Food Chemistry 115 (2009) 602–608

Contents lists available at ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem

Cold storage of porcine plasma treated with microbial transglutaminase under high pressure. Effects on its heat-induced gel properties

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ARTICLE INFO

Article history:
Received 23 April 2008
Received in revised form 27 October 2008
Accepted 19 December 2008

Keywords:
Porcine plasma
MTGase
HP
Setting time
Gelling properties

ABSTRACT

The objective of this work was to study the heat-induced gelling properties, at acid pH, of porcine plasma previously treated with microbial transglutaminase (*MTGase*) under high pressure (HP), when kept under refrigeration conditions for different times (setting time). The results indicated that, although the cross-linking activity of *MTGase* was enhanced under pressure, consequently, improving the thermal gel texture, the most significant effects, particularly on gel hardness, were obtained by keeping the treated plasma solutions under refrigeration for at least 2 h before gelation. On the whole, under such conditions, increases of approximately 60% of this textural parameter, calculated on the basis of the values corresponding to the heat-induced non-treated plasma gels at pH 5.5, were achieved. However, from the SDS–PAGE profiles, it can be suggested that mechanisms other than polymerisation by *MTGase* explain the beneficial effects of the treated plasma cold storage on gel texture. In contrast, the setting time had no effects on the water-holding capacity of heat-induced plasma gels at acid pH value, although this gel property was slightly enhanced by submitting porcine plasma solutions to the combined treatment (*MTGase* plus HP), with improvements being in accordance with the better-structured network of these heat-induced plasma gels.

1. Introduction

Interest in improving heat-induced gelling properties of plasma under acidic conditions, for use as a meat ingredient, has been previously reported (Fort, Carretero, Pares, Toldra, & Saguer, 2007; Saguer, Fort, Pares, Toldra, & Carretero, 2007). Porcine plasma shows good gelling properties under physiological conditions but, at acid pH, i.e., close to the p*I* of plasma proteins, soft and exudative gels are obtained (Parés, Saguer, Saurina, Suñol, & Carretero, 1998; Saguer, Fort, Alvarez, Sedman, & Ismail, 2008). Different approaches to tackle this question have recently been considered (Fort, Lanier, Amato, Carretero, & Saguer, 2008; Fort et al., 2007; Saguer et al., 2008), with the use of microbial transglutaminase (*MTGase*) before the thermal gelation of plasma (considered as a realistic way to solve this problem), especially when the enzymatic treatment is carried out under high pressure (HP) (Fort et al., 2008). The improvements achieved when this combined treatment is applied can probably be explained by a greater ability to form new intermolecular cross-links as a consequence of HP-induced protein unfolding, thus making more accessible the amino acids involved in the cross-linking reaction performed by *MTGase*, a baroresistant enzyme (Ashie & Lanier, 1999; Gilleland, Lanier, & Hamann, 1997; Lauber, Krause, Klostermeyer, & Henle, 2003; Lauber, Noack, Klostermeyer, & Henle, 2001a; Lauber, Noack, Klostermeyer, & Henle, 2001b; Lee & Park, 2002; Menendez, Rawel, Schwarzenbolz, & Henle, 2006; Nonaka, Ito, Sawa, Motoki, & Nio, 1997). However, in the study of Fort et al. (2008), the heat treatment necessary to induce gelation was not immediately applied after submitting the plasma solutions to the combined treatment; a short period of time under refrigeration conditions was used between both processing steps, during which time the enzymatic cross-linking should not be ignored. The efficacy of such a procedure has already been shown using HP-treated fish or turkey proteins, with or without added *MTGase*, to produce heat-induced gels. The gel strengthening is then attributed to the protein cross-linking induced by the added *MTGase* and/or by an endogenous calcium-dependent transglutaminase present in fish pastes (Ashie & Lanier, 1999; Gilleland et al., 1997; Uresti, Velazquez, Vazquez, Ramirez, & Torres, 2006). The optimal temperature and time conditions during this period (which can be referred to as "setting time", due to its similarity to the method used to produce harder products in the fish industry) depends on the substrate (Ashie & Lanier, 1999). Refrigeration conditions could be suitable for fish transglutaminase, while they could be considered too low for the cross-linking activity of *MTGase*. However, this enzyme still retains some residual activity near freezing point (Seguro, Nio, & Motoki, 1996; Yokoyama, Nio, & Kikuchi, 2004) and, in the meat processing industry, *MTGase* treat-

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doi:10.1016/j.foodchem.2008.12.083

ments for binding raw material are commercially performed under refrigeration conditions (Ajinomoto Food Ingredients LLC, 2007). Moreover, under these temperature conditions, microbiological spoilage of plasma can be minimised.

