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Properties of MTGase treated gluten film

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Abstract Water barrier and mechanical properties of gluten film affected by the treatment of microbial transglutaminase (MTGase) were evaluated with water vapor permeability (WVP), magnetic resonance imaging (MRI) T₂ map and mechanical properties of gluten films. The water barrier ability of gluten films was superior to the no film but inferior to the Parafilm[®] investigated by MRI T₂ maps in a two-gelatin-gel system. The WVP of gluten film increased, while the tensile force decreased, with increasing in the dosage of MTGase treated. The gluten films were also dyed with Acid Fuchsin and observed by a confocal laser scanning microscopy (CLSM) to localize the proteins in the film. The micrographs showed many pores and inhomogenous gluten network in MTGase treated gluten films. High WVP and weak mechanical properties of MTGase treated gluten films were attributed to the cross-link bonds formation, which created pores and inhomogenous gluten matrix as evidenced by CLSM.

Keywords Gluten film · Microbial transglutaminase (MTGase) · Water vapor permeability (WVP) · Magnetic resonance imaging (MRI) T₂ map · Confocal laser scanning microscopy (CLSM)

Introduction

Production and utilization of edible, biodegradable films prepared from agricultural resources, particularly plant proteins, have received great interests in recent years [1]. The films made from wheat gluten have gained much interest due to its unique cohesive and elastic properties

[2, 3]. Many researches have been reported to elucidate the preparation methods and properties of gluten-based films [4–10].

The chemical bonds responsible for the integrity of protein films involve hydrogen, ionic, hydrophobic and covalent bonds [11]. Although protein films generally have higher mechanical strength than lipid films, the plasticizers such as glycerol that are generally added to improve the flexibility of protein film will weakening the hydrogen bonding [12]. Most of protein films form good oxygen barriers, but they are poor moisture barriers because of the hydrophilic nature of protein molecules [13]. Hence, various modifications have been tried out, either by chemical [14–16], physical [2, 15, 17], or enzymatic methods [18–21], to improve the mechanical properties and water barrier properties of protein-based films. Introducing cross-linkages of proteins is a reasonable way to obtain stronger and less permeable films.

Two types of enzymes have been used to introduce cross-links in protein films: transglutaminase and peroxidase [21]. The effects of transglutaminase treatment on film properties have been studied for many proteins, including casein [18], whey proteins [11, 19], egg white proteins [20], soy proteins [22], zein proteins [11] and wheat gluten [21]. Laree et al. [21] indicated that transglutaminase was effective in introducing covalent bonds into films obtained from slightly deaminated gluten.

Microbial transglutaminase (MTGase, EC 2.3.2.13), produced mostly from *Streptoverticillum* spp., is one of the enzymes widely used for protein modification in recent years due to its lower cost than mammalian TGase [23]. It is an extracellular enzyme with a remarkable characteristic of Ca²⁺-independent catalytic property [24]. The MTGase catalyzes the formation of inter- and intramolecular ϵ -(γ -glutamyl)lysine cross-links (G-L bonds) in several food proteins. The polymerization and gelation of food proteins with MTGase have been reported having significant effects on the qualities of many foods by modifying its physicochemical properties, including viscoelasticity, gelation, emulsification, and foaming [25–27].

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The objective of this study was to produce films with wheat gluten and enzymatic-modified films by treating with transglutaminase in ethanol–water system. The properties of gluten films treated with MTGase, including water barrier and mechanical properties, and microstructure of films were investigated by using magnetic resonance imaging (MRI) system, textural analyzer and confocal laser scanning microscopy (CLSM).

Materials and methods

Materials

VITEN[®] wheat gluten (83% protein, 10% starch, 1.5% lipids, 1.5% ash, on dry bases) was supplied by Roquette Co. (Lestrem, France). The Ca^{2+} -independent MTGase with the activity of 746 units (U) was kindly provided by Ajinomoto Co., Inc. (Tokyo, Japan). One unit of enzyme activity is defined as the formation of 1 μmol of hydroxamic acid per minute at pH 6.0 and 37°C assayed by the method of Folk and Cole [28]. Absolute ethanol and glycerol was obtained from Merck Co. (Damstadt, Germany). Gelatin and sorbitol used for gel preparation of MRI measurements were purchased from Nacalai Tesque Inc. (Kyoto, Japan) and Showa (Tokyo, Japan). Acid Fuchsin, a fluorescence stain of protein, was purchased from Sigma Chemical Co. (St. Louis, MO).

