. A) 20 mg/ml, B) 30 mg/ml, and C) 40 mg/ml. Values represent means and standard deviations for five measurements. * Values are significantly different ($p < 0.05$). ** Values are very significantly different ($p < 0.01$).

temperature and a sharp thermal transitions as compared with those of un-cross-linked films. The cross-linked product suggests the capability of the MTGase to act as a unique agent for preparing gels with many different physical characteristics from commercial porcine collagen. For example, at the level of 40 mg/ml sample with 0.2% MTGase (Figure 5C), the melting point was 74°C; therefore, we could produce porcine collagen gel with the melting point around 70-80°C. The gels can be used as base materials in various processed foods requiring unique characteristics especially that of melting.

CONCLUSIONS

MTGase was able to improve the mechanical properties of the porcine collagen gel and to produce cross-linked porcine collagen through the ϵ -(γ -glutamyl) lysine bonds. Under SEM observation the collagen gel showed a compact net work structure. There was a close relationship between the structure of the gel and its mechanical properties. The thermal transition and melting point analysis gave more information about the functional properties of these gels. Findings suggest that applications could be aimed at the development of collagen gels with modified functional properties, that could be applied in complex systems such as foods. Knowledge of the structural-functional properties of porcine collagen gels and to their reactions with MTGase creates the opportunity to tailor them for use in food products.

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