Universitat de Girona

IMPROVEMENT OF HEAT-INDUCED GEL PROPERTIES OF PORCINE PLASMA

Nuri FORT FORT

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PhD Dissertation

IMPROVEMENT OF HEAT-INDUCED GEL PROPERTIES OF PORCINE PLASMA

Nuri Fort Fort

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Technology – Food and Agricultural Technology

Advisor:

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Dissertation submitted to apply for the Doctor degree by the University of Girona with the European mention

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Dra. Elena Saguer Hom, Lecturer in the Food Technology Area, in the Chemical and Agricultural Engineering and Food Technology Department, and Researcher in the Institute of Food and Technology at the University of Girona,

CERTIFY:

That she has supervised the work carried out by Nuri Fort Fort in the Food Technology Area at the University of Girona entitled *Improvement of heat-induced gel properties of porcine plasma*, which is submitted as a paper-compendium format in this dissertation to apply for the Doctor degree by the University of Girona. All the requirements to be submitted as a paper-compendium format and to get the European mention are complied.

Five papers are included in this dissertation, all of them published in the first quartile of Food Science category during 2007, 2008 and 2009. Nuri Fort Fort is the first author in three papers, and the second author in the other two. The maxim impact factors in Food Science category were 4.21 and 4.15 in 2007 and 2008, respectively; it is not still available the impact factor for 2009. Two papers were published in Food Chemistry in 2007 and 2009, respectively. This journal was in the 4th position (of 103) with an impact factor of 3.05 in 2007 and in the 9th position (of 107) with an impact factor of 2.70 in 2008. Two other papers were published in Food Hydrocolloids in 2007 and 2008, respectively; the journal was in the 8th position (of 103) with an impact factor of 2.49 in 2007 and in the 11th position (of 107) with an impact factor of 2.51 in 2008. The last paper was published in Meat Science in 2008, which was in the 17th position (of 107) with an impact factor of 2.18. All studies were focused on the loss of heat induced gel properties when porcine blood plasma is acidified, and in trying to recover this losses submitting plasma to an enzymatic treatment with microbial transglutaminase before heat-induced gelation at acid pH.

Dra. Elena Saguer Hom
Girona, November 2009

PUBLISHED WORKS

- Saguer, E., Fort, N., Alvarez, P.A., Sedman, J. & Ismail, A.A. 2008. Structure-functionality relationships of porcine plasma proteins probed by FTIR spectroscopy and texture analysis. *Food Hydrocolloids*, 22(3): 459-467.
- Saguer, E., Fort, N., Parés, D., Toldrà, M. & Carretero, C. 2007. Improvement of gelling properties of porcine blood plasma using microbial transglutaminase. *Food Chemistry*, 101(1), 49-56.
- Fort, N., Carretero, C., Parés, D., Toldrà, M. & Saguer, E. 2007. Combined treatment of porcine plasma with microbial transglutaminase and cysteine: Effects on the heat-induced gel properties. *Food Hydrocolloids*, 21(3): 463-471.
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SUMMARY

The amount of blood generated in slaughterhouses might have a major environment impact if not adequately treated. However, blood can be considered as a by-product because of the nutritive quality and functional properties of its proteins, with the valorisation possibilities being increased when blood fractions (red cell and plasma) are separated before use. Plasma shows excellent heat-induced gel properties at physiological conditions (pH 7.4), but they are gradually lost during its acidification. When plasma is acidified from pH 7.5 to pH 5.5, a ~53 % reduction in gel hardness and an ~8 % increase in released water after gel centrifugation are observed. These losses are related to changes not only in the protein interactions involved in the thermal gelation phenomena but also in protein structure. FT-IR measurements of protein secondary structure reveal the formation of non-native intermolecular β-sheet structure during thermal gelation of plasma, indicative of non-specific protein aggregation. At the end of the thermal gelation process the remaining native secondary structure diminishes as pH does, while the amount of the new-formed intermolecular β-sheet structure increases relatively. The lower the remaining native secondary structure and the faster the heat-induced aggregation with decreasing pH, the weaker and more exudative the gels obtained.

Plasma is frequently added to improve texture and syneresis properties of meat products, which usually show acid pH. So, the main objective of this thesis is to improve the heat-induced gel properties of porcine blood plasma at acid pH using microbial transglutaminase (*MTGase*), an enzyme capable of catalysing cross-linking reactions between glutamyl and lysil residues. The enzymatic treatment enhances textural properties and water holding capacity of plasma gels at pH 5.5, especially when incubated with 3 % of the enzymatic commercial product or MTG (equivalent to 43 $U \cdot g^{-1}$ of protein) for 3 h at 30 ºC and pH 7. This treatment increases ~0.4 N the hardness of gels, ~30 % higher than non-treated plasma gels at pH 5.5, and reduces ~3 % the released water after gel centrifugation respect to the control samples adjusted to pH 5.5. These improvements can be attributed to the enzymatic cross-linking of some plasma proteins, particularly globulins and fibrinogen. In spite of the importance of the achieved improvements, they are not enough to overcome loses on gelling properties of plasma proteins due to acidification.

The globular structure of the main plasma proteins can make the attack of the MTGase difficult; thus, proteins can become more reactive in front of the enzyme after suffering a partial unfolding.

Due to the abundance of disulfide bonds in plasma proteins, particularly serum albumin, the addition of a reducing agent like cysteine during the enzymatic treatment could result beneficial. However, the enzyme reactivity is not enhanced when 0.25 % cysteine is added to plasma treated with *MTGase* at the above mentioned conditions, although higher improvements on gel hardness are obtained, with increases ~0.9 – 1 N when compared to gels from untreated plasma at pH 5.5. Also, cysteine does not negatively affect the enzyme activity, despite the presence of a free –SH group in the active centre of the enzyme. The effects on gel texture of both *MTGase* and cysteine when applied together are additive more than synergistic, while the presence of the reducing agent nullifies the improvements of the water-holding capacity of plasma gels achieved by the enzyme.

High pressure is considered an alternative to provoke unfolding of plasma proteins in order to facilitate the *MTGase* activity. Treating plasma with *MTGase* (3 % MTG) under high pressure (400 MPa, 30 min, pH 7) leads to important increases of plasma gel hardness, achieving improvements of ~0.6 N relative to gels from untreated plasma at pH 5.5. However, water holding capacity is again only slightly modified. Under these conditions, the activity of *MTGase* is enhanced, and not only globulins and fibrinogen but also serum albumin are involved in the enzymatic cross-linking reactions.

The effects of treating plasma with *MTGase* under high pressure conditions can be enhanced by holding pressurised-plasma solutions at refrigeration conditions (setting time) for at least 2 h prior to its heat-induced gelation at pH 5.5. Under such conditions, increases of ~0.9 N in gel hardness compared to gels from untreated plasma at pH 5.5 are achieved. However, mechanisms other than *MTGase* polymerisation must take place during this cold storage. In contrast, the setting time has no effects on the water-holding capacity of heat-induced plasma gels at acid pH value.

Overall, it can be concluded that losses in texture of heat-induced plasma gels at acid pH are recovered to an important degree by treating plasma with *MTGase*, especially with added cysteine or under HP conditions. However, their water holding capacity is only slightly enhanced for the later.

RESUM

La quantitat de sang generada en els escorxadors pot comportar un important impacte ambiental si no es tracta adequadament. No obstant, la sang es pot considerar un subproducte degut a que les seves proteïnes presenten bones propietats nutricionals i funcionals, veient-se incrementades les possibilitats de revalorització quan les dues fraccions (cel·lular i plasmàtica) es tracten per separat. El plasma forma gels induïts per calor amb unes propietats excel·lents a pH fisiològic (pH 7,4), però aquestes propietats es van perdent gradualment a mesura que el pH s'acidifica. Una reducció de pH 7,5 a pH 5,5 del plasma comporta una disminució en la duresa del gel del ~53 % i un augment en la quantitat d'aigua alliberada després de la centrifugació del ~8 %. Aquestes pèrdues no només són degudes a canvis en les interaccions proteiques relacionades amb la gelificació per calor, també a canvis relacionats amb l'estructura proteica. Estudis de l'estructura secundària de les proteïnes amb FT-IR mostren la formació d'estructures intermolecular de làmina-β no nativa durant la gelificació tèrmica del plasma, indicativa d'agregació proteica no específica. Al final del procés de gelificació induïda per calor, la quantitat d'estructura secundària nativa disminueix a mesura que el pH ho fa, mentre que la quantitat d'estructura intermolecular de làmina-β no nativa va augmentant. Els gels obtinguts són més tous i exodatius a mesura que el pH disminueix, veient-se una disminució de la quantitat d'estructura secundària nativa i una agregació induïda per calor més ràpida.

El plasma s'utilitza freqüentment per millorar la textura i sinèresis de productes carnis, que solen presentar pH àcid. Així, l'objectiu d'aquesta tesis és millorar les propietats dels gels de plasma de sang porcina induïts per calor a pH àcid utilitzant transglutaminasa microbiana (*MTGasa*), un enzim capaç de catalitzar reaccions de polimerització entre residus de glutamina i lisina. El tractament enzimàtic millora la textura i la capacitat de retenció d'aigua dels gels a pH 5,5, especialment quan s'incuba amb 3 % de producte enzimàtic comercial o MTG (equivalent a 43 $U \cdot g^{-1}$ de proteïna) durant 3 h a 30 ºC i pH 7. Aquest tractament augmenta la duresa dels gels ~0.4 N, ~30 % superior a la duresa dels gels no tractats a pH 5,5, i redueix l'aigua alliberada després de la centrifugació ~3 % respecte a les mostres controls ajustades a pH 5,5. Aquestes millores es poden atribuir a la polimerització enzimàtica d'algunes proteïnes del plasma, especialment globulines i fibrinogen. Tot i que les millores obtingudes són importants, no són suficients per recuperar les pèrdues en les

propietats gelificants del plasma degut a la seva acidificació. L'estructura globular de les proteïnes majoritàries del plasma pot dificultar l'atac amb la *MTGasa*; així, les proteïnes poden esdevenir més reactives davant l'enzim després de ser parcialment desnaturalitzades.

Degut a que les proteïnes del plasma presenten molts ponts disulfur, especialment l'albúmina sèrica, l'addició d'un agent reductor com la cisteïna durant el tractament enzimàtic pot resultar beneficiós. No obstant, la reactivitat de l'enzim no millora quan s'afegeix 0,25 % de cisteïna a plasma tractat amb *MTGasa* a les condicions descrites anteriorment, encara que s'obtenen millores importants en la duresa dels gels, amb augments ~0,9 – 1 N respecte als gels obtinguts de plasma no tractat a pH 5,5. A més, la cisteïna no afecta negativament l'activitat enzimàtica, tot i la presència d'un grup –SH lliure en el centre actiu de l'enzim. L'efecte en la textura del gel quan es tracta conjuntament amb *MTGasa* i cisteïna és additiu més que sinèrgic, mentre que la presència de l'agent reductor anul·la la millora en la capacitat de retenció d'aigua aconseguida amb l'enzim.

L'alta pressió es considera una alternativa per desnaturalitzar les proteïnes del plasma i així facilitar l'activitat de la *MTGasa*. El tractament del plasma amb *MTGasa* (3 % MTG) sota alta pressió (400 MPa, 30 min, pH 7) comporta millores importants en la duresa dels gels, aconseguint així increments de ~0,6 N en comparació als gels control a pH 5.5. No obstant, la capacitat de retenció d'aigua només s'aconsegueix millorar lleugerament. Sota aquestes condicions, s'incrementa l'activitat de la *MTGasa*, i no només les globulines i el fibrinogen sinó també l'albúmina es veuen involucrades en la reacció de polimerització enzimàtica.