Preparation of gluten film

A film-forming solution was prepared by mixing 12.5 g of gluten in a premixed solution of 60 ml of 95% ethanol and 3.2 ml of glycerol. After dispersing gluten by using a magnetic stirrer, 40 ml of 0.1% Na_2SO_3 (Nacalai Tesque Inc., Kyoto, Japan) was gradually added to reduce the disulfide bonds of gluten into sulfur hydrogen bonds, which increased the reaction efficiency of MTGase later [29, 30]. The solution was then adjusted pH to 8.0 by using 5 N NH_4OH and kept at 70°C for 30 min. The heated solution was homogenized with a homogenizer (Nihonseiki Kaisha Ltd., Japan) at 3,000 rpm for 3 min followed by centrifuging at 1,000 rpm for 2 min to remove air bubbles. Different amount of MTGase (0–600 U/g gluten) was added into the homogenized film-forming solution and immediately poured 5 ml solution into a petri dish (8.8 cm in diameter), and then lid was covered. The film-forming solution with the addition of MTGase was incubated in an oven at 55°C for 2 h. After incubation, the lid was removed and film was dried for another 3 h at 55°C. This procedure resulted in the formation of a transparent film with a constant controlled thickness (0.091 ± 0.004 mm) that was measured at several positions of a film with a micrometer (TECLOCK, Japan). The casting films on petri dishes were peeled out, then conditioned for 72 h at a relative humidity (RH) of 52.9% and a temperature of $25 \pm 3^\circ\text{C}$ in a desiccator which contained saturated $\text{Mg}(\text{NO}_3)_2$ solution [31].

Moisture adsorption of gluten film

Moisture adsorption of gluten film was determined gravimetrically under various relative humidity (or a_w) conditions at 25°C. Film samples were cut into small pieces (20 mm, in diameter) and dried for 2 weeks in a desiccator containing P_2O_5 at 25°C. The dried gluten film was then equilibrated in a modified proximity equilibration cell (PEC) [32] maintained at a selected a_w using appropriate saturated salt solutions. The saturated salt solutions and their a_w at 25°C were LiCl – 0.11, MgCl_2 – 0.33, $\text{Mg}(\text{NO}_3)_2$ – 0.53, NaCl – 0.75, and K_2SO_4 – 0.97 (31). Equilibrium moisture contents [$\text{EMC} = (\text{gain in mass/dry mass}) \times 100\%$] were taken when no further weight changes was observed in the samples. The exception was the film equilibrated at $a_w = 0.97$, which had mold growth after equilibrating for 4 days.

Water vapor permeability of gluten film

To determine water vapor permeability of gluten films, water vapor transmission rate (WVTR) was determined by ASTM method E 96-00 [33] with some modifications. Each film specimen (42 mm i.d.) was mounted over a circular opening of $20 \times 20 \times 3.1416$ mm² in a poly(methyl methacrylate) cup, in which contained 37.5 ml distilled water and the distance between film and water surface was 3.5 cm initially. The film sealed cup was inserted in a larger PP cup, in which contained dried silica gel beads to adsorb water vapor transferring from the small cup. After storage for 24 h at 25°C the weight loss of small cup was measured. The WVTR of the film was calculated according to the equation of $\text{WVTR} = \Delta W / (\Delta t \times A)$, where ΔW is the weight loss of test cell, Δt the time of storage, and A the area of exposed film. Water vapor permeability (WVP, $\text{gPa}^{-1} \text{s}^{-1} \text{m}^{-1}$) of gluten film was calculated as $\text{WVP} = (\text{WVTR} \times d) / \Delta p$, where Δp was partial water vapor pressure (Pa) difference across the two sides of the film and d the film thickness (m). Under conditions of determination, the Δp was 1753.55 Pa.

Water barrier properties of gluten film investigated by MRI

A two-gelatin-gel system was used to investigate the water barrier properties of gluten film by using a MRI technique [34, 35]. The system included two Teflon cylinder moulds, which have a rectangular well inside each mould for setting the gelatin gel. The ratios of gelatin:water:sorbitol of two gelatin gels used in this study were 24:76:5 for the gel with $a_w = 0.968$ (a_{wH} -gel) and 24:76:100 for the gel with $a_w = 0.828$ (a_{wL} -gel). When performing the MRI measurements, two Teflon moulds with different a_w of gelatin gels were sealed face-to-face with or without gluten film in between. A set of two-gelatin gel system separated with Parafilm[®] (American National Can, IL, USA) was also investigated in this study. The T_2 maps of two-gelatin-gel

during storage was performed by using the multi-slice-multi-echo (MSME) pulse sequence, which was provided by the manufacturer (Bruker, Ettlingen, Germany) in a Bruker medspec 3T system. The T_2 maps of water protons were obtained from a serial of MR images acquired at different echo times and fitted by the following equation: $S = S_0 \exp(-TE/T_2)$, where S is the pixel signal intensity measured at echo time TE , while S_0 the signal intensity at $TE = 0$.

