Influence of Alginate and Microbial Transglutaminase as binding ingredients on restructured fish muscle processed at low temperature.

Helena M. Moreno, José Carballo and A. Javier Borderías.

Departamento de Ciencia y Tecnología de la Carne y productos cárnicos y del Pescado y productos de la pesca. Instituto del Frío (CSIC), C/ José Antonio Nováis, 10, 28040 Madrid, Spain.

***Corresponding author. Tel.: +3491 5492300; Fax; +3491 5493627. E-mail address: jborderias@if.csic.es (A.J. Borderías).**

Abstract: BACKGROUND: In view of the increasing demand for fresh products in Western countries recently, there is considerable interest in commercializing restructured fish products having the appearance of fresh fish. A number of methods have been studied for the purpose of inducing cold gelification. Two of the most widely-studied methods, namely addition of alginates and addition of transglutaminases, have been studied mainly in connection with meat products. This study deals with the use of alginate and transglutaminase as additives in cold gelification of minced hake *(Merluccius capensis)* muscle. The experiments were targeting the effects of concentration and combine effects of additional additives on physicochemical characteristics and mechanical properties.

RESULTS: As regards mechanical properties, the effectiveness of sodium alginate was improved by addition of the lowest concentration of $CaCl_2$ (1g kg^{-1}), whereas the highest concentration reduced the binding ability of the alginate. The presence of

sodium caseinate (15g kg^{-1}) in combination with MTGase was important in helping to increase the work of penetration in fish gels induced at low temperature.
Examination of the chemical properties of the muscle gels showed that sodium alginate did not establish covalent protein-protein bonds, while MTGase dramatically increased the number of covalent bonds formed between adjacent muscular proteins.
CONCLUSIONS:With both ingredients, thermostable fish gels of good quality were produced at temperatures below 10 ºC. In gels induced by sodium alginate, addition of small amount (1g kg^{-1}) of $CaCl_2$ considerably improved the gel. In gels induced by MTGase hence gels better suited for the preparation of restructured products.

Key words: Sodium Alginate; Calcium Chloride; Microbial Transglutaminase; Sodium casinate; Fish Restructured products; Gelification.

INTRODUCTION

Restructured fishery products are processed from minced and/or chopped muscle, usually with added ingredients, to make products with a new appearance and texture. For some time now there have been products in the form of fingers or other shapes produced by cutting from frozen or semi-frozen fish or mince blocks, which are covered in breadcrumbs or batter then re-frozen for using as fried products. The last 30 years have seen the development of a new generation of fishery products called analogues or substitutes. Most of them are formulated essentially from surimi.

In Europe today, there is considerable demand for “fresh products”. This demand has prompted attempts to comercialise chilled restructured products based on chopped or minced raw fish muscle, presenting eating-characteristics similar to cuts from intact muscles. Also, cold-set products are more versatile in that they can be processed in

many ways, for instance as marinated products, “sushi”, carpaccio, or as a ready-to-cook fish fillet analog.

Different bindings agents are available for the manufacture of added-valued fish products from mince or small pieces of fish muscle. Each of these binding agents works in a different way as the interacction could be different depending on the kind of ingredients and the type of fish muscle involved in the process.[1]

Alginate and Microbial Transglutaminase (MTGase) are very suitable as binding ingredients for fish, interacting with muscle particles to produce thermo-stable gels at temperatures bellow 30ºC. Alginates have been used extensively in meat products but not in fish products. Alginate is a polysaccharide extracted from brown seaweed and is used mainly in the form of sodium alginate. Although alginates are widely used in food systems as stabilizers and to modify the rheology of food, one of their more interesting properties is gelation. Alginate is different from other gel forming hydrocolloids in that it forms thermo-stable gels without thermal treatment. Alginate gels are formed by intermolecular association of polyvalent cations (calcium principally) with dimerically associated guluronic acid block regions of the polysaccharide molecule.[2] It is this gel that interacts with myofibrillar proteins. Shand [3] and Montero et al. [4], reported that these interactions are mainly electrostatic, between the anionic groups on the alginate and the positively charged groups on the protein. However, there is little information about the molecular mechanism whereby the functionality of myofibrillar proteins is improved by conjugation with alginates.[1] The interaction between alginates and proteins is determined by the concentration of hydrocolloids and calcium ion sources, which form thermo-stable gels capable of binding comminuted or ground fish muscle. The proportion of sodium alginate, the calcium ion source, the setting time and the reaction time are all variables used to achieve different kinds of texture. [5]

Another ingredient that can be used as a cold binder is Microbial Transglutaminase. Transglutaminase (protein-glutamine γ-glutamyltransferase) is an enzyme capable of catalyzing acyl-transfer reactions by cross-linking introducing proteins such as myosin, peptides and primary amine [6-8] and also by forming hydrophobic interactions. [9] It is well known that MTGase catalyzes covalent bonds between the ε-amino group of lysyl residues and the γ-carboxamide group of glutaminyl residues of adjacent proteins. [10,11] The role of MTGase in catalyzing the cross-linking of myosin heavy chains has been studied, but the mechanics of the reactions is still not clear. Furthermore, little research has been done into the formation of cross-linking between the individual myofibrillar proteins. [12] As an ingredient, MTGase is an extracellular enzyme produced by several microorganisms of the *Streptoverticillium* genus, *Bacillus subtillis*, and a variant of *Streptoverticillium morbraense*, and it differs from endogenous transglutaminase in that it is calcium independent. It is active over a fairly wide range of pH (4-9) and temperatures (0-70ºC). Its activity peaks at 55ºC, although the optimal temperature for setting when it is added to cold water fish muscle is in the range 25-30ºC. [13,14] Fish muscle has an endogenous transglutaminase of its own which is calcium sensitive, and enough calcium ions should be present for the endogenous TGase to be activated and setting induced at low or moderate temperatures [9]. A number of authors have used MTGase to improve gel properties in surimi [13,15] or to restructure seafood muscle. [6,16] MTGase activity is also independent of NaCl concentration. [14,17] Uresti et al. [14] reported the possibility of achieving good textural properties with fish muscle using a very low salt level. Kuraishi et al. [17] reported that sodium caseinate combines very effectively with MTGase for cross-linking in a cool state in meat products and suggested possible means of utilizing it in seafood products. Uresti et al. [14] confirmed these data making restructured products with fish muscle. Some previous work has already been done in