L'efecte de tractar el plasma amb *MTGasa* sota alta pressió es pot millorar mantenint les solucions de plasma pressuritzat en condicions de refrigeració almenys durant 2 h abans de la seva gelificació tèrmica a pH 5,5. Sota aquestes condicions, s'augmenta la duresa dels gels ~0.9 N comparat als gels control a pH 5,5. No obstant, durant el període de refrigeració succeeixen mecanismes diferents a la polimerització de la *MTGasa*. D'altra banda, la refrigeració no millora la capacitat de retenció d'aigua dels gels de plasma induïts per calor a pH 5,5.

En general es pot concloure que les pèrdues en la textura dels gels de plasma induïts per calor a pH àcid es poden recuperar parcialment tractant amb *MTGasa*, especialment afegint cisteïna o sota alta pressió. Encara que la seva capacitat de retenció d'aigua només es veu lleugerament millorada en el segon cas.

ABBREVIATIONS

AC	Aromatic compound
ACTIVA™	Microbial transglutaminase commercialized by Ajinomoto Food Ingredients LLC, containing 1 % of microbial transglutaminase and 99 % of maltodextrine
BSA	Bovine Serum Albumin
BSE	Bovine Spongiform Encephalopathy
DSC	Differential Scanning Calorimetry
DTT	Dithiothreitol
FA	Fatty Acids
FAO	Food and Agriculture Organization
FDA	Food and Drug Administration
HP	High Pressure
MBM	Meat and Bone Meal
MTG	ACTIVA™ or Microbial transglutaminase commercialized by Ajinomoto Food Ingredients LLC, containing 1 % of microbial transglutaminase and 99 % of maltodextrine
MTGase	Microbial Transglutaminase
PC	Phenolic compound
SEM	Scanning Electronic Microscopy
SDS-PAGE	Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis
SRMs	Specific Risk Materials
TGase	Transglutaminase
TSE	Transmissible Spongiform Encephalopathy
WHO	World Health Organization

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1. GENERAL INTRODUCTION

1.1. Food by-products: blood valorisation

The rapid world population growth results in an increasing demand for food, which runs parallel to an interest in identifying cheap and readily accessible alternatives of protein sources to those being frequently added in food formulation. For a long time, the possibilities of using whey (a protein-rich by-product of the cheese-making factories) as a food ingredient have been extensively studied because of both its nutritional and functional properties, including techno-functionality and bioactivity. Excellent reviews about it can be found in the literature (Foegeding *et al.*, 2002; Yalçin, 2006; Smithers, 2008).

However, there is a growing trend of searching new marketable protein by-product sources. In fact, many studies can be found focusing on tests for the real possibilities of protein concentrates and/or isolates as well as other derivatives coming from different food by-products or surpluses (Hsu *et al.*, 1982; Wang *et al.*, 1999; Wu, 2001; Sogi *et al.*, 2002; Rangel *et al.*, 2003; Regenstein, 2004; Chandi & Sogi, 2007; Shand *et al.*, 2007; Yu *et al.*, 2007; Hoogenkamp, 2008; Roldan *et al.*, 2008), which include blood from animal slaughtering, the main organic pollutant of abattoir effluents (Ockerman & Hansen, 1988; Pares *et al.*, 2000). Existing regulations restrict its direct dumping to wastewater treatment plants, the application of different elimination techniques like incineration being required to treat these effluents (Rendueles *et al.*, 1996). However, the good nutritional and functional properties of blood proteins (Ockerman & Hansen, 1988; Raeker & Johnson, 1995) make their recovery a feasible alternative, especially due to their use not entailing any consumer health risk if correctly handled and, in this way, contributing to sustainable development and minimizing the environmental impact (Pares & Carretero, 1997). In spite of their poor consumer acceptability, animal blood proteins could be actually cost-competitive because of the high volume generated and the easy recovery process of blood and its fractions (Satterlee *et al.*, 1973; Pearce & Kinsella, 1978; Caldironi & Ockerman,

1982; Autio & Mietsch, 1990; Ockerman & Hansen, 1994; Raeker & Johnson, 1995; Lu & Chen, 1999; Torres *et al.*, 2002; Veerman *et al.*, 2003). Bovine blood from healthy animals and its fractions have long been studied and used as food ingredients. Nowadays, there is an interest in recovering blood from other animals, particularly pigs and chickens because of the amount of these animals being slaughtered annually. However, the first recorded case of bovine spongiform encephalopathy (BSE, a type of transmissible spongiform encephalopathy or TSE) in 1986 in United Kingdom (Wells *et al.*, 1987) leds researchers to drastically diminish their interest for these topics, especially when the causal association between BSE and the new human variant of Creutzfeldt-Jakob disease was established in 1996 (Lasmezas *et al.*, 1996; Collinge *et al.*, 1996). BSE resulted from feeding cattle with scrapie-containing sheep meat and bone meal (MBM) (Wilesmith *et al.*, 1991), with the inter-species transmission occurring after applying incomplete inactivation procedures to meat by-products, especially in ruminant animals. Regulation 999/2001 and subsequent revisions (Regulations 1774/2002, 1923/2006, 828/2007 and 1069/2009) established the prohibitions concerning animal feeding and the classification of EU members and third countries into different categories depending on their TSE status to regulate the international trade.

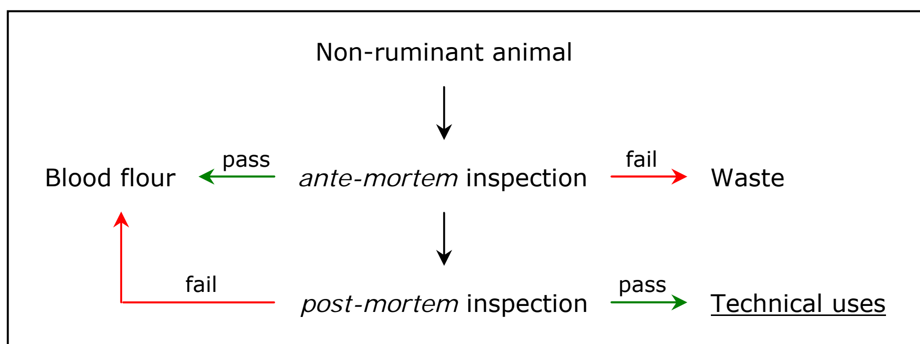

Figure 1. Possible uses of blood from non-ruminant animals in the EU

Regulations cover all animal by-products not intended for human consumption, like those used in the petfood production, as well as those for technical uses that

include blood and blood products, both of which are not considered as SRMs (Specific Risk Materials). The regulation stipulates that only materials from non-ruminant origin declared fit for human consumption following veterinary inspection and manipulated accordingly to hygienic-sanitary rules can be used for these purposes (Figure 1).

1.2. Animal blood in the food industry

Whole blood and the red cell fraction have very limited applications in the food industry due to the hemoglobin in the red cells gives an undesirable dark red colour to products, thus being only used in black pudding and blood sausages. Their potential as food ingredients could be enhanced by eliminating the heme group from hemoglobin, which is the responsible for the characteristic blood colour (Aubes-Dufau *et al.*, 1995; Yang & Lin, 1998) or, alternatively, by stabilizing the heme-iron in the reduced state to use hemoglobin as a natural red colorant (Saguer *et al.*, 2003; Salvador *et al.*, 2009). In contrast, plasma is actually used in the food industry to stabilize the water/protein/fat matrix in a large variety of comminuted meat products, such as frankfurters, bologna sausages, salami and mortadella (Sanina, 1971; Cironeanu *et al.*, 1973; Savostin, 1977; Breer, 1978; Rogov *et al.*, 1981; Nakamura *et al.*, 1983; Song *et al.*, 1984; Dolatowski, 1985; Dolatowski, 1986; Georgakis *et al.*, 1986). Moreover, many studies improving the already known plasma properties or searching for new possibilities –especially as fat or protein replacer– are being carried out for a long time (Satterlee *et al.*, 1973; Sabljak-Uglesic & Prilika, 1979; Vuono *et al.*, 1979; Torres *et al.*, 2002). Unlike other proteins used as food ingredients, plasma proteins do not show allergenic problems and cannot be regarded as foreign ingredients in formulated meat products because of the residual blood always present in meat and the similarity in protein composition between meat and blood.

1.3. Blood plasma: composition and functionality

Mammalian plasma is a complex straw-coloured fluid consisting mainly of water (92 % w/v) and high biological value-proteins (6 – 7 % w/v), with serum albumin (60 %), globulins (36 %, basically immunoglobulins) and fibrinogen (4 % w/v) being the most abundant ones. Plasma functionality is related to the intrinsic molecular properties of its main proteins, such as amino acid composition, primary sequence, structural arrangements, molecular size and shape, surface charge and hydrophobicity distribution patterns, and nature and extent of intra- and inter-molecular bonds (Kinsella, 1976; Kilara, 1984; Damodaran, 1996), with some of them being strongly affected by environmental conditions like pH, temperature and ionic strength.

For the main plasma proteins, their specific molecular characteristics are described below:

a) S*erum albumin* is a globular protein with a heart-shaped structure constituted by a polypeptide chain of 582 amino acids that has one highly flexible free sulfhydryl group in cysteine 34 (Cys^{34}), which has a relatively low pKa value compared to cysteines found in other molecules, and resulting in a high reactivity (Pedersen & Jacobsen, 1980; Clark *et al.*, 1981; Hermansson, 1982; Hermansson & Lucisano, 1982; Howell & Lawrie, 1984; Carter & Ho, 1994; Owusu-Apenten *et al.*, 2003). Moreover, the molecule is made up of three homologous domains (I, II, III), with nine loops (L1-L9) held by 17 disulphide bonds. Its secondary structure is predominantly helical (α-helix represents 55 – 68 % of its composition) with the remaining polypeptide occurring in turns and extended or flexible regions between subdomains with no or a very low amount of β-sheets (depending on the technique used or the physical state) (Carter & Ho, 1994). Its molecular weight is around 66 – 69 kDa and the isoelectric point (p*I*) is 4.8, although it is shifted to ~5.7 when fatty acids (FA)

are not bonded to the molecule (Carter & Ho, 1994). From DSC thermograms, it has been established that porcine FA-serum albumin shows a broad endothermic transition peak, with a minimum near 65 - 67 ºC and a broad shoulder between 82 - 85 ºC (Bleustein *et al.*, 2000; Davila *et al.*, 2007c).

b) *Globulins* are a vast group of proteins sub-classified into α, β and γ-globulins, which present different molecular weights (from ~40 to ~950 kDa) and p*I*s (from 2.7 to 7.6). γ-Globulins or immunoglobulins represent the most abundant ones and, although can differ structurally, they all display a Y-shaped structure composed of two light chains and two heavy chains, all basically constituted by β-sheet structure (Murray *et al.*, 2005), held together by both non-covalent interactions and disulfide bonds, but showing differences in types and/or number of interactions between molecules. They show a higher thermo-stability than serum albumin, exhibiting an endothermic transition around 75 ºC (Davila *et al.*, 2007c).

c) *Fibrinogen*, the fibrin precursor – the protein responsible for coagulation of blood –, is a hexamer containing two sets of three different chains (α, β and γ), linked to each other by disulfide bonds. In total, fibrinogen contains 29 disulfide bonds, but no free –SH groups are present (Henschen, 1964). Its molecular weight is ~340 kDa and its p*I* is ~5.5 (Putnam, 1975; Privalov & Medved, 1982; Weisel *et al.*, 1985). It melts in three different ranges of temperatures, i.e., near 59 ºC, 78 ºC and 96 ºC, with a particular thermal profile showing two sharp peaks separated by a broad transition (Davila *et al.*, 2007c). Thus, fibrinogen appears to be the most heat-sensitive of the main plasma proteins.