Thus, the objective of this research was to study how the functional properties (texture and water-holding capacity) and microstructure of heat-induced plasma gels at acid pH were affected by holding plasma solutions at chilled temperatures for a fixed period (setting time) after treatment by *MTGase* under HP.

2. Material and methods

2.1. Porcine blood plasma samples

Different porcine blood samples were hygienically collected from an industrial abattoir (Frigoríficos del Ter, SA, Salt, Spain). Samples obtained on different days were immediately mixed with sodium citrate solution (1%, w/v, final concentration in blood) and kept under refrigeration until they were centrifuged in the laboratory at 2530*g* at 5 °C for 15 min (SORVALL RC 5 C Plus, Dupton, Newtown, USA). Plasma was separated from the cellular fraction by decantation after centrifugation and immediately spray-dried using a Büchi Mini Spray-Dryer B-191 (Büchi Labortechnik AG, Flawil, Switzerland) at an air inlet temperature depending on the air outlet temperature, which was kept at 73 ± 5 °C; feed flow of the product was 670 ml h^{-1}; aspirator flow rate was 60 l h^{-1}; and spray air flow pressure was 5 bar. The protein content of spray-dried plasma measured using the Kjeldahl method (ISO R-937, conversion factor for nitrogen: 6.25) was 66.5 ± 3.1% (w/w).

2.2. Experimental design

Three spray-dried samples of porcine plasma were produced as indicated above. From each sample of spray-dried plasma, four different plasma solutions [7%, w/v, protein] were prepared and adjusted to pH 7 with 3 N HCl to assay the following conditions: (a) untreated or control solution (C), (b) *MTGase*-containing solution (M), (c) HP-treated solution (H), and (d) *MTGase*-containing solution treated under HP (HM). The samples with added enzyme had a final concentration of 3% (w/v) of ACTIVA® (1% *MTGase* and 99% maltodrextrin, Ajinomoto Co., Inc., Transglutaminase, Japan), corresponding to 43.3 *MTGase* activity units per gram of protein. Then, each plasma solution was refrigerated (4–5 °C) for 0, 2, 4 and 16 h (overnight) before its heat-induced gelation (this cold storage time will hereafter be referred to as setting time). After this refrigeration period, an aliquot of each solution was taken for determination by SDS–PAGE of degree of protein cross-linking. The rest of each solution was adjusted to pH 5.5 with 3 N HCl and submitted to heat-induced gelation to study the effects of the different treatments on texture, water-holding capacity and microstructure of the heat-induced plasma gels at this pH.

2.3. HP-treatment

Plasma solutions were individually vacuum-packed in Alcom vacuum bags (20 μm polyamide and 70 μm polyethylene, with a total thickness of 90 μm) (Alcom Srl, Campogalliano, Italy), using a Polar 80 Henkelman vacuum system (Hertogenbosch, Netherlands). The pressure vessel and the sample were preconditioned to a temperature of 20 °C prior to pressurisation at 400 MPa for 30 min in an Iso-Lab 900 Power High Pressure Food Processor (Stansted Fluid Power Ltd., Stansted, UK). Pressure-transmitting fluid (a mixture of ethanol–castor oil, 90:10) was pumped at the same temperature. The pressure unit took approximately 4 min to reach the pressure of 400 MPa, and 3 min to release the pressure.

2.4. Heat-induced gel treatment

An aliquot (75 ml) of each plasma solution was adjusted to pH 5.5 using 3 N HCl, and introduced into synthetic gut (Wieniepak® 2350/84, Teepak LLC, Belgium). Thermal gelation was carried out by submerging plasma solutions in a heated water bath at 80 °C for 45 min, after which they were immediately cooled to 20–25 °C in a water bath and kept under refrigeration conditions overnight (16 h). Gels were tempered at room temperature (30 min) before they were analysed.