our laboratory based on the above-cited research, and as a result the combination of 1g kg^{-1} microbial MTGase (10g kg^{-1} of commercial product) and 15g kg^{-1} of sodium caseinate has been determined to be one of the best for reestructuring with minced fish muscle.

The aim of this work was to determine the importance of different variables in the gelification of a minced fish muscle with a view to elaborating a raw restructured fish product. Another aim was to study the correspondence between mechanical properties and water retention of fish muscle cold-gelled with alginates and transglutaminase and the kind of physicochemical bonds formed.

MATERIALS AND METHODS

Raw materials and additives

The raw material was fillets of hake (*Merluccius capensis*) caught two-three months previously, frozen on board and stored at -20ºC.

Two kind of restructured products were studied, the first with alginate and the second with MTGase. The ingredients in the first case were sodium chloride, calcium chloride (Panreac Quimica, S.A.; Barcelona, Spain) and sodium alginate (Degussa Texturant Systems España, S.A.; Barcelona, Spain). The proportions of ingredients are shown in Table 1. In the second case, sodium chloride, sodium caseinate (Julio Criado Goméz, S.A. Madrid, Spain) and MTGase (Activa WM (990g kg^{-1} maltodextrine and 10g kg^{-1} MTGase) Ajinomoto Co.; Tokio, Japan) were used. Proportions of ingredients are shown in Table 2.

Sample preparation

Fillets of hake were minced in a meat mincer (FTS 11 Model, Van Dall SRL, Milano, Italy) with a hole size of 3 mm. The mince was then mixed with the others components in a refrigerated vacuum homogenizer (Stephan UM5, Stephan u. Söhne GmbH & Co., Hameln, Germany). The sequence of ingredient addition was, for the sample with alginate: sodium chloride, calcium chloride and sodium alginate; for the samples with MTGase: sodium chloride, sodium caseinate and MTGase dissolved in water. With each component the mixture was homogenized for 1 min. Finally, the full mixture was homogenized for 2 min. Round (diameter 96 mm) plastic plates 16 mm deep were filled with batters after mixing and vacuum-packed in plastic bags. They were then placed in a refrigerator (5ºC) overnight.

Proximate Analysis

Moisture, ash and fat in raw samples were determined in quadruplicate. [18] Crude protein content was measured in quadruplicate with a LECO FP-2000 Nitrogen Determinator (Leco Corporation, St Joseph, MI, USA).

Viscosity measurement

Apparent viscosity of muscle homogenate was measured according to Borderías et al.[19] with slight modifications, to determine the overall quality of frozen fillets. Thawed samples were homogenized with 50 g L^{-1} NaCl (1:4), pH=7 (phosphate buffer). The homogenate was filtered through gauze and centrifuged for 10 min at 345 x *g* for to remove air bubbles. Measurements were made at 12 rpm with a RV4 spindle, using a Brookfield model DV-III rheometer (Stoughton, MA) and the Rheocalc V 1.2 software system. Measurements were carried out in triplicate and results were expressed in centipoises (cP).

151

152 **Electrophoresis (SDS-PAGE)**

153 Approx. 0.05 g of muscle sample was treated with a denatured solution consisting of 5 g
154 L^{-1} 2-β-mercaptoethanol, 25 g L^{-1} sodium dodecyl sulphate (SDS), 10 mM Tris-HCl,
155 1mM ethyl-enediaminetetraacetic acid (EDTA) and 0.02 g L^{-1} bromophenol blue,
156 following Hames [20], and the final average concentration was adjusted to 2 g L^{-1} . The
157 samples were heated for 5 min at 100ºC. Electrophoresis assays were performed on a
158 PhastSystem apparatus (Pharmacia LKB Biothecnology AB, Uppsala, Sweden) using
159 12.5% polyacrylamide gels supplied by Pharmacia LKB Biotechnology AB.
160 Electrophoresis conditions were 10 mA, 250V and 3.0 W and temperature 15ºC. Protein
161 bands were stained with Coomassie brillant blue, commercialized by Pharmacia LKB
162 Biotecnology AB as "PhastGel Blue R" tablets. An aqueous solution of 300 g L^{-1}
163 methanol and 100 g L^{-1} acetic acid was used for destaining, and a solution of 50 g L^{-1}
164 glycerol and 100 g L^{-1} acetic acid as a preservative. The reference standard used for
165 molecular weights was a commercial high molecular weight (HMW) calibration kit
166 from Pharmacia LKB Biotecnology AB. The disappearing rate of the myosin heavy
167 chain band on the electrophoresis profile was analyzed with the programme ID-
168 Manager v. 2.0 (TDI S.A, Madrid, Spain).
169