The ability to form heat-induced gels with good textural properties and high water-holding capacity has been established as the most important functional property of plasma. Heat-induced gelation is a complex physicochemical process

involving successive events. Nowadays, it is accepted that proteins are only partially unfolded during heating, with the molecular size of denatured proteins being just mildly modified and with both hydrophobic and sulphydryl groups initially masked in the inner core of protein flipping to the molecule surface (Catsimpoolas & Meyer, 1970a). Under appropriate conditions, these exposed functional groups promote protein-protein aggregation through both covalent and non-covalent bonds and the formation of a three-dimensional network (Damodaran, 1996), characteristics of which are strongly dependent on the involved protein-protein interactions. The gel structure is then stabilized through hydrogen bonds during the subsequent cooling step (Xiong & Kinsella, 1990). At physiological conditions (pH 7.4), the relative abundance of serum albumin in plasma, together with the reactivity of its free –SH group (Cys^{34}), seem to be very important in enhancing the formation of a well-structured network by promoting intermolecular –SH/SS interchange reactions during thermal gelation (Catsimpoolas & Meyer, 1970a; Opstvedt *et al.*, 1984; Yasuda *et al.*, 1986; Shimada & Cheftel, 1988; Lee & Hirose, 1989; Legowo *et al.*, 1996). Moreover, not only serum albumin but also immunoglobulins and fibrinogen might participate in these intermolecular cross-linking reactions. In this sense, although in the past serum albumin was considered the main protein implicated in plasma gelation (Harper *et al.*, 1978), it is actually known that individually it is not able to develop strong gels; instead, the synergistic effect with globulins is responsible for achieving the desired gelation. Also, the presence or absence of fibrinogen leads to significant changes in the heat-induced plasma gel characteristics (Davila *et al.*, 2007a). Thus, under physiological conditions, the intermolecular covalent cross-linking through disulfide bonds can be considered the essential factor in defining the three-dimensional network characteristics, although other molecular binding mechanisms like hydrophobic interactions, H-bonding, and/or electrostatic interactions forces also participate in the gel structure formation (Hermansson,

1982; Hermansson & Lucisano, 1982; Howell & Lawrie, 1984; Pares *et al.*, 1998a).

The importance of the type of protein-protein interactions participating in the gel formation has been established for a long time. However, they are strongly affected by environmental conditions like pH, protein concentration, temperature, ionic strength, type of salt and/or pressure (Totosaus *et al.*, 2002). It is known that plasma gels become softer and more exudative as pH reduces from physiological conditions to values near the p*I* of the serum albumin (~4.8) (O'Riordan *et al.*, 1989; Pares *et al.*, 1998a; Davila *et al.*, 2007a). A similar behaviour has been observed for other proteins (Matsudomi *et al.*, 1991; Doi *et al.*, 1994; Ju & Kilara, 1998), which has to a great extent been attributed to the decrease in the electrostatic repulsion and the increase in the exposition of the hydrophobic groups that lead to a high unspecific aggregation through non-covalent interactions, in this way forming random aggregates which typically result in a decrease in gelling ability (Matsudomi *et al.*, 1991; Doi *et al.*, 1994; Ju & Kilara, 1998). In contrast, at pH values far from the protein p*I*, the electrostatic repulsions allow a more ordered protein network with better gel properties to be obtained (Egelandsdal, 1980; Dalgleish & Hunt, 1995). However, pH also affects the thiol group reactivity, with a positive correlation being observed between both parameters (Kella & Kinsella, 1988; Xiong & Kinsella, 1990). Thus, the serum albumin-Cys^{34} reactivity significantly decreases at low pH (~4 – 6), limiting the intermolecular –SH/SS interchange reactions (Svenson & Carlsson, 1975; Pedersen & Jacobsen, 1980; Stewart *et al.*, 2005).

Also, these losses in gel properties as pH reduces from physiological conditions are strongly related to changes in gel microstructure, which becomes more heterogeneous and with higher porous (Figure 2). A close relationship between gel microstructure and its properties has long been established (Hermansson &

Lucisano, 1982; Verheul & Roefs, 1998). Gel microstructure depends on degree of unfolding, kinetics of the aggregation step and the type of involved interactions, all of which are strongly affected by environmental conditions (Mulvihill *et al.*, 1990; Renard & Lefebvre, 1992). In general, gels with fine-stranded microstructure are obtained from globular proteins under strong electrostatic conditions; in contrast, their microstructure becomes coarser and particulate in conditions of weak electrostatic repulsion (Clark *et al.*, 1981; Doi & Kitabatake, 1989).

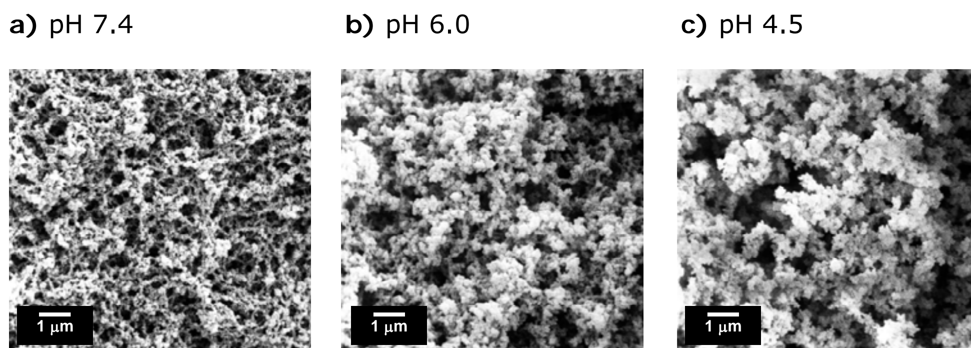

Figure 2. Scanning Electronic Microscopy (SEM) images of heat-induced plasma gel microstructure as a pH function. Source: Adapted from Pares *et al.* (1998a)

1.4. Improving of heat-induced gelation of plasma

In terms of uses of plasma in the food industries, the loss of its heat-induced gelation properties as pH is reduced from physiological conditions is particularly relevant. There is actually an interest in enhancing these properties under more realistic conditions, i.e., closer to those in the food products (Torres *et al.*, 1997; Makala, 1998; Klettner & Stiebing, 2002). Thus, different strategies have been used to improve the heat-induced gelling properties of different protein sources, which include chemical, physical and enzymatic treatments.

Chemical reactions

Several chemical reactions can be applied to modify plasma protein functionality, which are mostly based on the reactivity of the individual amino acid side chains (Table 1), as well as on the hydrolysis of the peptide bonds. However, their applications are very limited due to concerns about: cultural, legal and economic aspects; possible reversibility of the achieved modification; and consumer acceptance and safety, including toxicity, loss of nutritional value, deterioration of sensorial or functional properties, and interactions with other consumed foods (Richardson, 1985; Howell, 1996; Means & Feeney, 1998).

Table 1. Commonly modified amino acid side chains in proteins

Side Chain	Amino acid	Commonly used modifications
Amino	Lysine	Alkylation, acylation
Carboxyl	Glutamate, Aspartate	Esterification, amide formation
Disulfide	Cystine	Reduction, oxidation
Imidazole	Histidine	Oxidation, alkylation
Indole	Tryptophan	Oxidation, alkylation
Phenolic	Tyrosine	Acylation, electrophilic substitution
Sulfhydryl	Cysteine	Alkylation, oxidation
Thioether	Methionine	Alkylation, oxidation
Guanidino	Arginine	Condensation with dicarbonyls

Source: Feeney (1977)

Physical treatments

In terms of physical treatments for proteins, high pressure (HP) processing is notable, especially for these products which easily undergo thermal coagulation, like blood and its fractions. Protein molecular changes caused by the application of elevated pressure (typically in the range from 100 to 600 MPa for foods and food ingredients) are governed by the principle of Le Châtelier, which says that pressure favours reactions resulting in a volume decrease and inhibits those provoking the opposite effect (Silva *et al.*, 2001; Meersman & Heremans, 2008). Specifically, it has been shown that electrostatic and hydrophobic interactions are more pressure sensitive than hydrogen and disulfide bonds (Heremans, 1992;

Balny & Masson, 1993; Galazka & Ledward, 1998). The pressure-induced conformational changes under appropriate conditions of HP, temperature and time can allow the functionality of protein to improve or to open new possibilities of application (Totosaus *et al.*, 2002). However, changes are also dependant on protein structure, as well as other environmental factors like pH, ionic strength and protein concentration (Pares & Ledward, 2001; Boonyaratanakornkit *et al.*, 2002; Lullien-Pellerin & Balny, 2002).

Enzymatic treatments

The application of enzymatic technologies has several advantages over the use of chemicals in protein modification, especially regarding the high reaction specificity and, in many cases, the mild conditions required for the enzyme activity (Hamada, 1992). Enzymes with different mechanisms have been largely used for the modification of food proteins. Protein cross-linking enzymes are one of the most promising tools to modify protein functionality (Nio *et al.*, 1986; Nonaka *et al.*, 1989; Kuraishi *et al.*, 2001). Oxidative enzymes like lipoxygenases, protein disulfide isomerases, sulfhydryl oxidases, protein disulfide reductases, peroxidases, laccases, lysyl oxidases and tyrosinases (Table 2) from different sources can be used for such purposes. However, transglutaminases (*TGases*) are the most intensively studied and applied cross-linking enzymes in the field of food processing because of their broad application range relative to potential substrates. For a long time, the *TGase* commercialized by the Japanese company Ajinomoto Food Ingredients LLC has been the only one actually available for industrial applications in the European market. However, currently Ajinomoto's *TGase* patent has expired all over the world, and other companies are producing *TGase* for food applications, including the Yiming Biological Products Co., Ltd (previously Yiming Fine Chemicals Co., Ltd), Chemaster International Inc., Zhengzhou Longxiangtianhua Biotechnology Co., Ltd, and Shanghai Kinry Food Ingredients Co., Ltd, all from China.

Table 2. Protein cross-linking oxidative enzymes applicable in food technology

Enzyme	Catalyzed reaction	Cross-linkage type	Partici-pation	Uses	Literature
Lipoxy-genase	Lipid oxidation: peroxy free radical formation	Disulfide bond	Indirect	Improver in bread making (better mixing tolerance and loaf volume)	Frazier *et al.*, 1973; Hoseney *et al.*, 1980
Disulfide isomerase	-SH/SS interchange	Disulfide bond	Direct	Improver in baking industry	Watanabe *et al.*, 1998; van Oort, 2000
Sulfhydryl oxidase	-SH group oxidation	Disulfide bond	Direct	Improve dough's rheological properties	Haarasilta & Vaeisaenen, 1989; Vignaud *et al.*, 2002
Protein disulfide reductase	-SH/SS interchange	Disulfide bond	Direct		
Peroxi-dase	Tyrosine oxidation	Dityrosine bond	Direct/ Indirect	Improve foaming properties of ovalbumin Improver in baking industry Increase film tensile strength	Singh, 1991; Faergemand *et al.*, 1998; Michon *et al.*, 1999
Tyrosinase	Tyrosine oxidation	Dityrosine-bond Tyr-Cys bond Tyr-Lys bond	Direct	Increase gel firmness in meat products Improve emulsifying properties Cross-linking gluten proteins Cross-linking dairy proteins	Ito *et al.*, 1984; Matheis & Whitaker, 1987; Takasaki *et al.*, 2001; Thalmann & Loetzbeyer, 2002; Lantto *et al.*, 2007b; Selinheimo, 2008; Mattinen *et al.*, 2008
	Low M_w phenolic compound (PC) oxidation	$Protein_1$-PC-$Protein_2$	Indirect		
Laccase	Tyrosine oxidation	Dytirosine bond	Direct	Improve dough handling, loaf volume and crumb structure of bread Improve gel formation of meat products at high salt concentration, but decreased with high dosages Cross-linking dairy proteins Kamaboko manufacturation	Faergemand *et al.*, 1998; Figueroa-Espinoza & Rouau, 1998; Mattinen *et al.*, 2005; Mattinen *et al.*, 2006; Selinheimo, 2008
	Aromatic compound (AC) oxidation, including mono- and polyphenols	$Protein_1$-AC-$Protein_2$ Disulfide bond	Indirect		
Lysyl oxidase	Aldehyde formation from Lys	Aldehyde cross-linking	Indirect	Not yet been applied	Matheis & Whitaker, 1987

In spite of the low number of *TGase* manufacture companies, their potential applications in different areas may result in an important industrial expansion. Studies searching for new sources and for the optimization of the process, including both enzyme production and recovery (Zhu & Tramper, 2008), are being

carried out. In this regard, the relatively recent interest in solid-state fermentation assays should be particularly mentioned (Barros Soares *et al.*, 2003; Souza *et al.*, 2008; Nagy & Szakacs, 2008).