2.5. Texture analysis

Texture profile analysis (TPA) was performed on gel portions (14 mm length and 24 mm diameter), using a TA XT2 texturometer (Stable Micro Systems Ltd., Surrey, UK). An aluminium cylindrical probe of 50 mm diameter was used to compress samples, twice, to 30% deformation, 2 s being the time established for gel recovery between cycles. Tests were undertaken at room temperature at a uniaxial compression rate of 1 mm s^{-1}. For each sample, measures were taken on three different portions. Hardness, springiness and cohesiveness of protein gels were determined from the TPA curve, as previously described by Fort et al. (2007).

2.6. Water-holding capacity (WHC) analysis

A technique combining filtration and centrifugation, proposed by Parés et al. (1998) and slightly modified by Fort et al. (2007), was used. Gel cylinders (14 mm length and 8 mm diameter) were placed into cylinders of PVC with polyester mesh (100 μm aperture; Henry Simon, Cheshire, UK) in the bottom, which were suspended inside centrifuge tubes and then centrifuged at 4000*g* and 15 °C for 10 min. The results are reported as percentages (w/w) of water released after centrifugation; four replicates were measured for each sample.

2.7. Scanning electron microscopy (SEM)

The microstructure of the heat-induced gels was determined using SEM, as described previously by Fort et al. (2007). A fixation step with 2.5% (v/v) glutaraldehyde in 0.2 M phosphate buffer (pH 7.2), followed by post-fixation with 1% osmium tetraoxide in 0.1% sodium cacodylate buffer, was carried out on gel samples. They were then rinsed in distilled water before being dehydrated in ethanol with increasing serial concentrations from 50% to 100% (v/v). Samples were dehydrated by critical-point drying, with CO_2 as the transition fluid in a dryer, mounted on a bronze stub and sputter-coated with gold (Emitech K550, EM Integrated Technology, Kent, UK). A Zeiss DSM 960 A scanning electron microscope (Carl Zeiss; Electron Optics Division, Oberkochem, Germany), at an acceleration voltage of 15 kV was used, to view the gel samples.

2.8. SDS–polyacrylamide gel electrophoresis analysis (SDS–PAGE)

Plasma solutions were analysed by reducing SDS–PAGE electrophoresis in a Mini-PROTEAN® 3 Electrophoresis Cell (BioRad, Hercules, CA, USA). Plasma solution (10 μl) which contained 50 μg of total protein, was mixed with the same volume of a SDS solution (0.5 M tris–HCl buffer, pH 6.8, containing 4% SDS, 40% glycerol, 0.2% β-mercaptoethanol and 31% bromophenol blue) to give a final protein concentration of 2.5 μg μl^{-1}. The mixture was incubated at 70 °C for 5 min and then applied to the electrophoresis wells. Gels (3.9% stacking gel and 12% separating gel) were prepared in the

laboratory, following the Laemmli method (Ausubel et al., 2002). Acrylamide (40%) (A4058) and bisacrylamide (2%) (M1533), both purchased from Sigma–Aldrich (St. Louis, MO, USA), were used to prepare the gels while the BenchMark™ Protein Ladder (Invitrogen, Carlsbad, CA, USA) was used as molecular weight standards from 20 to 220 kDa. Electrophoresis was carried out at 70 V for 30 min and then at a constant voltage of 120 V for 2 h. The gels were treated with 2.5% glutaraldehyde in distilled water and then stained with 0.1% Coomassie Brilliant blue G-250 in methanol:acetic acid:distilled water (3:1:6). Destaining was performed in 30%:10% methanol:acetic acid. Afterwards, gels were soaked in 10% acetic acid containing 10% glycerol. The images of the electrophoresis gels were captured and analysed using the documentation system UVItec UVIPro Gold (UVItec Ltd., Cambridge, UK).

2.9. Statistical analyses

Statistical analyses were carried out with the SPSS 14.0 for Windows (SPSS Inc., Chicago, IL). In all cases, data were submitted to ANOVA using the general linear model procedure (Proc GLM) and Tukey's test was used to compare means. The significance level for all tests was $\alpha = 0.05$.