a_w and moisture content of gelatin gels during storage

Two gelatin gels in the two-gelatin-gel system were carefully removed after different storage times. The a_w of gelatin gels were measured by using the AquaLab CX-3 (Decagon Devices Inc., WA, USA). The moisture content of gelatin gel was determined by pre-dried it at 60°C for 24 h, then dried it by P_2O_5 in a desiccator for one week. The moisture content of gelatin gel after storage was calculated by taking the weight differences before and after drying divided by the weight of total solids in gelatin gels. The a_w and moisture content of each sample was analyzed three replicates.

Mechanical properties

A texture analyzer with a 5 kg cell load (model TA-XT2i, Stable Micro Systems, UK) with a tensile grip probe (A/TG) was used to measure the tensile strength (TS) and elongation at break (EL) of film samples (70 mm×30 mm) by using a modified method of ASTM D882-91 [36]. Initial grip separation was 20 mm, with a crosshead speed of 1 mm/s. Force and elongation were recorded during extension, where TS was calculated from stress at break divided by the film cross-sectional area (600 mm²) and EL was expressed in percentage of the initial length (20 mm) of the elongating part of the specimen.

Microstructure of gluten film observed by CLSM

Microstructure of gluten film was observed with a Leica TCS SP2 CLSM mounted on an upright DM RXE fluorescence light microscope (Leica Lasertechnik GmbH, Heidelberg, Germany). Gluten film was stained with Acid Fuchsin as described by Fardet et al. [37]. The film (6 mm×6 mm) stained with Acid Fuchsin were illuminated with the krypton laser at 488 nm and the maximum of the emission peak was determined with the spectrometer of the microscope. The emission maximum was 620 nm for gluten film. Therefore, the bandwidth for recording the fluorescence images was set from 570 to 620 nm. Samples were observed both in the epi-fluorescence mode and reflection mode. For each film, four images were taken from 1 mm away from four corners and 30 nm underneath the surface of cover glass. The parameters of measurements were: scan mold-XYZ active, PMT 1-500 (HV), PMT 2-

650 (HV), PMT Trans-257.9 (HV), and the magnification of image was 400X.

Statistical analysis

The mechanical property, water adsorption, and water vapor permeability of gluten films were measured at least in triplicate. For the measurements of tensile strength and elongation at break of gluten film, seven films of each replicate were analyzed. The overall F -test was run using the general linear model procedure of SAS [38]. The differences between means were analyzed using least significant difference procedures and the significance level was defined as $p < 0.05$.

Results and discussion

Cross-linkage of gluten with MTGase under film forming conditions

Since gluten is a water insoluble protein, co-solvent and ethanol–water systems had been used to disperse protein for preparing the film-forming solution and for the enzymatic reaction [4, 5, 10, 39–41]. To examine the activity of MTGase under the conditions of gluten film forming procedure, the activity of MTGase was determined by measuring the formation of hydroxyamate [28] and the results were shown in Table 1. The activity of MTGase was significantly influenced by the solvent and pH. The formation of hydroxyamate under the conditions (ethanol:water=1:1 at pH=8.0) of gluten film formation solutions was only 1/170 of that under the tri-acetate buffer solution (water at pH=6.0) during the first 10 min of reaction. After 2 h incubation, the reaction activity of MTGase in ethanol:H₂O=1:1 (pH=8.0) solution was only 0.4% of that in tri-acetate buffer solution (pH=6.0). Increases of MTGase dosage or reaction time slightly increased the formation of hydroxyamate and the degree of reaction. Compared to the reaction under tri-acetate buffer solution, the cross-linked modification of gluten by MTGase occurred, but with much less reaction activity, as investigated by the formation of much less