170 **Water Binding capacity (WBC)**

171 A frozen sample (2 g) was cut into small pieces and placed in a centrifuge tube
172 (diameter 10 mm) with enough pipet filter (Gilson, Inc. Middenton, WI 53562 USA) as
173 absorber. The muscle was thawed in a tube and then centrifuged in a Jouan MR1812
174 centrifuge (Saint Nazaire, France) for 10 min at 3000 x *g* at room temperature. Water

175 Binding capacity (WBC) was expressed as per cent water retained per 100g water
176 present in the muscle prior to centrifuging.
177
178 **Mechanical properties**
179 Puncture Tests were carried out at room temperature (20-22 ºC). Samples (round plastic
180 plate 16 mm deep and 96 mm in diamater) were penetrated to breaking point seven
181 times at separate points. Puncturing was performed using a 5 mm diametrer cylindrical
182 stainless steel plunger attached to a 50 N cell connected to the crosshead on a TA-XT
183 plus Texture Analyzer (Texture Technologies Corp., Scarsdale, NY, USA). Breaking
184 force (N), Breaking Deformation (mm) and Work of Penetration (N mm) were
185 determinated in the force-deformation curves. Force-deformation curves were obtained
186 at 0.2 mm/s crosshead speed.
187 Texture Profile Analysis (TPA) was performed using a TA-XT plus Texture Analyzer
188 as described by Bourne. [21] Seven cores (diameter 19 mm; height 12 mm) of samples
189 were axialy compressed to 40% of their original height. Force-time deformation curves
190 were derived with a 50 N load cell applied at a crosshead speed of 0.8 mm s^{-1}.
191 Attributes were calculated as follows: hardness (Hd) = peak force (N) required for the
192 first compression; cohesiveness (Ch) = ratio of active work done under the first
193 compression curve (dimensionless); springiness (Sp) = distance (mm) the sample
194 recovers after the first compression and adhesiveness (Adh) = negative force area of the
195 first compression bite (N seg). Measurements were performed seven times at room
196 temperature (20-22 ºC).
197
198 **Protein solubility in different buffers**

The protein concentration after solubilization with different buffers was determined as an indirect way of analysing the kind of physicochemical links established among proteins due to the addition of alginate and MTGase. The protein analysis was determined in triplicate according to their solubility in 0.6 M NaCl (Solution A) and then in a 50 % (v/v) mixture of this and 8 M urea (Solution B). The protocol was as described by Fernández-Martín et al. [22]: 10 g of the sample was homogenized in an Omni-Mixer (ES Homogenizer, OMNI International INC., Gainsville, VA, USA) for 1 min at 2-4 ºC with solution A, then centrifuged (Beckman 2JMC, Fullerton, CA, USA) for 30 min at 20000 x *g* and 4 ºC. The supernatant was removed and 50 ml of solution A was added to the precipitate, which was stirred for 1 h in a cold room at 4 ºC. The sample was centrifuged again at 20000 x *g*, 4 ºC and 30 min and the two supernatants were mixed to constitute fraction 1 (S1). Solution B (50 ml) was then added to the resulting precipitate, which was homogenized (1 min, 2-4 ºC), stirred (24 h, 4 ºC) under the conditions described above and then centrifuged (20000 x *g*, 4 ºC, 30 min) also by twice. The combination of the two supernatants was fraction 2 (S2). To summarize, the following fractions were collected: salt-soluble fraction (S1), urea-soluble fraction (S2) and an insoluble residue (S3). Soluble protein concentration (%) in S1 and S2 was determined by the Lowry method. [23] According to Kauzman [24], and others, S1 is associated with proteins remaining in a native-like conformation, intermolecularly associated by electrostatic bonds; S2 is considered to be composed of aggregates intermolecularly linked by hydrophobic interactions and hydrogen bonds; and fraction S3 is considered to be composed of muscle protein aggregates linked by covalent, disulphide and other bonds and other insoluble proteins, mainly collagen.

Statistical Analysis

One way ANOVA was conducted using Statgraphics 2.1 (STSC Inc., Rockville, MD). The difference among means was analysed using a Tukey HSD test ($p<0.05$).

RESULTS AND DISCUSSION

Proximate and functional analysis of fish muscle

The major constituents of the raw sample were: moisture 80.82 ± 0.14 %, protein 16.25 ± 0.77 %, fat 1.22 ± 0.2 % and ashes 1.62 ±0.08 %. As the proximate analysis shows, the muscle had very little fat and a high proportion of protein.

The apparent viscosity determined in a muscle homogenate was 4989 cP. This correlates with the functional quality of fish muscle protein. [25,26] On the basis of these viscosity data, the quality of this frozen fish muscle may be rated between high and good. [27]

Mechanical properties of restructured products

Role of alginates

Breaking force, breaking deformation and work of penetration in samples restructured with alginate are presented in Table 3. Penetration work was significantly higher (attributed to both breaking force and deformation) in all samples with alginates than in control sample without alginate, but it was especially higher in samples in which the level of calcium chloride was lower (1g kg^{-1}) (A1 and A3). In relation to this fact, Trout [28] reported that use of a high-calcium source lowered the binding ability of the alginate. Different amounts of added alginate (0.5 and 5g kg^{-1}) did not significantly change any of the puncture test data. This finding does not agree with Clarke et al. [29], who reported that in a beef muscle-alginate restructurate, penetration force increased when binding agents increased.