3. Results and discussion

3.1. Textural properties

Fig. 1 shows the effects of the different tested treatments (*MTGase*-added and/or submitted to HP, as well as a control) on the hardness of heat-induced plasma gels at pH 5.5 as a function of the storage time under refrigerated conditions (setting time) before gelation. Statistical analysis indicated that, when plasma solutions were gelled just after treating (i.e., no setting time), the gel hardness significantly ($P < 0.05$) depended on the treatment applied. As expected, the addition of *MTGase* (M), just before the heat-induced gelation, did not significantly change this parameter compared with the control gels (C), while significantly ($P < 0.05$) softer gels, in comparison to these latter, were obtained from HP-treated plasma. In contrast, hardness was significantly ($P < 0.05$) increased, compared to all of the other treatments (including control), when plasma solutions with added *MTGase* were treated at HP (HM), with increases of around 0.6 N (38%) being obtained compared to control samples.

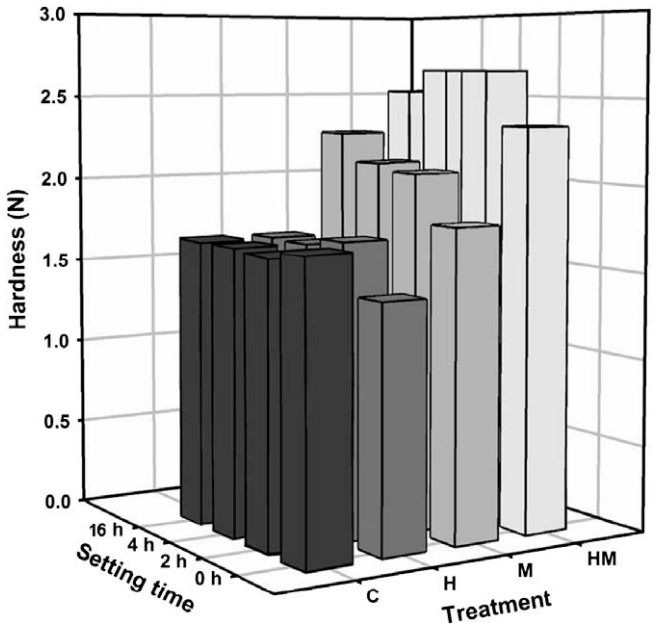

Fig. 1. Hardness of heat-induced gels at pH 5.5 obtained from different plasma solutions (non-treated, C, treated under HP, H, *MTGase*-added, M, and *MTGase*-added treated under HP, HM) and kept at chilled temperature for different times (0, 2, 4 and 16 h). Data are means values for triplicate samples.

When the setting time was also considered as a main factor in the model for statistical analysis, a significant ($P < 0.05$) interaction between treatment and setting time was detected. Thus, the effect of duration of this time was separately analysed for each treatment. In the absence of *MTGase* (C- and H-plasma solutions), the gel hardness was not significantly affected by keeping the plasma solutions under chilled conditions for at least 16 h, although H-gels tended to be slightly harder when refrigerated. Moreover, no significant differences were obtained between C- and H-gels when submitting plasma solutions to refrigeration before gelation. These results agree with those obtained in a previous study, in which thermal gels from pressurised samples, kept for 3 h under chilled conditions after pressure treatment (400 MPa – 30 min), showed a texture similar to those of gels derived from non-treated plasma samples (Fort et al., 2008). Overall, these results seem to indicate that reversibly modified proteins can be partially refolded after releasing pressure if a setting time is applied before inducing thermal gelation (Montero, Lopez-Caballero, Perez-Mateos, Solas, & Gomez-Guillen, 2005; Pothakamury, Barbosa-Canovas, Swanson, & Meyer, 1995; Royer, 2002; Tabilo-Munizaga & Barbosa-Canovas, 2004).