Enzymatic reactions can be favoured under pressure. In this sense, the application of *TGase* under HP has been used to enhance the enzyme-catalyzed modifications, especially in the case of globular proteins which are poor substrates for enzyme activity in their native state (Nonaka *et al.*, 1997; Gilleland *et al.*, 1997; Ashie & Lanier, 1999; Lauber *et al.*, 2001a; Lauber *et al.*, 2001b; Lauber *et al.*, 2003; Partschefeld *et al.*, 2007).

1.5. Transglutaminases (*TGases*)

Transglutaminases or *TGases* have been defined as protein-glutamine-γ-glutamyl-transferases (E.C. 2.3.2.13) that catalyse the acyl-transfer reaction between the γ-carboxylamide group of a glutamine residue and a primary amine, frequently the ε-amino group of a lysine residue, although water can also act as acyl acceptor in the absence of primary amines, resulting in the glutamine residue deamidation (Figure 3) (Ikura *et al.*, 1980a; Ikura *et al.*, 1980b; Ikura *et al.*, 1981).

However, more recently it was shown that these reactions are not the only ones carried out by *TGases*; some of them could also be able to carry out lipid esterification reactions with the formation of ester bonds between hydroxyl groups of polar lipids and glutamine side chains (Nemes *et al.*, 1999), or acting as protein disulfide isomerases (Hasegawa *et al.*, 2003).

Reactions involving protein-bound lysine residues as acyl acceptors (Figura 3A), which result in the formation of high molecular weight polymers because of the ε-(γ-glutamyl)-lysine intermolecular cross-linking (Folk & Finlayson, 1977), are

particularly interesting from the viewpoint of changes in the nutritional and techno-functional properties of food proteins.

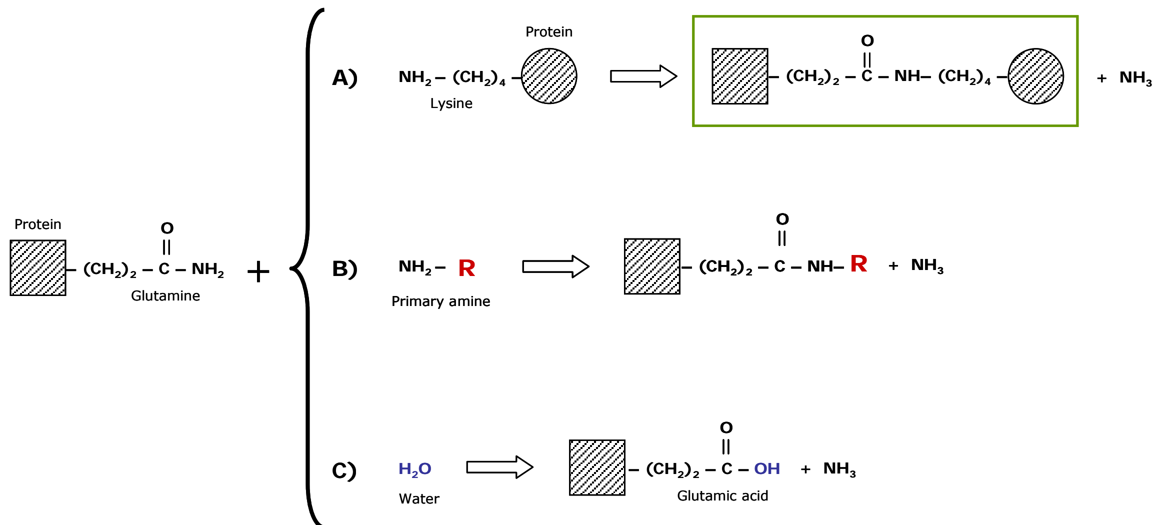

Figure 3. Reactions catalyzed by *TGases*: A) acyl-transfer reaction to a protein-bound lysine residue or cross-linking reaction; B) acyl-transfer reaction to primary amine; and C) acyl-transfer reaction to water or deamidation reaction. Source: Adapted from Folk (1980)

In fact, in the 1980s it was observed that milk caseins and soybean globulins are good substrates for *TGase* (Ikura *et al.*, 1980a; Ikura *et al.*, 1980b).

From then, many industrial applications using this enzyme have been described:

- increasing the nutritive value of foods by introducing limiting essential amino acids like lysine and methionine into proteins (Ikura *et al.*, 1981; Iwami & Yasumoto, 1986; Nonaka *et al.*, 1996), which could be cleaved by a γ-glutamylamine cyclotransferase (Fink *et al.*, 1980) and a γ-glutamyltransferase (Meister *et al.*, 2004), in spite of the resistance of glutamine-lysine bonds to gastrointestinal enzymes;

- improving the functional properties of proteins like solubility, thermal stability, emulsifying properties, water binding or gel texture (Nio *et al.*, 1986; Zhu *et al.*, 1995; Nielsen, 1995; Motoki & Seguro, 1998; Kuraishi *et al.*, 2001; de Jong & Koppelman, 2002; Yokoyama *et al.*, 2004; Jaros *et al.*, 2006b; Dube *et al.*, 2007);
- decreasing the protein allergenicity (Watanabe *et al.*, 1994);
- forming edible films (Motoki & Seguro, 1998);
- protecting lysine from Mallaird reactions (Aalami & Leelavathi, 2008; Gan *et al.*, 2009a).

Moreover, the cross-linking reaction with *TGase* is, apparently, free of undesirable side reactions producing off-flavours, bitter peptides or toxic products (Dickinson, 1997). However, the ammonia released during the *TGase* activity could participate in Maillard reactions and contribute to changes in sensorial properties (Wu & Corke, 2005).

1.5.1. *TGases*: sources and biological functions

First studies on applications of *TGase* in food industry came after the isolation of enzymes from mammalian tissues and body fluids. However, *TGases* are widely distributed among animals (including mammals, fish, birds, amphibians and some invertebrate), plants and microorganisms, fulfilling a great variety of biologic functions.

Animal transglutaminases

To date, eight different mammalian *TGases* (Factor XIII and TG1-TG7) have been characterised at genomic level, forming a group of structurally and phylogenetically related multidomain enzymes for which protein post-translational

modification activity is strictly dependent on Ca^{2+} (Beninati & Piacentini, 2004; Stenberg *et al.*, 2008). They are involved in several biological processes, including blood-clotting and wound-healing (Factor XIII); skin differentiation and epidermal keratinisation (TG1, TG3 and TG5); apoptosis, cell differentiation, intracellular signalling, cell-matrix interactions and cell migration (TG2); and semen coagulation (TG4) (Pisano *et al.*, 1968; Ichinose *et al.*, 1990; Aeschlimann & Paulsson, 1994; Nemes & Steinert, 1999; Akimov *et al.*, 2000; Piacentini *et al.*, 2000; Candi *et al.*, 2005; Mangala *et al.*, 2005; Sarang *et al.*, 2005; Arrizubieta, 2007). The physiological functions for TG6 and TG7 remain unclear. Also, they are related to several diseases like coeliac disease, thus increasing their potential as therapeutic targets or diagnostic aids (Griffin *et al.*, 2002; Gerrard & Sutton, 2005). In addition, a *TGase*-like protein with no enzymatic activity located at the red blood cell membrane and called erythrocyte band 4.2 has been also described (Korsgren *et al.*, 1990).

The guinea pig liver *TGase* –a cytoplasmatic TG2– was the first identified and purified (Clarke *et al.*, 1957; Sarkar *et al.*, 1957) and the only commercially available enzyme until the late 1980s (Folk & Cole, 1965; Folk & Cole, 1966). Although its potential use as texture-enhancer in foods was established, its scarce availability, as well as the extensive purification procedure, entailed extremely high prices on the market, resulting in a low attractiveness for potential industrial applications (Zhu *et al.*, 1995). In addition, the requirement of Ca^{2+} for its activation could entail protein precipitation in some food systems containing casein, soybean globulin or myosin (Seguro *et al.*, 1996). In the early 1970s, the research was focused on blood coagulation factors, specifically on the human Factor XIII (Chen & Doolittle, 1971). Some years later, *TGases* from pork and cattle blood were also obtained (Wilson, 1992). However, although commercially available, they have not been practically used in food industry because of the

complexity of requirements for their activation, which include thrombin (Yokoyama *et al.*, 2004).

The effects of *TGase* found in fish muscle (endogenous *TGase*) on surimi quality have been intensively studied due to its direct relationship with the setting phenomenon of salted and ground fish flesh taking place during its production (Yasueda *et al.*, 1994). Industrialized surimi-making process was developed in 1960s by Nishitani Yosuke of Japan's Hokkaido Fisheries Experiment Institute to process the increased catch of fish and to revitalize Japan's fish industry. From then, a large amount of papers have been published concerning to it as well as on the effects of adding other *TGases*. However, until now, fish *TGases* have not been used as food additives. Recently, *TGases* from different fish species have been purified and characterized (Yasueda *et al.*, 1994; Noguchi *et al.*, 2001; Hemung & Yongsawatdigul, 2008). The potential uses in food industry can be related to their ability to act at relatively low temperatures.

Other described animal *TGases* include that from chicken erythrocyte (Weraarchakul-Boonmark *et al.*, 1992) or those from several invertebrates (Myhman & Bruner-Lorand, 1970; Brozen *et al.*, 1987), although neither of them have been used in industry.

Plant transglutaminases

In plants, *TGases* have been located in different compartments such as chloroplasts, mitochondria, cell wall and cytoplasm (del Duca & Serafini-Fracassini, 2005). Their functions are related to plant growth, cell division, differentiation, programmed cell death, fertilization and stress. The first identified plant-*TGase* was this one from pea seed (Icekson & Apelbaum, 1987), but up to now *TGase*-activity has been reported at different rates in higher plants and algae (Signorioni *et al.*, 1991; Kuehn *et al.*, 1991; Kang & Cho, 1996; Serafini-

Fracassini *et al.*, 2002; Villalobos *et al.*, 2004; Della Mea *et al.*, 2004a; Della Mea *et al.*, 2004b; Carvajal-Vallejos *et al.*, 2007; Serafini-Fracassini *et al.*, 2009). The available data indicate that plant *TGases* are similar in overall structure and catalytic mechanism to those of mammals (Villalobos *et al.*, 2004), also being Ca^{2+}-dependent (Lilley *et al.*, 1998), despite the fact that it was at first proposed that their activity did not require exogenous Ca^{2+}. At present, no plant *TGases* are commercially available for industrial applications.

Microbial transglutaminases

Microbial *TGase* from *Streptoverticillium mobaraensis*, which was later classified as *Streptomyces mobaraensis* (Date *et al.*, 2004), was firstly reported by Ando *et al* (1989). Several *TGases* from strains of the genus *Streptoverticillium* have been described, all showing a high activity and obtained from bacterial fermentation. Since then, other *TGases* have been isolated from different microorganisms, including moulds like *Physarum polycephalum* (Klein *et al.*, 1992), in this case a mammalian-type *TGase* (Wada *et al.*, 2002); yeasts like *Candida albicans* (Ruiz-Herrera *et al.*, 1995); and bacteria like *Bacillus subtilis* (Kobayashi *et al.*, 1998; Suzuki *et al.*, 2000) and *Bacillus circulans* (Barros Soares *et al.*, 2003), as well as other *Streptomyces* isolates (Zhu *et al.*, 1995). As above-mentioned, the most commonly used at an industrial level is that from *Streptomyces mobaraensis*, commercialized by Ajinomoto Food Ingredients LLC (Japan) as ACTIVA™ TG through different powder formulations depending on specific applications (Ajinomoto Food Ingredients LLC, 2009).