Table 1 Formation of hydroxyamate under the conditions of film forming solution

Reaction time (h)	Formation of hydroxyamate (μM)		
	Tri-acetate (pH=6.0) ^a ; 150 U/ml soln ^b	EtOH/H ₂ O=1.5 (pH=8.0) ^a ; 150 U/ml soln ^b	EtOH/H ₂ O=1 (pH=8.0) ^a ; 600 U/ml soln ^b
0.17	0.67271	0.00368	0.02077
0.5	2.53654	0.00368	0.02077
1.0	3.83517	0.00368	0.05231
1.5	3.18849	0.00368	0.05494
2.0	3.66036	0.01551	0.05626

^aReaction solution

^bEnzyme concentration

Fig. 1 Water adsorption isothermal curves of gluten films with the treatment of MTGase (0–600 U/g vital gluten), which were determined by equilibrating gluten film in PEC containing a series of saturated salt solutions for 24 h at 25°C

amount of hydroxyamte in the conditions of film forming solution.

Water adsorption and water barrier properties of gluten film

Figure 1 is the water adsorption isothermal curves of gluten films with the treatment of MTGase (0–600 U/g vital gluten), which were determined by equilibrating gluten films in PEC containing a series of saturated salt solutions for 24 h. It was found that the patterns of water adsorption curves of gluten film were not significantly affected by the treatment of MTGase below $a_w=0.75$, whereas the water adsorption increased with increasing the dosage of MTGase at $a_w=0.97$ after equilibrated for 24 h.

Table 2 is the water vapor permeability of gluten film. It was found that no significant differences between gluten film with and without the treatment of MTGase (150 ppm) on both WVTP and WVP. However, WVTP and WVP of gluten films modified with MTGase increased with increasing the dosage of MTGase. This is attributed to the changes of gluten network due to the formation of ϵ -(γ -

glutamyl)lysine cross-links introduced by the reaction of MTGase. The cross-links of gluten might create larger pores in gluten network and facilitate transferring the water vapor through the film than the films prepared without the modifications by MTGase.

In order to investigate the water barrier properties of gluten film in actual food systems, two gelatin gels with different a_w were separated with gluten film. The water status of gelatin gels in the assembly two-gelatin-gel system was investigated by MRI during storage of 10 days. Based on the changes of brightness of images in T_2 maps, the moisture migration from a_{wH} -gel to a_{wL} -gel and redistribution of moisture in each gel were clearly revealed (Fig. 2a, c, e, and g). As MRI T_2 values along the central line of cross-section of two gels were monitored, the T_2 profiles were significantly differences among the systems separated with and without films in between two gels (Fig. 2b, d, e, and h). The gradients of T_2 values observed in both gels was attributed to the chemical environmental differences of water molecules in gels, which were the function of spatial position and storage time. The T_2 maps of the two-gelatin-gel system separated by Parafilm[®] showed no significant changes in the brightness and physical structure of both gels during the storage period (Fig. 2a). Besides, the MRI T_2 values along the central line of cross-section of two gels were maintain the same (Fig. 2b). This indicated that the Parafilm[®] had a good water resistance that prevented from water migration between two gels, and so is suitable to be used as a control system. The water barrier properties of both gluten films (Fig. 2e–h) without and with MTGase treatment were superior to the no film (Fig. 2c and d) but inferior to the Parafilm[®] in between two gels. After 10 days' storage, the T_2 profile showed a gradient in a_{wH} -gel and kept consistent in a_{wL} -gel although the a_w was the same in both gels, in which two gels was not separated with any film. According to the results of MRI, the water barrier property of MTGase treated gluten film was not significantly different from no MTGase treated gluten film when they were stored for 7 and 10 days (Fig. 2f and h).

Table 3 is the changes of water activity and moisture content of two gels in the two-gelatin-gel system with or without film in between during storage. Once two gels had been attached each other, water continuously migrated from a_{wH} -gel into a_{wL} -gel until no removable water molecules were available in the a_{wH} -gel or the a_w of two gels were approached to equilibrium. It is found that the a_w of two-gelatin-gel system without film in between reached the same a_w after the storage of 10 days, whereas the a_w of two-gelatin-gel systems separated with gluten film were getting close but not the same after the storage of 10 days. As observed in T_2 maps and T_2 profile, there was no water migration occurred in the system which two gels were separated by Parafilm[®].