249 Values of hardness, cohesiveness, springiness and adhesivenes (Table 3) were higher in
250 samples with alginate and calcium chloride than in control sample although there were
251 no significant differences among samples containing alginate. Clarke et al. [29] and
252 Devatkal and Mendiratta, [30] also reported increased hardness values when binding
253 agents were added. It is interesting to note that samples containing different levels of
254 sodium alginate but smaller proportions of calcium chloride (A1 and A3) presented
255 more work of penetration and were more adhesive. As in the case of the puncture test,
256 TPA values were the same irrespective of the proportion of alginate.
257
258
259
260 *Role of MTGase*
261 Breaking force, breaking deformation and work of penetration in samples restructured
262 with MTGase are presented in Table 4. Work of penetration was greater in samples with
263 10g kg^{-1} MTGase, due to increased breaking force but not breaking deformation. The
264 presence of sodium caseinate in combination with MTGase is important to increase
265 work of penetration in fish gels made at cold temperatures. This was reported by
266 O´Kennedy and Kelly [31] and Kuraishi et al. [17] in substrates other than fish muscle .
267 Sodium caseinate by itself, without the presence of MTGase, is also able to increase the
268 work of penetration by increasing both breaking deformation and breaking force. This
269 does not agree with Uresti et al. [14] who reported that at a similar salt concentration the
270 breaking force was higher but not the breaking deformation, but that the resulting work
271 of penetration was similar; in any event, in the present work the puncture test was
272 performed up to the breaking point while in the work of Uresti et al. [14] it was performed
273 at a fixed deformation.

Hardness values, analysed with TPA (Table 4), increased in the presence of MTGase, and much more so when MTGase was added in combination with sodium caseinate. Kuraishi et al. [17], stated that when caseinate is added with MTGase, the ε-(γ-glutamyl)lysine groups increase dramatically. In that connection, Beltrán-Lugo et al. [32] observed by light microscopy that the sodium caseinate/MTGase matrix produced a solid continuous phase with a relatively compact matrix. Sodium caseinate is able by itself to increase hardness, although not significantly. Adhesiveness was much lower when MTGase was added (both with and without sodium caseinate). Also, cohesiveness was greater in samples with added MTGase, but addition of sodium caseinate in the same sample did not change this value .

Water binding capacity (WBC)

Role of alginate

Values of water binding capacity (WBC) analysed by centrifugation in the samples with alginate are presented in Table 3. In samples with added alginate, WBC was higher when samples contained 1g kg^{-1} of calcium chloride (A1 and A3) than when they contained 10g kg^{-1} (A2 and A4). This is consistent with the fact that these samples scored higher in the puncture test.

Role of MTGase

Values of WBC are presented in Table 4. Samples with 15g kg^{-1} sodium caseinate (Mt1 and Mt3) presented better water retention values and the sample containing 10g kg^{-1} MTGase (Mt3) presented the best water retention values, which correlated with the values of work of penetration. Kuraishi et al. [33] stated that transglutaminase had considerable potential to improve WBC, as had previously been claimed by Fisher. [34]

When MTGase was not accompanied by sodium caseinate (Mt2), the gels made by cool setting presented better WBC than the control although this difference was not significant. Sodium caseinate by itself (Mt1) helped to increase water retention as compared to the control (MtC), which is also consistent with high breaking force and breaking deformation.

Chemical properties of restructured products

Role of alginates

The values of solubility in different buffers, that reflects the types of bonds formed between alginates and proteins, are presented in Figure 1a. Samples A1 and A3, with low calcium contents, presented solubility values in solutions S2 and S3 which did not differ significantly from the control sample (CA), which had no added alginate or calcium. At the same time, as reported above, these samples presented higher work of penetration and better WBC than AC, A2 and A4 (Table 3) and could therefore be considered better gels. These results suggest that the alginate established its own gel-net, which somehow enhanced the gelification of the minced muscle, but not by establishing protein-protein or protein-alginate bonds. There are some contradictions in the literature regarding the kinds of bonds formed between proteins and alginates. Some researchers have reported that ionic bonds are established between negative groups of alginates and positive groups of myofibrillar proteins and amino acids; [35] others, among them Montero et al. [4], reported some interaction between alginates and proteins after analysing the microstructure by scanning microscopy. Urdangarin et al. [1] reported that alginate was covalently attached to myosin heavy chain through the Maillard reaction between the ε-amino group of protein lysine and the reducing terminus of alginate; however, in the present work, given the storage temperatures it does not seem possible

that Maillard reactions could have been established. In previous unpublished work carried out in our laboratory, in which fresh hake (*Merluccius capensis*) muscle was restructured with added alginate, covalent protein-protein or alginate–protein bonds were established. What has probably happened in the present work, is that in the raw material the protein has aggregated to some extent as apparent viscosity values show. As a consequence the myofibrillar proteins would be poorly suitable for bonding to alginates. Solubility S2, consisting of protein molecules linked by hydrophobic interactions and hydrogen bonds, was higher in samples A2 and A4 than in the rest, while S3, consisting of protein molecules bound by covalent bonds, was lower in the same samples. This means that these were the most significant of the bonds implicated in gelification.