Recombinant transglutaminases

It should be also mentioned that efforts have been made to obtain different *TGases* by genetic manipulation. Some examples include: human Factor XIIIa expressed in *Saccharomyces cerevisiae* (Bishop *et al.*, 1990); *Streptoverticillium TGase* expressed in *Escherichia coli* (Takehana *et al.*, 1994), *Streptomyces*

lividans (Washizu *et al.*, 1994) and *Corynebacterium glutamicum* (Date *et al.*, 2003); fish *TGase* expressed in *Escherichia coli* (Yasueda *et al.*, 1995); guinea pig *TGase* expressed in *Escherichia coli (Ikura et al., 1998)*; *TGase* from *Streptoverticillium ladakanum* expressed in *Streptomyces lividans* (Lin *et al.*, 2004); *TGase* gene from *Streptomyces platensis* cloned and expressed in *Streptomyces lividans* (Lin *et al.*, 2006b); or maize chloroplast *TGase* overexpressed in *Escherichia coli* (Carvajal-Vallejos *et al.*, 2007). However, none of these enzymes have been commercialized because of the lack of food regulations and consumer acceptability (Motoki & Seguro, 1998).

An interest has emerged in expressing animal *TGases* in plants due to the benefits in terms of cost, safety, productivity and convenience (del Duca & Serafini-Fracassini, 2005). For example, Claparols *et al*. (2004) used rice to express mammalian *TGase*.

1.5.2. Microbial transglutaminase from *Streptomyces mobaraensis* or *MTGase*

TGase from *Streptomyces mobaraensis* (formerly classified as *Streptoverticillium mobaraense*) and referred to as microbial transglutaminase or *MTGase*, is a monomeric protein with 331 amino acids in a simple polymeric chain (Ando *et al.*, 1989; Kanaji *et al.*, 1993), with the secondary structure consisting in eight β-strands surrounded by eleven α-helices. The enzyme adopts a disk-like shape with a deep cleft at the edge of the disk, with a cysteine residue (position 64) located in the active site, the thiol group of which is involved in the enzymatic reaction (Kashiwagi *et al.*, 2002). The molecular weight of *MTGase* has been reported to be about 40 kDa, as judged from SDS-PAGE and gel chromatography (Ando *et al.*, 1989). Mass spectrometry revealed a molar mass of 37869.2 ± 8.8 Da, which is in good agreement with the value of 37 863 Da estimated from amino acid sequence

(Kanaji *et al.*, 1993). *MTGase* shows a p*I* around 8.9, and an optimum pH range from 6.0 to 7.0, but with some residual activity at pH 4.0 and 9.0, pointing towards stability over a wide pH range (Ando *et al.*, 1989; Seguro *et al.*, 1996; Motoki & Seguro, 1998; Yokoyama *et al.*, 2004). Ando *et al*. (1989) determined an optimum temperature of 50 ºC at pH 6.0, although the enzyme retained some residual activity near freezing point. However, at 70 ºC it loses its activity within a few minutes (Seguro *et al.*, 1996; Yokoyama *et al.*, 2004). It is obtained by aerobic fermentation at 25 – 35 ºC and, because the bacteria excrete the enzyme into the cultural broth, cell disruption is not necessary, this way making the purification really easy.

According to Kashiwagi *et al.* (2002), *MTGase* is not phylogenetically related to mammalian *TGase*; the similarities between the active site structures and the differences in the overall structure between *MTGase* and the FXIII and other similar *TGases* suggest that the relationship between these enzymes is a special case of convergent molecular evolution (Arrizubieta, 2007). *MTGase* shows a novel catalytic mechanism, which confers some advantages for its industrial applications, in comparison to other *TGases* such as: Ca^{2+} independent activity (Ando *et al.*, 1989), lower molecular weight (Ohtsuka *et al.*, 2001), lower substrate specificity (De Jong *et al.*, 2001), lower deamidation activity (Ohtsuka *et al.*, 2001; Kashiwagi *et al.*, 2002), higher reaction rate (Shimba *et al.*, 2002), and catalytic cysteine residue being sufficiently exposed (Kashiwagi *et al.*, 2002). Also, no allergenic concerns of *MTGase* commercialized by Ajinomoto Food Ingredients LLC itself have been identified after applying the 2001 FAO/WHO Decision Tree (Pedersen *et al.*, 2004). To date, the FDA does not disagree with its consideration as GRAS established for an expert scientific panel (Ajinomoto Food Ingredients LLC, 2009). However, it has recently been shown that *TGase* can enhance the gluten immunogenicity through the deamidation of glutamine to

glutamic acid; for this reason, its use is not recommended in food products intended for coeliac disease patients consumption (Dekking *et al.*, 2008).

All these advantages and the potential of cross-linked proteins for developing novel foodstuffs of products with high convenience, improved sensory and nutritional-physiological properties are reflected in the apparition of many patent applications and research papers in recent years. Specific applications have been reported for dairy, seafood and fish, meat, tofu, noodles and pasta and baked goods (Table 3). Dube *et al.* (2007) reported that from 1990 to 2007 highlighted 165 relevant patent applications covering general applications of *MTGase* (34), processing of dairy (24), fish (18), meat (15), bread and bakery (17), noodles and pasta (17), soybean (24), and other vegetables or animal foods (14). Also, in 2008 and 2009, 17 new patents concerning *TGase* applications in food industry have appeared: dairy: 8; fish: 2; meat: 2; vegetables proteins: 5 (Freepatentsonline, 2009).

However, it must be considered that *MTGase* cross-linking activity depends not only on the presence of the amino acids implicated in the reaction but also on the protein structure (Dickinson & Yamamoto, 1996; Ohtsuka *et al.*, 2001). Usually, the reactive glutamine residues are in the loops and high flexible zones of the polypeptide chain, making *MTGase* react weakly with globular proteins in their native state. This poor reactivity of *MTGase* on globular proteins has been demonstrated for bovine and human serum albumin (Nonaka *et al.*, 1989); globular whey proteins (Ikura *et al.*, 1984; Nonaka *et al.*, 1989; Aboumahmoud & Savello, 1990; Traore & Meunier, 1992); and 11S pea seed globulin (Larre *et al.*, 1992). A relatively low reactivity of plasma proteins with *MTGase* could be expected because of the globular structure of its main protein (Crumpton & Wilkinson, 1963; Triantaphyllopoulos & Triantaphyllopoulos, 1967; Denko *et al.*, 1970).

Table 3. *MTGase* applications in food industry

	Product	**Effect**	**Reference**
Milk	Yogurt	Increases strength, viscosity, firmness and water binding; dry, smooth and whiter shining surface; reduces syneresis; middle taste and more palatable by the consumer	Jaros *et al.*, 2007; Ozer *et al.*, 2007; Boenisch *et al.*, 2007; Oner *et al.*, 2008; Gauche *et al.*, 2009
	Fresh cheese	Reduces syneresis and improves consistency and general appearance	Grindstaff & Chappell, 2006; Radosevic *et al.*, 2007; Gustaw *et al.*, 2008
Seafood/Fish: carp flounder hairtail kamaboko lizard mackerel pollack sardine shrimp snapper etc.	Surimi gels and restructured fish products	Improves texture and water-binding	Jirawat & Penprapha, 2005; Moreno *et al.*, 2008; Karayannakidis *et al.*, 2008b
	Low-salt restructures fish products (surimi, patties…)	Improves texture and water binding	Ramirez *et al.*, 2006; Min & Green, 2008
	Pieces of fish	Binding together to produce restructures fish	Huemer, 2005
	Packaging	Increases bloom strength of gelatine used as biomaterial-based packaging product	Jongjareonrak *et al.*, 2006; Yi *et al.*, 2006; Piotrowska *et al.*, 2008
Meat: beef pork chicken	Sausages/Comminuted meat	Improves texture and water-binding	Katayama *et al.*, 2005; Lin *et al.*, 2006a; Ahhmed *et al.*, 2007; Kawahara *et al.*, 2007; Nefedova *et al.*, 2007; Wu & Wang, 2007
	Low-salt products	Produces meat products with similar physicochemical properties to salt-containing products	Jimenez-Colmenero *et al.*, 2005
	Pieces of meat	Binding together to produce restructured meat	Huemer, 2005; Dimitrakopoulou *et al.*, 2005
	Frozen diced broiler breast	Prevention of freeze cracking	Sungtong *et al.*, 2006
Soybean	Flour	Improves functional and thermal properties	Ahn *et al.*, 2005; Hyun-Joo *et al.*, 2008
	Tofu	Improves texture, sensory properties and shelf life	Chuan-He, 2007; Chuan-He *et al.*, 2007; Yasir *et al.*, 2007
	Films	Improves mechanical and surface hydrophobic properties of cast films, used as wrapping of food products requiring a packaging allowing low gas exchange with the environment	Chuan-He *et al.*, 2005; di Pierro *et al.*, 2005; Guocheng *et al.*, 2007

	Product	Effect	Reference
Wheat	Flour	Improves functional and thermal properties Restore the functionality from insect-damaged wheat Reduction of its immunoreactivity	Ahn *et al.*, 2005; Bonet *et al.*, 2005; Leszczynska *et al.*, 2006; Hyun-Joo *et al.*, 2008
	Bread/Bakery	Gives texturised products with improved elasticity, water binding, crumb strength, firmness and heat stability Increases characteristics of deep-frozen products	Young-Hyun *et al.*, 2005; Collar *et al.*, 2005; Collar & Bollain, 2005; Dube *et al.*, 2007; Yamazaki *et al.*, 2007; Huang *et al.*, 2008; Bong-Kyung & Ng, 2008; Steffolani *et al.*, 2008
	Noodles/Pasta	Changes in properties, dry quality, cooking quality characteristics, and microstructure of cooked pasta	Basman *et al.*, 2006; Aalami & Leelavathi, 2008; Takacs *et al.*, 2008; Gan *et al.*, 2009b
Other vegetables[1]**:** barley buckwheat corn oat pea peanut rice sorghum teff etc.	Flour	Improves functional and thermal properties	Ahn *et al.*, 2005; Gharst *et al.*, 2007; Takacs *et al.*, 2007; Marcoa & Rosell, 2008
	Bread/Bakery	Improves microstructure of gluten-free bread	Moore *et al.*, 2006; Dube *et al.*, 2007; Renzetti *et al.*, 2008
	Noodles/Pasta	Improves mechanical and sensory properties of gluten-free pasta	Takacs *et al.*, 2007

[1] With the increasing demand for vegetarian foods, the application of *MTGase* for the production of plant protein-based foodstuffs as functional ingredients utilising novel proteins, e.g. from peas, lupins, sesame, and sunflower, seems promising (Dube *et al.*, 2007). Also, the production of alternative dietary source for individuals suffering from coeliac disease or wheat allergy is another promising field (Takacs *et al.*, 2007).

However, globular proteins may become more susceptible to polymerization by *MTGase* following partial unfolding, due to an increase in the accessibility of glutamine and lysine residues (Nonaka *et al.*, 1997; Gilleland *et al.*, 1997; Ashie & Lanier, 1999; Lauber *et al.*, 2001a; Lauber *et al.*, 2001b; Lee & Park, 2002; Lauber *et al.*, 2003; Menendez *et al.*, 2006). Serum albumin cross-linking by *MTGase* after partial unfolding has been observed by several authors (Chanyongvorakul *et al.*, 1997; Kang *et al.*, 2003). Protein unfolding can be achieved by modifying some extrinsic factors like temperature, pH or Ca^{2+}

content, or by disrupting S-S bonds through enzymatic treatment (adding a disulfide isomerase) or adding a reducing agent. Dithiothreitol (DTT) has shown to be very efficient in the breaking down S-S bonds (Motoki & Nio, 1983; Jocelyn, 1987; Tanimoto & Kinsella, 1988; Mahmoud & Savello, 1992; Siepaio & Meunier, 1995; Yildirim & Hettiarachchy, 1997; Faergemand *et al.*, 1997), although its use is not allowed in food applications. Alternatively, other compounds as sodium bisulfite, acid ascorbic, glutathione or cysteine have also been tested (Traore & Meunier, 1991; Jiang *et al.*, 2000; Truong *et al.*, 2004). HP has also been used to make some proteins like casein, lactoglobulin, BSA, ovalbumin, lysozyme and meat proteins, some of which normally do not react with *MTGase* at atmospheric pressure, more accessible to the active site of the enzyme. *MTGase* has been shown to be stable to pressures up to 400 MPa at room temperature (Nonaka *et al.*, 1997; Lauber *et al.*, 2001b; Lee & Park, 2002; Menendez *et al.*, 2006). Menendez *et al.* (2006) proposed that its remarkable stability under HP can be explained by the characteristics of its active site, which is located within an extended β-strand region of the protein. Regions containing β-sheet structures are nearly incompressible and more stable against HP than α-helix structures.