Conversely, gels from plasma solutions containing *MTGase* (M- and HM-solutions) showed a significantly ($P < 0.05$) greater hardness after refrigeration for at least 2 h before gelation, but with no further significant increases on further refrigeration (4 or 16 h). It should be noted that similar increases (around 37%) in gel hardness relative to non-treated gels (not considering the setting time) were obtained for both HM-solutions, following gelation, just after the combined treatment and after keeping M-solutions under refrigeration for 16 h. These results indicate that the enzyme may actually act at low temperatures. Similar increases were obtained in a previous study in which plasma was treated with microbial transglutaminase at 30 °C for 3 h (Saguer et al., 2008), thus suggesting that the effects of *MTGase* on hardness of heat-induced plasma gels at acid pH are equivalent under both 30 °C and refrigeration conditions. However, the highest increases in hardness were obtained when HM-solutions were kept under chilled conditions for relatively short periods. The most beneficial effects on this textural parameter were obtained by refrigeration for up to 4 h, with no significant differences after holding for 2 or 4 h. Overall, increases of around 60–61% relative to values for control gels at pH 5.5 were achieved. Unlike M-solutions, a significantly ($P < 0.05$) negative effect on gel hardness from HM-solutions, held overnight under refrigeration, was obtained, relative to the values achieved for the shortest setting times (2 and 4 h).

All plasma gels showed a very high springiness (>0.9) while cohesiveness values around 0.5 were obtained (Table 1). In spite of this, gel springiness was significantly ($P < 0.05$) increased in *MTGase*-added plasma samples, with the best improvements (increases of ~2.5% compared to control gels) being obtained when the enzymatic treatment was carried out under HP. However, no effects on this textural parameter were detected as a function of the refrigeration time. In contrast, cohesiveness was not significantly changed compared with the C-gels under any conditions. Both springiness and cohesiveness are indicative of the damage to the gel structure from the first compression (Handa, Takahashi, Kuroda, & Froning, 1998); thus, these results suggest that the treatments applied had essentially no effects on gel structure.

Table 1
Springiness and cohesiveness of heat-induced plasma gels at pH 5.5 obtained from different plasma solutions (non-treated, C, treated under HP, H, *MTGase*-added, M, and *MTGase*-added treated under HP, HM), and kept at chilled temperature for different times (0, 2, 4 and 16 h). Results are means ± SD ($n = 3$). Different lower case letters indicate significant differences in springiness between treatments according to Tukey's test ($\alpha = 0.05$).

Setting time (h)	Springiness				Cohesiveness			
	C	H	M	HM	C	H	M	HM
0	0.92 ± 0.00 a	0.92 ± 0.00 a	0.93 ± 0.01 b	0.94 ± 0.01 c	0.54 ± 0.01	0.54 ± 0.02	0.55 ± 0.01	0.56 ± 0.00
2	0.92 ± 0.01 a	0.93 ± 0.02 a	0.93 ± 0.01 b	0.95 ± 0.01 c	0.54 ± 0.01	0.54 ± 0.01	0.55 ± 0.00	0.55 ± 0.00
4	0.92 ± 0.01 a	0.93 ± 0.01 a	0.94 ± 0.00 b	0.94 ± 0.00 c	0.54 ± 0.00	0.55 ± 0.02	0.55 ± 0.00	0.55 ± 0.00
16	0.92 ± 0.00 a	0.92 ± 0.01 a	0.94 ± 0.00 b	0.94 ± 0.01 c	0.53 ± 0.01	0.54 ± 0.01	0.54 ± 0.01	0.55 ± 0.01

3.2. Water-holding capacity (WHC)

The effects of the treatments and setting times tested on the WHC of heat-induced plasma gels at acid pH, expressed as water released after centrifugation, are shown in Table 2. Overall, it can be seen that exudative gels were obtained in all cases. There was a slight but significant ($P < 0.05$) reduction in the percentage of released water, compared with control samples, when plasma solutions containing *MTGase* were submitted to HP before thermal gelation, with the setting time not showing a significant effect on this gel parameter. Other studies have previously shown that texture and water retention are affected differently by *MTGase* treatment, this last property being more difficult to enhance (Fort et al., 2007; Saguer et al., 2007), probably due to its dependence on gel microstructure.