Role of MTGase

As regards the analysis of solubility in different buffers to determine what bonds were established in samples with added MTGase (Figure 1b), there was a clearly observable increase of Fraction S3 in samples with added MTGase (Mt2) and added MTGase/caseinate (Mt3), which means that covalent bonds were formed. Ikura, [36] Motoki and Nio [37] and others, reported that cross-linking occurs when MTGase acts on protein molecules so that ε-(γ-glutamyl)lysine crosslinks are formed; these bonds are more abundant when MTGase is accompanied by sodium caseinate. [17] The mechanical properties were consistent with the increase of S3, and hence also with the number of covalent bonds formed. When sodium caseinate was added to mince without MTGase (Mt1), S1 increased significantly, which means that the bonds, even the weak ones, were solubilized; on the other hand, puncture test values show that the work of penetration was higher. The presence of Na^+ ions probably helps to achieve better

solubilization and the formation of a more orderly net during setting, stabilized with weak bonds. Hydrophobic bonds did not change with respect to the control (MtC); in this connection Imm and Regenstin [38] likewise found no increase of hydrophobicity when casein was added to a myosin dispersion.

Electrophoretic analyses

Role of alginates

The myosin heavy chain (MHC) band decreased when different proportions of sodium alginate were added to sample (Figure 2a). In samples A1 and A3, with different proportions of alginate and a minimal proportion of $CaCl_2$, the reduction of the MHC band with respect to the control (AC) was greater (A1, 26.5% and A3, 27.2%) than in samples with more $CaCl_2$ (A2, 48.6% and A4, 35.9%) (Figure 2a). These data suggest that a smaller amount of $CaCl_2$ favours the diminishment of MHC, probably due to protein-protein or alginate-protein polymerization. This assertion is consistent with the higher insoluble fraction (S3) found in the solubility test, indicating the formation of a larger number of covalent bonds (Figure 1a). It is also consistent with a higher breaking force and breaking deformation in samples A1 and A3 than in A2 and A4. Also, the higher polymerization of samples A1 and A3 correlates with higher WBC.

Role of MTG-ase

A glance at Figure 2b shows that there was a per cent decrease in MHC bands when MTGase was added (Mt2, 54.9% and Mt3, 53.6% with respect to the control MtC). This decrease was accompanied by an increase in the number of polymer bands in the stacking gel, but not entering the resolving gel. This was also reported by Lee et al. [8] in fish samples with MTG-ase with setting at 25ºC.

The sample containing only sodium caseinate (Mt1) also presented a MHC band which was a little lower than the control (79.4%). This could be because there are some kinds of links between casein and myosin, but that is not consistent with what was found in protein solubilization (Figure 1b). Irrespective of casein/myosin bonding, it was seen earlier on that the values for mechanical properties, especially breaking deformation, were higher. The reason for this could lie in the high protein flexibility demonstrated by casein even when it has not been heated. [38]

CONCLUSIONS

Within the range of concentrations considered, mechanical properties depend more on amount of $CaCl_2$ added than on the amount of sodium alginate. Texturizing is more effective when 1g kg^{-1} is used rather than a higher amount (10g kg^{-1}).

MTGase presents more intense texturizing action when it is added to minced fish in combination with sodium caseinate and is allowed to set for about 24 hours so that more covalent bonds can be formed. Sodium caseinate by itself presents some texturizing action .

ACKNOWLEDGEMENTS

This research was supported by the Consejo Superior de Investigaciones Científicas for the Predoctoral Scholarship under program I3P-2004.

399 **REFERENCES**

400 (1) Urdangarin M, Shigeru K, Ryo S and Hiroki S, Improved solubility and stability of
401 carp myosin by conjugation with alginate oligosaccharide. *Fish Sci* **70**:896-902 (2004).

402 (2) Means WJ and Schmidt GR, Algin Calcium Gel as a Raw and Cooked Binder in
403 Structured Beef Steaks. *J Food Sci* **51**(1):60-65 (1986).

404 (3) Shand PJ, Sofos JN and Schmidt GR, Properties of Algin Calcium and Salt
405 Phosphate Structured Beef Rolls with Added Gums. *J Food Sci* **58**(6):1224-1230
406 (1993).

407 (4) Montero P, Hurtado JL and Pérez-Mateos M, Microstructural behaviour and gelling
408 characteristics of myosystem protein gels interacting with hydrocolloids. *Food*
409 *Hydrocolloids* **14**(5):455-461 (2000).

410 (5) Tolstoguzov VB, Functional properties of protein-polysaccharide mixture. In:
411 Michell JR, Ledward DA, editors. Functional properties of food macromolecules.:
412 *Elsevier Applied Science Publishers Ltd.;* p. 385-415 (1986).

413 (6) Suklim K, Flick GJ, Marcy JE, Eigel WN, Haugh CG and Granata LA, Effect of
414 cold-set binders: Alginates and microbial transglutaminase on the physical properties of
415 restructured scallops. *J Texture Stud.* **35**(6):634-642 (2004).

416 (7) Pérez-Mateos M, Solas T and Montero P, Carrageenans and alginate effects on
417 properties of combined pressure and temperature in fish mince gels. *Food*
418 *Hydrocolloids* **16**(3):225-233 (2002).

419 (8) Lee HG, Lanier TC, Hamann DD and Knopp JA, Transglutaminase effects on low
420 temperature gelation of fish protein sols. *J Food Sci* **62**(1):20-24 (1997).

(9) Park JW, Ingredient technology for surimi and surimi seafood. In: Park JW, editor. . second edition ed. FL. USA.: *CRC Press. Boca Raton.;* p. 649-707 (2005).

(10) Seki N, Nozawa H and Ni SW. Effect of transglutaminase on the gelation of heat-denatured surimi. *Fish Sci* **64**(6):959-963 (1998).