1.6. Objectives

The **main objective** of the studies reported in this thesis was to improve the heat-induced gel properties of porcine blood plasma at acid pH. As previously described, plasma shows good gelling capacity under physiological conditions, but this property is lost when the environment is acidified.

To achieve the main objective it is proposed:

1. to study in depth the effects of pH on the heat-induced gel properties of porcine plasma and to elucidate the structure-functionality relationship;

2. to investigate if treating porcine plasma with microbial transglutaminase (*MTGase*) enhances its heat-induced gel properties at acid pH;
3. to determine the effects of treating porcine plasma simultaneously with *MTGase* and cysteine as a reducing agent on the heat-induced gel properties at acid pH;
4. to improve the heat-induced gelation properties of porcine plasma at acid pH by treating plasma with *MTGase* under high pressure and to determine how keeping treated plasma solutions at chilled temperature can affect the heat-induced plasma gel properties.

2. STRUCTURE-FUNCTIONALITY RELATIONSHIPS OF PORCINE PLASMA PROTEINS PROBED BY FTIR SPECTROSCOPY AND TEXTURE ANALYSIS

E. Saguer, N. Fort, P.A. Alvarez, J. Sedman, A.A. Ismail. "Structure-functionality relationships of porcine plasma proteins probed by FTIR spectroscopy and texture analysis". *Food hydrocolloids*. Vol 22, issue 3 (May 2008) : p. 459-467

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Abstract

FTIR spectroscopic and texture analysis studies were undertaken to elucidate the molecular basis of structure–functionality relationships of porcine plasma proteins in solution and the gel state at varying pH. At room temperature, porcine plasma proteins aggregated as a function of decreasing pH. A parallel decrease in the intensity of amide I′ bands at 1652 cm^{-1} in the infrared spectra of the protein solution (assigned to α-helix, predominant in serum albumin) and 1688/1638 cm^{-1} (assigned to intramolecular antiparallel β-sheet, predominant in immunoglobulins) along with an increase in the band at 1644 cm^{-1} (assigned to unordered or random structure) was observed to take place with decreasing pH. Bands assigned to intermolecular antiparallel β-sheet structures (1683 and 1617 cm^{-1}) were observed in infrared spectra of porcine plasma protein solutions heated to the point of gel formation. Texture and water holding capacity were also very sensitive to pH. The results indicate that the lower the remaining native secondary structure and the faster the heat-induced aggregation (observed by FTIR spectroscopy) with decreasing pH, the weaker and more exudative the gels.

Keywords: Porcine plasma proteins; pH; Aggregation; FTIR spectroscopy; Gelling properties

3. IMPROVEMENT OF GELLING PROPERTIES OF PORCINE BLOOD PLASMA USING MICROBIAL TRANSGLUTAMINASE

E. Saguer, N. Fort, D. Parés, M. Toldrà, C. Carretero. "Improvement of gelling properties of porcine blood plasma using microbial transglutaminase". *Food chemistry*. Vol. 101, issue 1 (2007) : p. 49-56

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Abstract

The effect of microbial transglutaminase (*MTGase*) on the texture and water-holding capacity (WHC) of heat-induced gels prepared from porcine blood plasma at pH 5.5 was investigated. Different concentrations of commercial *MTGase* were added to plasma and incubated for several times under specific conditions of temperature and pH. From the results obtained, it can be postulated that enzymatic treatment enhances textural properties and WHC of plasma gels at pH 5.5, especially when incubated with 3% of the commercial product for 3 h at 30 °C and pH 7. This treatment increased by 0.4 N the hardness of gels and reduced by 3% the water released after gel centrifugation with respect to the control samples. SDS–PAGE confirmed that cross-linking took place when *MTGase* was added to plasma solutions. However, the results suggest that the sole addition of *MTGase* was not effective enough to improve the gelling properties of plasma proteins under acidic conditions.

Keywords: Porcine plasma; *MTGase*; Cross-linking; Texture; Water holding capacity

4. COMBINED TREATMENT OF PORCINE PLASMA WITH MICROBIAL TRANSGLUTAMINASE AND CYSTEINE: EFFECTS ON THE HEAT-INDUCED GEL PROPERTIES

N. Fort, C. Carretero, D. Parés, M. Toldrà, E. Saguer. "Combined treatment of porcine plasma with microbial transglutaminase and cysteine: effects on the heat-induced gel properties". *Food hydrocolloids*. Vol. 21, issue 3 (May 2007) : p. 463-471

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Abstract

The effects of the treatment of porcine plasma with microbial transglutaminase (MTGase) and cysteine on its heat-induced gelation at pH 5.5 were studied. Four different conditions, besides the control samples, were considered in this work: cysteine addition; MTGase alone treatment; MTGase and cysteine simultaneously treatment; and MTGase treatment first, adding then cysteine just before the thermal gelation. Texture (hardness, springiness and cohesiveness) and water-holding capacity (WHC) were the measured gel properties. Scanning electron microscopy images were also taken in order to evaluate the effects on gel microstructure, and differential scanning calorimetry (DSC) and SDS-polyacrylamide gel electrophoresis analysis (SDS-PAGE) analyses were carried out to get information about the implied proteins in the cross-linking reactions. The results suggest that gel properties were differently affected due to of the applied treatment. All of them improved the texture of plasma gels with respect to the control samples; however, in the case of the combined treatments the order of addition of cysteine did not affect the obtained results and their effects seemed to be additive more than synergic. The MTGase treatment was the only one capable of improving WHC. Cysteine alone did not improve this parameter but, on the contrary, the improvements achieved with MTGase were lost when treating also with cysteine. From DSC and SDS-PAGE we postulate that at least fibrinogen and globulins would be proteins participating in the MTGase reaction.

Keywords: Porcine plasma; Gelling properties; MTGase; Cysteine; Acidic conditions

5. SIMULTANEOUS APPLICATION OF MICROBIAL TRANSGLUTAMINASE AND HIGH HYDROSTATIC PRESSURE TO IMPROVE HEAT INDUCED GELATION OF PORK PLASMA

N. Fort, T.C. Lanier, P.M. Amato, C. Carretero, E. Saguer. "Simultaneous application of microbial transglutaminase and high hydrostatic pressure to improve heat induced gelation of pork plasma". *Meat science*. Vol. 80, issue 3 (November 2008) : p. 939-943

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Abstract

The effects of treating porcine plasma with microbial tranglutaminase (*MTGase*) under high hydrostatic pressure (HHP) were studied as a means of improving its gel-forming properties when subsequently heated at pH 5.5, near the pH of meats. Plasma containing varying levels of commercial *MTGase* was pressurized (400 MPa, room temperature, pH 7) for different times, and adjusted to pH 5.5 prior to heating to induce gelation. *MTGase*-treatment under HHP led to greater enhancement of heat-induced plasma gel properties as compared to control samples. The greatest improvements were achieved by pressurising plasma with 43.3 U *MTGase*/g protein for 30 min, thereby achieving recoveries of 49% and 63% in fracture force (gel strength) and fracture distance (gel deformability) of the subsequently heat-induced gels, respectively, relative to gel properties obtained by heating untreated plasma at physiological conditions (pH 7.5).

Keywords: Porcine plasma; *MTGase*; HHP; Gelling properties; Acidic conditions

6. COLD STORAGE OF PORCINE PLASMA TREATED WITH MICROBIAL TRANSGLUTAMINASE UNDER HIGH PRESSURE. EFFECTS ON ITS HEAT-INDUCED GEL PROPERTIES

N. Fort, J.P. Kerry, C. Carretero, A.L. Kelly, E. Saguer. “Cold storage of porcine plasma treated with microbial transglutaminase under high pressure. Effects on its heats-induced gel properties”. *Food chemistry*. Vol. 115, issue 2 (July 2009) : p. 602-608

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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.

Keywords: Porcine plasma; *MTGase*; HP; Setting time; Gelling properties

7. GENERAL DISCUSSION

Porcine plasma forms heat-induced gels with excellent texture and water holding capacity at pH 7.5, i.e., very close to physiological conditions (pH 7.4). At these pH conditions, the formation of intermolecular disulfide bonds through –SH/SS interchange reactions probably plays an important role in governing the mechanisms implied in the plasma thermal gelation and, consequently, in the gel properties (Catsimpoolas & Meyer, 1970b; Pedersen & Jacobsen, 1980; Opstvedt *et al.*, 1984; Shimada & Cheftel, 1988; Lee & Hirose, 1989; Legowo *et al.*, 1996; Owusu-Apenten *et al.*, 2003; Visschers & de Jongh, 2005). In this sense, disulfide bonds and free –SH groups –both implied in the -SH/SS interchange reactions– are relatively abundant in plasma proteins. Serum albumin shows 17 disulfide bonds and 1 free -SH group at Cys^{34} (Carter & Ho, 1994), while fibrinogen contains 29 disulfide bonds and no free –SH groups (Henschen, 1964). Globulin fraction exhibits changeable amounts of them. In fact, at neutral pH, albumin and globulins show a synergistic effect with regard to major physical attributes of gels, being mainly disulfide bonds but also some hydrophobic residues the interactions involved in protein aggregation. Fibrinogen also entails a positive effect on texture and water holding capacity of plasma gels; in this sense, the presence of fibrinogen increase the formation of disulfide bonds between plasma proteins (Davila *et al.*, 2007a; Davila *et al.*, 2007b).

However, gelling properties of plasma are negatively affected as pH is reduced. At pH 5.5 –typical for lots of meat products– a ~53 % reduction in gel hardness and an ~8 % increase in released water after centrifugation relative to gels at pH 7.5 are obtained (see Section 2). These losses can be partially attributed to the lower reactivity of the -SH groups at these pH conditions (Kella & Kinsella, 1988; Xiong & Kinsella, 1990), thus limiting the intermolecular –SH/SS interchange reactions, i.e., the number of covalent bonds between plasma proteins. In contrast, the electrostatic repulsion is diminished because of the proximity of the pH value to the p*I* of plasma proteins, particularly of serum albumin, and the exposition of

hydrophobic groups is increased, promoting a high non-specific aggregation through non-covalent interactions. Thus, random aggregates are formed, which typically result in a decrease in gelling ability (Matsudomi *et al.*, 1991; Doi *et al.*, 1994; Ju & Kilara, 1998; Pares *et al.*, 1998a; Davila *et al.*, 2007a). This is consistent with the fact that an increase in intermolecular β-sheet structure –related to protein aggregation– relative to the amount of remaining intramolecular structures is actually observed during heat-induced gelation by decreasing pH (see Section 2). This extensive formation of non-native intermolecular β-sheets leads to compact structures that limit proteins-water interactions. Also, interactions between different plasma protein fractions have been shown to be altered as pH reduces, being covalent bonds decreased and hydrophobic interactions increased (Davila *et al.*, 2007a). Moreover, as pH is acidified, fibrinogen has a negative effect at least on gel texture (Davila *et al.*, 2007a).