3.3. Gel microstructure

Microstructure of the thermal plasma gels at acid pH was investigated using SEM at a magnification of 5000× (Fig. 2). Gels from non-treated plasma solutions showed relatively large aggregates and irregular voids. Such microstructure completely agrees with their poor WHC. Similar results had been previously reported (Fort et al., 2007; Parés et al., 1998). Although no great changes in the gel microstructure could be observed by submitting the plasma solutions to the different treatments and setting times before thermal gelation in this work, some minor differences were detected. More homogeneous gels with a minor pore size were obtained when enzymatic treatment with *MTGase* was carried out under HP; however, an effect of the setting time cannot be deduced (Fig. 2g and h). These results could also suggest that, at pH close to the p*I* of plasma proteins, the dominant mechanism governing the gel network development is the low level of electrostatic repulsion.

3.4. Degree of cross-linking

Fig. 3 shows the electrophoretic pattern obtained under reducing conditions of the non-treated plasma solution (C), as well as treated samples (*MTGase*-added and/or HP-treated), as a function of the setting time (0 and 4 h). No differences between protein profiles corresponding to both C-solutions, kept or not for 4 h under chilled conditions, were detected. The polymerisation of plasma proteins was also neither enhanced under pressure (H-solutions) nor by keeping these solutions at chilled temperature for 4 h after treating. In contrast, for M-added plasma solutions (M), a significant decrease or disappearance in some bands was observed, along with the appearance of new bands corresponding to newly-formed polymers. The bands with a molecular weight in the range from ~90 to ~100 kDa reacted, during the time spent to mix the enzyme in the plasma solution, with this cross-linking activity not having effects on gel hardness (Fig. 1). In contrast, the reduction in intensity of the other bands (molecular weight ~ 110–180 kDa) was especially evident when these plasma solutions were kept for 4 h under chilled conditions after adding the enzyme, indicating that the cross-linking activity actually takes place at temperatures much lower than its optimum. It is also important to note that no changes were detected in the intensity of the band corresponding to the serum albumin when compared with that in the non-treated samples (C), confirming that *MTGase* cannot attack serum albumin when it is in the native state.

On the other hand, a more notable increase in the cross-linking of *MTGase* was observed when the enzymatic reaction was carried on *MTGase*-added plasma submitted to HP (HM-solutions), with its electrophoretic profile showing that, in this case, the most of the protein bands, with molecular weights ranging from ~90 to ~180 kDa, practically disappeared after the combined treatment. Moreover, reduction (or disappearance) of some other bands, compared with M-solutions under refrigeration conditions, was also observed, specifically the band of molecular weight ~55 kDa and the slower migrating native bands (>220 kDa). The formation of high molecular weight polymers is clear from the appearance of new bands, some of them retained in the stacking gel and others not penetrating the running gel. Regarding the molecular weights of plasma proteins (Howell & Lawrie, 1983), fibrinogen and globulins seem to be cross-linked proteins when plasma containing *MTGase* is treated under pressure. However, the participation of plasma serum albumin in the *MTGase* reaction, when carried out under HP, must be considered, due to a small decrease in the intensity of its band on SDS–PAGE (~66 kDa). These results seem to confirm that serum albumin becomes more susceptible to polymerisation after partial unfolding under pressure. However, the other main plasma proteins seem to be more susceptible to effects of HP on increasing *MTGase* cross-linking activity. When the effect of the setting time for HM-treated samples was analysed, no differences in the protein patterns were observed between plasma held at chilled temperature for 4 h before its heat-induced gelation (HM-4) and HM-treated plasma gelled by heat immediately after treatment (HM-0), suggesting that protein polymerisation due to the *MTGase* activity was achieved during the HP-treatment. Thus, beneficial effects of the refrigeration time on gel texture, particularly hardness, were only obtained in the presence of *MTGase*. However, the results seem to suggest that different phenomena took place during the setting time period, depending on the prior

Table 2
Water-holding capacity (WHC), expressed as water released after centrifugation (%), of heat-induced plasma gels at pH 5.5 obtained from different plasma solutions (non-treated, C, treated under HP, H, *MTGase*-added, M, and *MTGase*-added treated under HP, HM), kept at chilled temperature for different times (0, 2, 4 and 16 h). Results are means ± SD ($n = 3$). Different lower case letters indicate significant differences between treatments according to Tukey's test ($\alpha = 0.05$).