(11) Uresti RM, Velázquez G, Vázquez M, Ramírez JA and Torres JA, Effects of combining microbial transglutaminase and high pressure processing treatments on the mechanical properties of heat-induced gels prepared from arrowtooth flounder (*Atheresthes stomias*). *Food Chem* **94**(2):202-209 (2006).

(12) Nakahara C, Nozawa H and Seki N, A comparison of cross-linking of fish myofibrillar proteins by endogenous and microbial transglutaminases. *Fish Sci* **65**(1):138-144 (1999).

(13) Ramírez JA, Santos IA, Morales OG, Morrisey MT and Vázquez M, Application of Microbial Transglutaminase to improve mechanical properties of surimi from silver carp. *Sci Tech Aliment* **3**(1):21-28 (2006).

(14) Uresti RM, Téllez-Luis SJ, Ramírez JA and Vázquez M, Use of dairy proteins and microbial transglutaminase to obtain low-salt fish products from filleting waste from silver carp (*Hypophthalmichthys molitrix*). *Food Chem* **86**(2):257-262 (2004).

(15) Jiang ST, Hsieh JF, Ho ML and Chung YC, Microbial transglutaminase affects gel properties of golden threadfin-bream and pollack surimi. *J Food Sci* **65**(4):694-699 (1989).

441 (16) Vácha F, Novik I, Špička J and Podola M, Determination of the effect of microbial
442 transglutaminase on technological properties of common carp (*Cyprinus carpio L.*)
443 meat. *Czech J Anim Sci* **51**(12):535-542 (2006).

444 (17) Kuraishi C, Sakamoto J, Yamazaki K, Susa Y, Kuhara C and Soeda T, Production
445 of restructured meat using microbial transglutaminase without salt or cooking. J Food
446 Sci **62**(3):488-490 (1997).

447

448 (18) AOAC, *Association of official of Analytical Chemistry* (17th edn). Maryland
449 (2000).

450 (19) Borderías AJ, Jiménez-Colmenero F and Tejada M, Parameters Affecting Viscosity
451 as a Quality-Control for Frozen Fish. *Mar Fish Rev* **47**(4):43-45 (1985).

452 (20) Hames BD, An introduction to polyacrylamide gel electrophoresis. *Oxford: IRL*
453 *Press.;* p. 1-86 (1985).

454 (21) Bourne MC, Texture Profile Analysis. *Food Technol* **32**(7):62-70 (1978).

455 (22) Fernández-Martin F, Cofrades S, Carballo J and Jiménez-Colmenero F, Salt and
456 phosphate effects on the gelling process of pressure/heat treated pork batters. *Meat Sci*
457 **61**(1):15-23 (2002).

458 (23) Lowry OH, Rosebrough NJ, Farr AL and Randall RJ, Protein Measurement with
459 the Folin Phenol Reagent. *J Biol Chem* **193**(1):265-275 (1951).

460 (24) Kauzman W, Some factors in the interpretation of protein denaturation, in
461 *Advances in protein chemistry*, ed. by Anson ML, Edsall JT, Bailey K. Academic Press,
462 New York, pp. 1-64 (1959).

463 (25) Jiménez-Colmenero F and Borderías JA, A study of the effect of frozen storage on
464 certain functional properties of meat and fish protein. *J Food Tech* **18**:731-737 (1983).

465 (26) Kim B and Park JW, Rheology and texture properties of surimi gels, in *Surimi and*
466 *surimi sea food*, ed by Park JW, Dekker M. Taylor & Francis Group, New York,
467 (2000).

468 (27) Barroso M, Careche M and Borderías AJ, Quality control of frozen fish using
469 rheological techniques. *Trends Food Sci Technol* **9**(6):223-229 (1998).

470 (28) Trout RG, Color and bind strength of restructured pork chops:The effect of
471 Calcium carbonate and sodium alginate concentration. J Food Sci **54**(6):1466-1470
472 (1989).

473 (29) Clarke AD, Sofos JN and Schmidt GR, Effect of Algin Calcium, pH and Muscle
474 Proteins on Gelation and Microstructure of Gels and Structured Beef. *Lebensmittel-*
475 *Wissenschaft & Technologie* **21**(1):46-53 (1988).

476 (30) Devatkal S and Mendiratta SK, Use of calcium lactate with salt-phosphate and
477 alginate-calcium gels in restructured pork rolls. *Meat Sci* **58**(4):371-379 (2001).

478 (31) O'Kennedy BT and Kelly PM, Evaluation of milk protein interactions during acid
479 gelation using a simulated yoghurt model. *Milchwissenschaft* **55**(4):187-190 (2000).

480 (32) Beltrán-Lugo AI, Maeda-Martínez AN, Pacheco-Aguilar R, Nolasco-Soria HG and
481 Ocaño-Higuera VM, Physical, textural, and microstructural properties of restructured

adductor muscles of 2 scallop species using 2 cold-binding systems. J Food Sci **70**(2):E78-E84 (2005).

(33) Kuraishi C, Yamazaki K and Susa Y, Transglutaminase: Its utilization in the food industry. *Food Rev Int* **17**(2):221-246 (2001).

(34) Fisher RA, Seafood restructuring using cold-set binding technology. VSGCP-G-99-002:16 (1999).

(35) Rourke TJ, Clarke AD, Bailey ME and Hedrick HB, Ionic interactions in algin/calcium/myofibrillar protein gels. *J Muscle Foods* **8**(1):33-46 (1997).