Thus, incorporating new intermolecular covalent interactions others than disulfide bonds between plasma proteins could be expected to compensate for the low participation of these last ones in the development of a protein network at acidic conditions. In fact, intermolecular cross-linking resulting from microbial transglutaminase (*MTGase*) activity improves the ability of several food proteins to form heat-induced gels (Sakamoto *et al.*, 1994; Muguruma *et al.*, 2003; Truong *et al.*, 2004; Jongjareonrak *et al.*, 2006; Jaros *et al.*, 2006b; Ahhmed *et al.*, 2007; Kawahara *et al.*, 2007; Gharst *et al.*, 2007; Boenisch *et al.*, 2007; Karayannakidis *et al.*, 2008b). However, adequate incubation conditions during the enzymatic treatment –including temperature, time, pH and enzyme concentration– must be established for each particular substrate.

For porcine plasma treated with *MTGase*, temperature is a key factor in improving its thermo-gelling ability at acid pH. According to Fort *et al*. (2004), plasma should be treated at ~30 ºC for 3 h to enhance gel properties, in spite of a >50 %

reduction in the enzyme activity at this temperature (Seguro *et al.*, 1996; Ajinomoto Food Ingredients LLC, 2009). As the temperature is increased up to the optimal for the *MTGase* activity (50 ºC, Ajinomoto Food Ingredients LLC, 2009), the beneficial effects are progressively diminished, to the point that thermal gels at pH 5.5 obtained from plasma treated with *MTGase* at 50 ºC has poorer properties than those ones from untreated plasma (Fort *et al.*, 2004).

Although no effects of temperature on protein plasma structure are expected based on their thermo-stability at the pH conditions (pH 7.0) at which the enzymatic treatment is carried out (Pares *et al.*, 1998b; Davila *et al.*, 2007b), we must bear in mind that the folding–denaturing transition in proteins is now considered as a multi-step, not as a 2-state approach where proteins have a native or denatured structure (Schellman, 1997; Lazaridis & Karplus, 2003; Scharnagl *et al.*, 2005). Thus, we hypothesize that for plasma the relatively long incubation times applied could induce little alterations in the native molecular structure even at 30 ºC, which could favour non-specific aggregation during gelation. The negative effects would increase as temperature did, so when the enzymatic treatment is carried out at temperatures above 30 ºC, the enzymatic treatment is not effective enough to overcome the effects of temperature itself.

On the other hand, although *MTGase* still retains some residual activity near freezing point (Seguro *et al.*, 1996; Yokoyama *et al.*, 2004), as temperature is reduced from the optimal for its activity, this becomes gradually slower (Ajinomoto Food Ingredients LLC, 2009), with the relationship between temperature and time needing to be established for each substrate. For plasma, similar improvements on heat-induced gel texture are achieved at 30 ºC and under refrigeration conditions, but longer incubation times are required at the latter case (3 h as much *versus* more than 4 h, respectively). However, it is important to note that water-holding capacity of *MTGase*-treated gels is only

improved at 30 ºC (see Section 3 and Section 6). Differences in the molecular processes taking place during the enzymatic treatment can be expected, especially when considering that effects of temperature itself on the gel properties are detected. In this sense, it is observed that, on hold plasma without *MTGase* at 30 ºC for 3 h, gel hardness tends to increase slightly relative to untreated plasma, although no other properties are modified (see Section 3). In contrast, when plasma is held under chilled conditions for different time periods (from 2 h to overnight), no effects on gel properties are observed (see Section 6). This also means that the effects on gel texture attributed to the enzyme activity could be, in some cases, partially due to the incubation conditions.

Surprisingly, most studies of improvement of protein functionality through treatment with *MTGase* have been carried out under a specific temperature, without taking into account the contribution of varying this parameter during enzymatic treatment. This could be especially important because of the relatively broad range of temperatures (from chilled conditions to 55 ºC) overall applied on proteins from different sources (Aluko & Yada, 1999; Babiker, 2000; Gomez-Guillen *et al.*, 2001; Siu *et al.*, 2002; Pietrasik & Li-Chan, 2002b; Jimenez-Colmenero *et al.*, 2005; Wang *et al.*, 2007; Lantto *et al.*, 2007a; Karayannakidis *et al.*, 2008a; Norziah *et al.*, 2009). However, more recently several authors are considering the importance of establishing the optimum temperature during the enzymatic treatment for each specific protein substrate (Tammatinna *et al.*, 2007; Lorenzen, 2007; Tang *et al.*, 2007; Agyare *et al.*, 2009; Castro-Briones *et al.*, 2009a; Castro-Briones *et al.*, 2009b).

In contrast, no differences in the improvements of the heat-induced plasma gel properties at acid pH are observed after its treatment with *MTGase* in the pH range 5.5 – 8.0 (Fort *et al.*, 2004). However, effects of pH have been reported on both *MTGase* activity, which optimum is at pH 6 – 7 and has an activity above 80

% between pH 5 – 9 (Ando *et al.*, 1989; Seguro *et al.*, 1996; Motoki & Seguro, 1998; Yokoyama *et al.*, 2004), and on plasma protein structure as pH is reduced from physiological conditions to acid pH (Pares *et al.*, 1998a; Davila *et al.*, 2006; Davila *et al.*, 2007a). At neutral pH, i.e., close to physiological conditions and to the optimum for the *MTGase* activity, the globular structure of most plasma proteins could make the enzymatic attack more difficult, which would compensate for the higher enzyme reactivity; as pH is either increased or reduced, a greater susceptibility of substrate to enzyme attack due to protein denaturation can be expected but, at the same time, reduced enzyme activity.

Improvements in heat-induced gel properties previously treated with *MTGase* are also highly dependent on enzyme concentration. Use of levels of *MTGase* in the range 0.1 – 300 $U \cdot g^{-1}$ of protein can be found in the literature depending on the protein substrate, but most studies used concentrations from 0.1 to 20 $U \cdot g^{-1}$ of protein (Faergemand & Murray, 1998; Jiang *et al.*, 2000; Siu *et al.*, 2002; Pietrasik & Li-Chan, 2002a; Pietrasik & Li-Chan, 2002b; Jaros *et al.*, 2007; Boenisch *et al.*, 2007; Jiang *et al.*, 2007; Guangfeng, 2007; Tang *et al.*, 2007; Agyare *et al.*, 2008; Gan *et al.*, 2008; Moreno *et al.*, 2009; Lin *et al.*, 2009; Castro-Briones *et al.*, 2009a). Also, from these studies, it can be established that low levels (0.1 – 1 $U \cdot g^{-1}$ of protein) are more often used to treat fish proteins (Jiang *et al.*, 2000; Ramirez *et al.*, 2001; Tammatinna *et al.*, 2007; Perez-Mateos & Lanier, 2007; Karayannakidis *et al.*, 2008b; Moreno *et al.*, 2009), probably due to the contribution of the endogenous transglutaminase present in the raw material (Kimura *et al.*, 1991; Kamath *et al.*, 1992), while the level of *MTGase* used for plant, meat or milk proteins varies between studies (Faergemand & Murray, 1998; Ramirez *et al.*, 2002; Siu *et al.*, 2002; Pietrasik & Li-Chan, 2002a; Pietrasik & Li-Chan, 2002b; Jaros *et al.*, 2006a; Jaros *et al.*, 2007; Yamazaki *et al.*, 2007; Boenisch *et al.*, 2007; Jiang *et al.*, 2007; Guangfeng, 2007; Mohamed & Gordon, 2007; Bonisch *et al.*, 2007; Marco *et al.*, 2007; Tang *et al.*, 2007; Oner

et al., 2008; Agyare *et al.*, 2008; Gan *et al.*, 2008; Moon *et al.*, 2009; Medina-Rodriguez *et al.*, 2009; Lin *et al.*, 2009; Castro-Briones *et al.*, 2009a).

As stated in Section 3, for porcine plasma, 43 $U \cdot g^{-1}$ of protein –the equivalent to 3 % of commercial product ACTIVA or MTG in this work– is established as the adequate concentration when the enzymatic treatment is carried out at 30 ºC for 3 h. Higher enzyme concentrations reduce the achieved improvement on heat-induced gel properties, probably because of an excess of cross-linking covalent bonds as a consequence of the *MTGase* activity, which would prevent a correct protein unfolding during the subsequent thermal gelation step and, this way, making the formation of an homogeneous protein network more difficult (Sakamoto *et al.*, 1994; Asagami *et al.*, 1995; Tsai *et al.*, 1996; Kuraishi *et al.*, 1997; Imm *et al.*, 2000; Jiang *et al.*, 2000).

A negative effect is also obtained for incubation times longer than 3 h (Fort *et al.*, 2004), which can be also attributed to an excess of covalent bonds or to more temperature-induced molecular changes taking place as incubation period is increased, which could have adverse effects for the formation of a better structured network. Other authors have observed increases in gel properties with increasing time, until reaching a constant value or a decrease when optimum time is exceeded (Soeda *et al.*, 1996; Arciszewska & Cegielka, 2003; Hwang *et al.*, 2008; Castro-Briones *et al.*, 2009a).

At the conditions above mentioned (3 % MTG / 3 h / 30 ºC / pH 7), increases of ~0.4 N in gel hardness, i.e., ~30 % higher than the control at pH 5.5, and with a slight protective effect on gel structure, as indicated by the springiness and cohesiveness values, along with reductions ~3 % (w/w) of released water after gel centrifugation are achieved when compared to control plasma adjusted to pH 5.5 (see Section 3). However, bearing in mind the texture and water-holding capacity

of the heat-induced plasma gels at pH 7.5, the improvements achieved are not enough to considerably counteract the negative effects of acidification of plasma (see Section 2 and Section 3).

From the SDS-PAGE patterns, it is clearly evident that globulins and fibrinogen participate in the *MTGase* cross-linking reactions. However, serum albumin –one of the most important proteins in thermal gelation of plasma through a synergistic behaviour with globulins (Davila *et al.*, 2007a)– seems to not participate in such reactions, at least to an appreciable degree (see Section 3). This would be in agreement with the fact that no evidences of isolated serum albumin polymerization are observed when treated with *MTGase* at the same conditions than whole plasma (unpublished data). The lack of albumin polymerisation is probably because of its globular and compact structure, making it very resistant to enzymatic attack (Traore & Meunier, 1992). However, its reactivity could be increased through its molecular unfolding before or during the enzymatic treatment.

Treating globular proteins with *MTGase* in the presence of DTT –a strong reducing agent which is able to break disulfide bonds– has been shown to be effective in enhancing the cross-linking activity of *MTGase* (Motoki & Nio, 1983; Jocelyn, 1987; Tanimoto & Kinsella, 1988; Mahmoud & Savello, 1992; Siepaio & Meunier, 1995; Yildirim & Hettiarachchy, 1997; Faergemand *et al.*, 1997). Due to the fact that use of DTT is not allowed in food applications, cysteine was chosen for our studies. Cysteine, a reducing agent with the ability to interact with free -SH groups and disulfide bonds -thus also favouring the –SH/SS interchange reactions– has been shown to be able to unfold bovine serum albumin (Boye *et al.*, 1996). However, it is important to consider that adding reagents with these abilities can alter the heat-induced gel properties (Pour-El & Swenson, 1976; Schmidt *et al.*, 1979; Yasuda *et al.*, 1986; Shimada & Cheftel, 1988; Matsudomi

et al., 1991; Legowo *et al.*, 1996; Hoffmann & Vanmil, 1997; Hongsprabhas & Barbut, 1997; Boye & Alli, 2000). Indeed, our studies provide evidence that increases in plasma gel hardness at acid pH take place as added cysteine concentration does in the range 0.05 - 0.25 %. Particularly, adding 0.25 % cysteine increases plasma gel hardness by ~0.4 – 0.5 N, ~40 % higher than the control at pH 5.5, but unfortunately without positive effects on the water-holding capacity at the tested concentration range (Saguer *et al.*, 2004). The microstructure of gels formed from plasma solutions with added cysteine, which is shown to be very heterogeneous and particulate, with large aggregates, are consistent with the effects on gel functional properties (see Section 4).