Setting time (h)	WHC (Percentage of released water)			
	C	H	M	HM
0	75.8 ± 1.8 ab	79.9 ± 1.8 a	75.3 ± 2.4 bc	74.4 ± 2.4 c
2	76.4 ± 0.8 ab	76.2 ± 0.3 a	73.3 ± 1.1 bc	74.0 ± 0.7 c
4	74.4 ± 1.6 ab	76.3 ± 3.1 a	72.1 ± 0.7 bc	73.0 ± 3.8 c
16	80.4 ± 0.4 ab	75.8 ± 1.8 a	75.1 ± 4.4 bc	72.1 ± 1.8 c

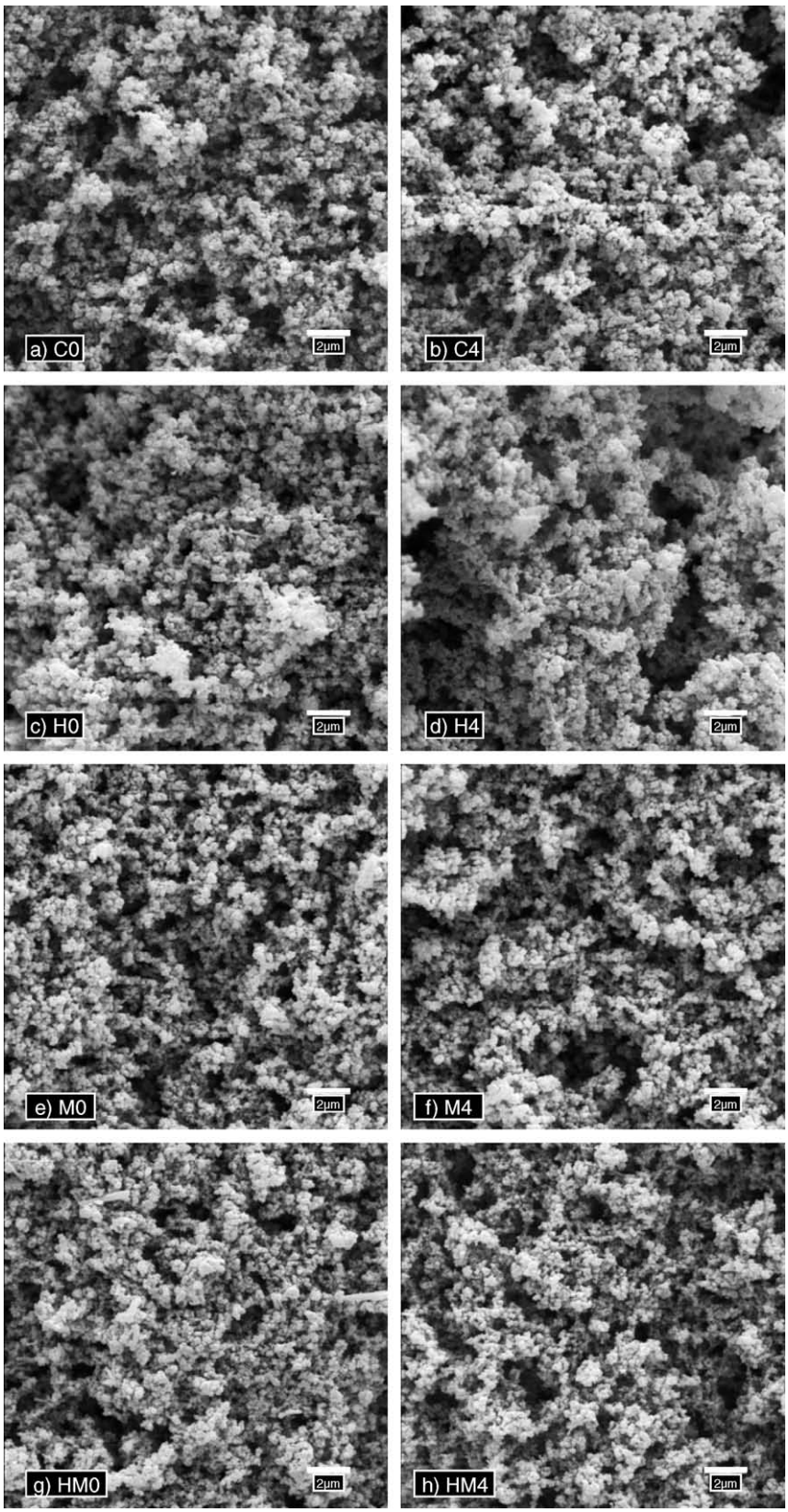

Fig. 2. Microstructure of heat-induced plasma gels at pH 5.5 obtained from different plasma solutions (non-treated, C, treated under HP, H, *MTGase*-added, M, and *MTGase*-added treated HP pressure, HM), and kept at chilled temperature for different times (0 or 4 h).

treatment. In M-gels, the improvements seem to be related to the *MTGase* cross-linking during the cold storage period. In contrast, in HM-gels other mechanisms should explain the hardness behaviour as a function of the duration of setting time.

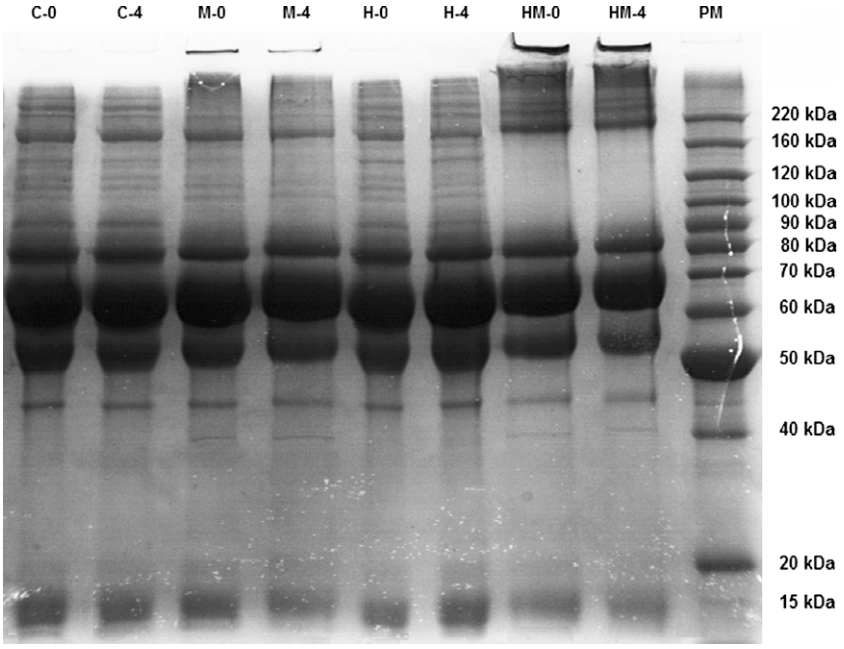

Fig. 3. SDS–PAGE patterns of different plasma solutions: non-treated (C), *MTGase*-added (M), treated under HP (H), and *MTGase*-added treated under HP (HM) as a function of the refrigeration time (0 and 4 h).

4. Conclusions

Holding porcine plasma treated simultaneously with *MTGase* and high pressure for 2 h under refrigerated conditions (setting time) is actually very effective in enhancing the textural properties of gels obtained after heating at acid pH, especially hardness. However, the water-holding capacity is only slightly improved, probably due to subtle effects on gel microstructure. From SDS–PAGE profiles, it can be proposed that cross-linking by *MTGase* is actually occurring under HP, but that other mechanisms taking place during the setting time could also be responsible for these improvements, although protein polymerisation during HP-treatment seems to have an important influence on the efficacy of the setting time on gel hardness. More studies, clarifying these points, should be carried out in the future.

Acknowledgments

This work was financially supported by the MEC Project of the Spanish Government (Ref. AGL2004-06077/ALI), the Universitat de Girona (Spain), the Departament d'Universitats, Recerca i Societat de la Informació de la Generalitat de Catalunya (Spain), and University College Cork (Ireland). We would like to thank Mr. Eddie Beatty for his helpful technical assistance. We also are very grateful for the laboratory work carried out by Anna Mª Aymerich, Eva Espigulé and Núria Carbó.

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