(36) Ikura K, Kometani T, Yoshikawa M, Sasaki R and Chiba H, Crosslinking of Casein Components by Transglutaminase. *Agric Biol Chem* **44**(7):1567-1573 (1980).

(37) Motoki M and Nio N, Crosslinking between Different Food Proteins by Transglutaminase. *J Food Sci* **48**(2):561-566 (1983).

(38) Imm JY and Regenstein JM, Interaction of commercial dairy proteins and chicken breast myosin in an emulsion system. *J Food Sci* **62**(5):967-971 (1997).

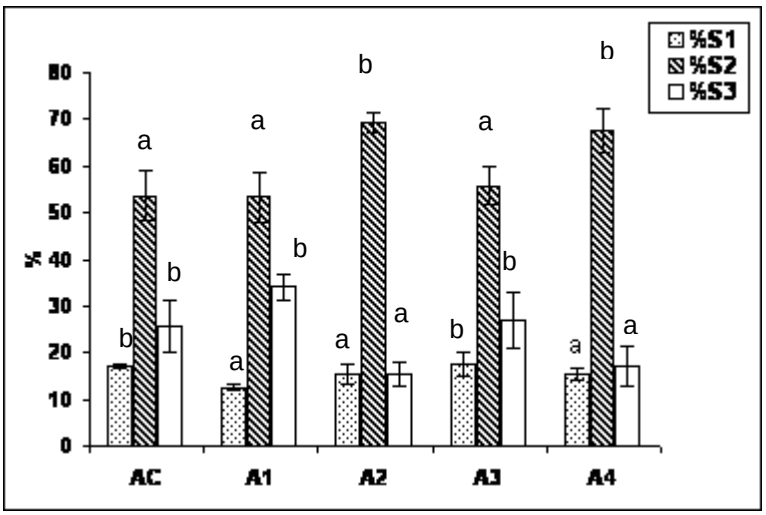

Figure 1a: % of bonds of samples with Alginate. Different letters a, b, indicate significant differences (P≤0.05).
*Sample codes in Table 1.

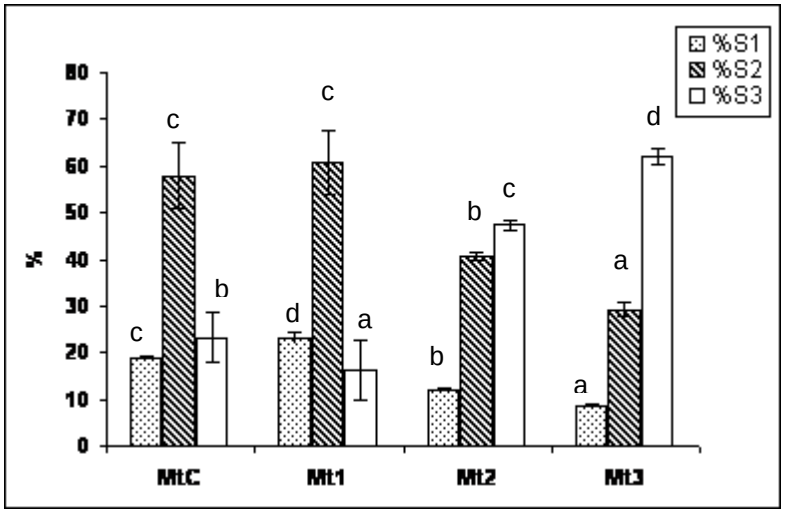

Figure 1b: % of bonds of samples with MTGase. Different letters a, b, c, etc indicate significant differences (P≤0.05).
*Sample codes in Table 2.

516

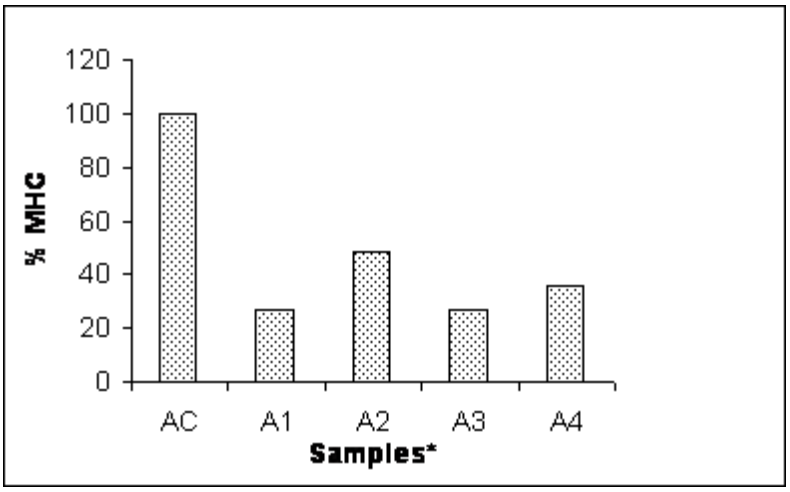

Figure 2a: % of Miosyne Heavy Change of samples with Alginate
*Sample codes in Table 1