When plasma is submitted to a combined treatment with *MTGase* (3 % MTG / 3 h / 30 ºC / pH 7.0) and cysteine (0.25 %), increases on texture of plasma gels ~0.9 – 1 N with respect to the control samples at pH 5.5 are achieved. However, the observed improvements result from an additive effect more than synergic between both *MTGase* and cysteine. Such behaviour is observed not only when treating cysteine-added plasma with *MTGase* but also adding cysteine after the enzymatic treatment (see Section 4). Thus, cysteine has no effects on *MTGase* activity, neither positive nor negative, in spite of the presence of a –SH group in the active center of the enzyme. Again, globulins and fibrinogen are clearly the proteins mainly involved in the cross-linking reaction (see Section 4). The low accessibility of the disulfide bonds in serum albumin to reducing agents in the pH range of 5 – 7 (Katchalski *et al.*, 1957), and the decrease in –SH/S-S interchange reaction rate due to the pK_a of the -SH group of cysteine (between 8.5 – 9.5, depending on its location in the structure) (Swaisgood, 2005) could be related to the noted lack of effects of cysteine on the reactivity of *MTGase* towards serum albumin. In this sense, it must be noted that the –SH group reactivity depends on the protonation state of the cysteine residue, being less reactive below its pK_a, when protonated (Visschers & de Jongh, 2005).

Regardless of the positive effects of cysteine –both in the presence and absence of *MTGase*– on gel texture, the water-holding capacity is only improved after the enzymatic treatment without cysteine (see Section 4). In spite of this, from the SEM micrographs, no clear differences in the heterogeneous gel microstructure could be detected between *MTGase*-treated gels with or without cysteine (see Section 4). In the conditions carrying out the experiments, added cysteine would tend to form more easily disulfide bonds with plasma proteins, especially through its reaction with the free –SH group of serum albumin (Gabaldon, 2002), due to the fact that, at low pH, intermolecular –SH/SS interchanges reactions are limited (Mckenzie *et al.*, 1972; Monahan *et al.*, 1995). In such situations, it can be suggested that the interaction between cysteine and the -SH group of Cys^{34} of albumin would lead to a higher unspecific aggregation.

The high stability of *MTGase* to pressures up to 400 MPa at room temperature (Nonaka *et al.*, 1997; Lauber *et al.*, 2001b; Lee & Park, 2002; Menendez *et al.*, 2006; Menendez *et al.*, 2009), makes us to consider treating plasma under high pressure (HP) as an alternative way to unfold its proteins. HP is able to increase reactivity of *MTGase* towards proteins that effectively do not react at atmospheric pressure (Nonaka *et al.*, 1997; Gilleland *et al.*, 1997; Ashie & Lanier, 1999; Lauber *et al.*, 2001a; Lauber *et al.*, 2001b; Lauber *et al.*, 2003; Gomez-Guillen *et al.*, 2005; Hsieh *et al.*, 2009). Due to the fact that pressure itself can modify protein functionality (Heremans, 1992; Balny & Masson, 1993; Galazka & Ledward, 1998; Pares & Ledward, 2001; Silva *et al.*, 2001; Totosaus *et al.*, 2002; Boonyaratanakornkit *et al.*, 2002; Lullien-Pellerin & Balny, 2002; Meersman & Heremans, 2008), its effects on the heat-induced gelation ability of plasma proteins have been also studied regardless of the *MTGase* treatment. In fact, HP treatment of plasma at 400 MPa for 30 min before its heat-induced gelation at pH 5.5 lead to slightly softer gels compared to non-pressurised samples, although no

effects on water-holding capacity are observed. However, hardness tends to recover when pressurised-plasma solutions are kept at refrigeration temperatures for at least 2 h prior to their thermal gelation (see Section 6). These results seem to indicate that HP treatment can unfold plasma proteins, although these are partially refolded after releasing the pressure. Other authors have observed a similar behaviour (Pothakamury *et al.*, 1995; Royer, 2002; Tabilo-Munizaga & Barbosa-Canovas, 2004; Montero *et al.*, 2005). However, submitting plasma with 3 % MTG to pressures of 400 MPa for 30 min at pH 7.0 before heat-induced gelation at pH 5.5 lead to increases of ~0.6 N in hardness compared to control heat-induced gels at pH 5.5, with slight positive effects on water-holding capacity. Prolonging HP-treatment time or increasing enzyme concentration doesn’t improve the recovery of plasma gels achieved (see Section 6).

It is important to note that the previously mentioned improvements in *MTGase* gels treated under HP are enhanced after a holding period under refrigeration (or setting time) between pressurisation treatment and heat-induced gelation. When plasma was kept under refrigeration for 2 h, there were no effects on elasticity and cohesiveness, nor on water-holding capacity; however, improvements of ~0.9 N on hardness compared to non-treated gels at pH 5.5 are achieved (see Section 6). It has been proved that the enzymatic cross-linking activity happens only during pressurization, and the beneficial effects of keeping the treated plasma under refrigeration conditions should be attributed to other phenomena (see Section 6). On the other hand, it seems that, under these conditions, not only globulins and fibrinogen but also serum albumin are involved in the cross-linking reactions (see Section 6).

According to the results, texture of heat-induced plasma gels at acid pH can be improved when submitted to *MTGase* treatment, especially when adding cysteine or under HP conditions. However, losses in gel texture due to plasma acidification

are not completely recovered relative to untreated-plasma gels at pH 7.5. On the other hand, water holding capacity of the gels is only improved –though slightly– under HP conditions. Bearing in mind that this parameter affects product stability and quality, as well as its yield, HP treatment is, as a whole, considered the most adequate one.

The poor effects of *MTGase* treatment on gel microstructure under any of the assayed conditions are consistent with the lack of improvements obtained in the water holding capacity of plasma heat-induced gels at pH 5.5. So, future studies could be focused on finding alternatives to get gels with more ordered and structured microstructures. Different approaches can be considered in order to get better results:

- regarding *MTGase* reactivity, proteins which are highly reactive in front of the enzyme like myosin, meat gelatine, soy globulins, casein, etc. (Ajinomoto Food Ingredients LLC, 2009) could show a synergetic effect with the reactivity of plasma proteins. In this sense, Pietrasik *et al.* (2007) observed increases in both texture and water holding capacity of gels from muscle proteins when dairy proteins were added during the enzymatic treatment;
- plasma proteins could be treated with enzymes showing a cross-linking activity different from that one showed by *MTGase*. Tyrosinase, laccase or lysyl oxidase could be considered (Ito *et al.*, 1984; Matheis & Whitaker, 1987; Faergemand *et al.*, 1998; Figueroa-Espinoza & Rouau, 1998; Takasaki *et al.*, 2001; Thalmann & Loetzbeyer, 2002; Mattinen *et al.*, 2005; Mattinen *et al.*, 2006; Lantto *et al.*, 2007b; Selinheimo, 2008; Mattinen *et al.*, 2008). Different types of intermolecular interactions can confer modified characteristics to plasma heat-induced gels. Also, combinations of enzyme treatments – including or not *MTGase* – should be considered;

- mixing plasma with proteins from other sources with different characteristic. Focusing on the acid nature of the plasma proteins, basic proteins like lysozyme, clupeine, modified β-lactoglobulin or egg yolk low density lipoprotein could promote gelation through attractive electrostatic forces (Kojima & Nakamura, 1985; Poole *et al.*, 1987; Phillips *et al.*, 1989; Arntfield & Bernatsky, 1993; Yang & Choi, 1995; Friedli & Howell, 1996). Plasma gelation could also be improved by adding proteins rich in disulfide bonds and/or free -SH groups to favour –SH/SS interchange reactions. There are different proteins that could be considered like proteins from milk (whole milk, whey or isolated proteins), egg (especially ovalbumin), soy (like glycinin), etc. (Swaisgood, 2005; Visschers & de Jongh, 2005).

- combinations of the above-mentioned individual treatments should be considered, which include mixtures of different proteins and/or enzymes at different conditions (including concentration, temperature, pH, ionic strength, pressure conditions,….)

8. CONCLUSIONS

According to the studies enclosed in this thesis, it can be concluded:

1. Porcine plasma forms heat-induced gels with excellent texture and water holding capacity at pH 7.5 (very close to physiological conditions), although they are severely diminished when plasma is acidified. Specifically, decreasing pH of plasma from 7.5 to 5.5 entails reductions of ~53 % in gel hardness and increases of ~8 % in released water after gel centrifugation. Losses on gel properties are strongly related with changes in its microstructure.

2. The losses on heat-induced gel properties are related to changes in the protein structure, with a higher extent of the formation of new antiparallel intermolecular β-sheet structures relative to the amount of remaining native intramolecular structures being observed as pH of plasma is acidified.

3. The incorporation of new intermolecular covalent interactions between plasma proteins resulting from microbial transglutaminase (*MTGase*) activity prior to its thermal gelation at pH 5.5 partially counteracts the negative effects of acidification of plasma on its heat-induced gel properties. The best conditions for the enzymatic treatment are: 3 % MTG (equivalent to 43 $U \cdot g^{-1}$ of protein) for 3 h at 30 ºC and pH 7, which allows us to obtain increases of ~0.4 N in gel hardness, ~30 % higher than the untreared-plasma gels at pH 5.5, and reductions of ~3 % in released water after gel centrifugation when compared to untreated heat-induced gels at pH 5.5.

4. The thermal conditions (30 ºC for 3 h) associated with the proposed enzymatic treatment slightly increases gel hardness –but not the water-holding capacity– to values not significantly different to those obtained with the enzymatic treatment. This fact suggests that the achieved improvements in hardness after the enzymatic treatment are partially related to the associated thermal conditions.

5. *MTGase* concentrations or incubation times above the optimal have detrimental effects on the improvements achieved by the enzymatic treatment.

6. Adding cysteine to plasma as a reducing agent does not enhance the cross-linking activity of *MTGase*. Even so, the combined treatment (3 % MTG for 3 h at 30 ºC and pH 7.0 of plasma containing 0.25 % cysteine) enhances gel texture, with increases of ~0.9 – 1 N on gel hardness when compared to non cysteine-added plasma heat-induced gels at pH 5.5. This improvement results from an additive more than synergistic effect between both, *MTGase* and cysteine. However, cysteine nullifies the positive effects of *MTGase* on water-holding capacity of heat-induced plasma gels at pH 5.5.

7. Treating plasma containing *MTGase* (3 % MTG) under high pressure (400 MPa for 30 min at pH 7) increases the *MTGase* cross-linking activity, entailing improvements of ~0.6 N in hardness of heat-induced gels at pH 5.5 compared to untreated-plasma gels at pH 5.5. However, water-holding capacity is only slightly improved. Prolonging pressure treatment time or increasing enzyme concentration does not improve the recovery of plasma gels achieved.

8. Globulins and fibrinogen are the main proteins involved in the *MTGase* cross-linking reactions; albumin participates –and in a minor degree– only when the enzymatic treatment is performed under high pressure.

9. The high pressure (400 MPa for 30 min at pH 7) itself has a slightly negative effect on texture of heat-induced plasma gels at pH 5.5 but not water holding capacity, although it tends to recover after holding pressured plasma at refrigeration temperatures up to 2 h prior to the thermal gelation.

10. Holding pressurized-porcine plasma containing *MTGase* (3 % MTG / 400 MPa / 30 min / pH 7.0) for 2 h under refrigeration (setting time) enhances hardness of heat-induced gels at pH 5.5, with increases of ~0.9 N being obtained relative to gels from untreated plasma at pH 5.5. Setting time has no effect on the water-holding capacity. During setting time, other mechanisms than *MTGase* cross-linking are responsible of the improvements on texture, although the cross-linking previously achieved under high pressure has an important influence on these improvements.

According to the above mentioned conclusions resulting from the studies reported in this thesis, it can be **mainly concluded** that losses in texture of heat-induced plasma gel at acid pH are partially recovered –especially carrying out the *MTGase* treatment under high pressure (HP)–, although their water holding capacity is only slightly enhanced. The results are consistent with the slightly modification on gel microstructure achieved.

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