Mechanical properties of gluten film

The mechanical properties of gluten film affected by the dosage of MTGase were shown in Table 4. It is found that

Table 2 Water vapor permeability of gluten film modified with MTGase

MTGase (U/g protein)	WVTP ¹ (g/m ⁻² hr ⁻¹ kPa ⁻¹)	WVP ¹ (g mm/m ⁻² hr ⁻¹ kPa ⁻¹)
0	19.76±4.81a	2.11±0.23a
150	18.09±2.83a	2.15±0.15a
300	31.50±0.18b	3.48±0.33b
450	35.59±7.07b	3.63±0.21b
600	41.65±6.73c	4.78±0.23c

^aMean value in the same column followed by different letters are significantly different ($p<0.05$)

Fig. 2 The MRI T_2 maps (a, c, e, and g) and T_2 profiles (b, d, f, and h) along the central line (dotted line) of two-gelatin-gel system ($a_{wH}=0.965$ and $a_{wL}=0.815$) during storage. (a, b) with paraffin, (c, d) without film, (e, f) no MTGase treated gluten film, and (g, h) with MTGase (600 U/g protein) treated gluten film in between two gels

the elongation of gluten film decreased when treated with MTGase above 450 U/g vital gluten, whereas the tensile strength of MTGase modified gluten films were not significantly affected by enzyme dosage. The results of mechanical properties of MTGase modified gluten film are opposite to the hypothesis proposed in this study. The hypothesis is the introducing cross-link of proteins by MTGase might strengthen the protein network, then stronger and less permeable protein films should be obtained. That means the gluten film modified with MTGase should improve film's mechanical properties by introducing cross-

linkages of gluten molecules and reduce the water vapor permeability because denser and closer network is formed. The opposite results of mechanical and water barrier properties were found in this study indicated that the undesirable microstructural changes of gluten film network might be occurred when MTGase was applied.

Microstructure of gluten film

To reveal the effects of MTGase treatment on the microstructure of gluten film network, the film was stained

Table 3 Changes of a_w and moisture content of gels in the two-gelatin-gel system that two gels were separated without or with a gluten film during storage

Storage time (day)	No film		Parafilm [®]		Gluten film		MTGase treated gluten film (600 U/g vital gluten)	
	H	L	H	L	H	L	H	L
Water activity (a_w) ^a								
0	0.968d	0.828a	0.968b	0.828a	0.968e	0.828a	0.968f	0.828a
3	0.964d	0.888b	ND	ND	0.955d	0.875b	0.956e	0.891b
7	0.931c	0.914c	ND	ND	0.948d	0.927c	0.953e	0.913c
10	0.914c	0.913c	0.970b	0.828a	0.928c	0.922c	0.931d	0.919c
Moisture content (%) ^b								
0	332g	60a	332b	60a	332f	60a	332f	60a
3	226f	85b	ND	ND	245e	76b	262e	69a
7	220ef	98c	ND	ND	222d	98c	239d	89b
10	210e	107d	331b	61a	212d	104c	229d	99c

Note. ND, not determined

^aMean (n=3). The values followed by the same letters are not significantly different ($p \leq 0.05$) for every two-gelatin-gel system

^bMean (n=3). The moisture content of gelatin gel before and after storage was calculated based on the weight of total solids (gelatin and sorbitol) under the assumption that the water molecules were the only molecules which migrated during storage

Fig. 3 Microstructure of gluten film observed by fluorescence micrograph of CLSM and the intensity profile of fluorescence along the line in micrographs (a, b) without MTGase treatment, (c, d) treated with 300 U/g vital gluten, and (e, f) treated with 600 U/g vital gluten

Table 4 Mechanical properties of gluten film modified with MTGase

MTGase (U/g protein)	Elongation (%)	Tensile strength (kPa)
0	218.99 ± 22.74a	1.573 ± 0.428a
150	210.27 ± 18.80a	1.525 ± 0.306a
300	198.88 ± 21.03a	1.430 ± 0.286a
450	195.11 ± 15.96b	1.436 ± 0.286a
600	195.01 ± 20.02b	1.381 ± 0.199b

Note. Mean value in the same column followed by different letters are significantly different ($p < 0.05$)

with Acid Fuchsin and then observed by CLMS. Both fluorescence micrograph and its intensity profile showed that the discontinuous protein network was formed when gluten film was treated with MTGase (Fig. 3). The discontinuity of protein network was significant when the dosage of MTGase increased. The CLSM micrographs showed that many large pores were found when 600 U MTGase/g vital gluten was applied during film-forming and resulted in discontinuous gluten matrix, especial for high dosage of MTGase. High WVP and weak mechanical properties of MTGase treated gluten film as observed in this study was attributed to the localized cross-link bonds formation by MTGase, which created pores and inhomogenous gluten matrix locally as evidenced by CLSM.

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