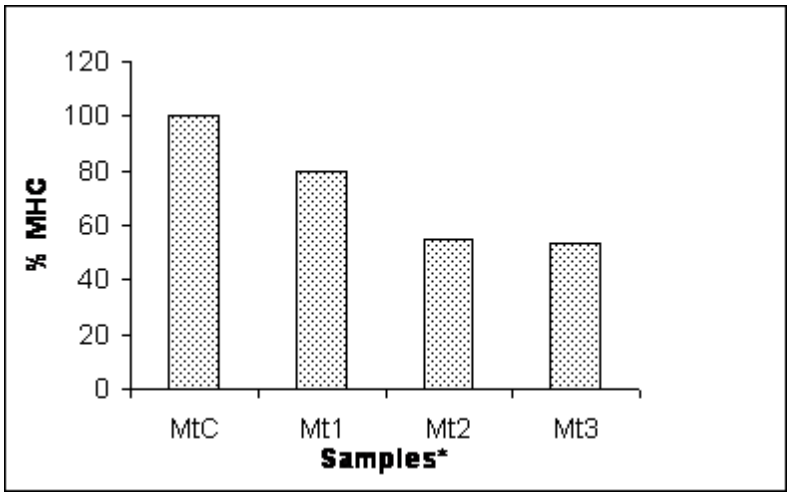

Figure 2b: % of Miosyne Heavy Change of samples with MTGase
*Sample codes in Table 2.

517

518

519

520

Table 1: Composition of samples made of sodium alginate

Samples	NaCl (g kg^{-1})	Sodium alginate (g kg^{-1})	Calcium chloride (g kg^{-1})
AC	15	0.0	0
A1	15	0.5	1
A2	15	0.5	10
A3	15	5.0	1
A4	15	5.0	10

521
522
523

524

Table 2: Composition of samples made of MTGase

Samples	NaCl (g kg^{-1})	Sodium caseinate (g kg^{-1})	MTGase (g kg^{-1})	H_20 (g kg^{-1})
MtC	15	0	0	50
Mt1	15	15	0	50
Mt2	15	0	10	50
Mt3	15	15	10	50

525

Table 3: Mechanical properties (TPA and Puncture Test) and WBC of restructured product made of Sodium Alginate

Samples*	Hardness (N)	Springeness (mm)	Cohesiveness	Adhesiveness (N seg^{-1})**	Breaking Force (N)	Breaking Deformation (mm)	Work Penetration (N mm)	WBC (%)
AC	5.66 ± 0.76^{a}	3.56 ± 0.02^{a}	0.63 ± 0.01^{a}	0.004 ± 0.05^{a}	0.46 ± 0.04^{a}	5.08 ± 0.19^{a}	2.37 ± 0.26^{a}	85.96 ± 1.65^{b}
A1	7.48 ± 0.54^{b}	4.30 ± 0.08^{b}	0.75 ± 0.01^{b}	0.011 ± 0.22^{c}	1.20 ± 0.10^{d}	6.17 ± 0.24^{c}	7.40 ± 0.83^{d}	92.06 ± 2.00^{c}
A2	7.00 ± 0.90^{b}	4.35 ± 0.05^{b}	0.75 ± 0.01^{b}	0.009 ± 0.10^{c}	0.88 ± 0.08^{bc}	5.55 ± 0.16^{ab}	4.90 ± 0.53^{bc}	77.78 ± 2.95^{a}
A3	7.08 ± 0.43^{b}	4.32 ± 0.02^{b}	0.75 ± 0.01^{b}	0.014 ± 0.10^{b}	1.07 ± 0.10^{cd}	5.99 ± 0.34^{bc}	6.44 ± 0.87^{cd}	87.87 ± 1.41^{c}
A4	6.64 ± 0.36^{b}	4.38 ± 0.05^{b}	0.76 ± 0.02^{b}	0.009 ± 0.04^{c}	0.75 ± 0.04^{b}	5.39 ± 0.16^{a}	4.05 ± 0.34^{ab}	84.18 ± 1.72^{b}

Different letters a, b, c, indicate significant differences (P≤0.05).

* Sample codes in Table 1. ** Absolute values.

526
527

528

Table 4: Mechanical properties (TPA and Puncture Test) and WBC of restructured product made of MTGase

Samples*	Hardness (N)	Springeness (mm)	Cohesiveness	Adhesiveness (N seg^{-1})**	Breaking Force (N)	Breaking Deformation (mm)	Work Penetration (N mm)	WBC (%)
MtC	4.43 ± 0.77^{a}	4.26 ± 0.03^{a}	0.70 ± 0.01^{a}	0.014 ± 0.07^{a}	0.44 ± 0.055^{a}	4.12 ± 0.27^{a}	1.82 ± 0.27^{a}	83.78 ± 1.70^{a}
Mt1	5.12 ± 0.64^{a}	4.21 ± 0.14^{a}	0.72 ± 0.02^{a}	0.014 ± 0.13^{a}	0.69 ± 0.11^{b}	5.68 ± 0.93^{b}	3.95 ± 1.37^{b}	89.39 ± 1.02^{b}
Mt2	12.65 ± 0.52^{b}	4.26 ± 0.04^{a}	0.79 ± 0.01^{b}	0.005 ± 0.06^{b}	1.44 ± 0.12^{c}	5.08 ± 0.36^{b}	7.35 ± 1.15^{c}	85.88 ± 1.18^{a}
Mt3	13.66 ± 1.08^{c}	4.33 ± 0.11^{a}	0.79 ± 0.03^{b}	0.007 ± 0.39^{a}	1.91 ± 0.19^{c}	5.57 ± 0.33^{b}	10.66 ± 1.64^{d}	94.22 ± 0.67^{c}

Different letters a, b, c, etc indicate significant differences (P≤0.05).
*Sample codes in Table 2. **Absolute values.

529
530

531