ADDITION OF MICROBIAL TRANSGLUTAMINASE AND PROTEASE INHIBITORS TO IMPROVE GEL PROPERTIES OF FROZEN SQUID MUSCLE.

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ABSTRACT

Thermal gelation profiles of batters from newly frozen and long-term (five months) frozen squid with added protease inhibitors, microbial transglutaminase (MTG) or a combination of the two, revealed notable differences as a consequence of frozen storage, attributed in part to decreased autolytic activity in the muscle. Puncture and compression-relaxation tests in gels revealed that the incorporation of protease inhibitors in addition to MTG considerably improved gel elasticity; however, MTG alone produced higher breaking deformation than in other samples. SDS-PAGE showed a higher prevalence of covalent cross-linking in gels containing MTG, where MHC and paramyosin were mainly involved. The combination of protease inhibitors and MTG produced the hardest gels, which achieved the maximum score in the folding test.

Keywords: gels, squid, proteolytic activity, inhibitors, microbial transglutaminase, frozen storage.

INTRODUCTION

Squid represents an abundant source of marine protein, with many species of varying commercial value. The advantages of cephalopod muscle include high post-processing yield and very low fat content, a bland flavour and very white flesh. Although achieving increasing consumer acceptance, it is still unappreciated as such in many areas. Therefore, transformation into gel based products may be a means of increasing its consumption. However, gels from squid muscle are weak and brittle, with low gel strength and low water holding capacity [1]. Combined addition of gelling ingredients such as non-muscle proteins and hydrocolloids has been reported to improve gel forming in giant squid *Dosidicus gigas* [2].

One of the reasons most frequently adduced for the poor gel-forming ability of cephalopod muscle is its rate of proteolytic activity, which is much higher than that of most fish species [3,4]. The addition of protease inhibitors to surimi or minced fish has proved successful in fish species with weak gel-forming ability or slow setting properties. Examples include carp [5,6], chum salmon [7] and highly parasited Pacific whiting [8]).

On the other hand, cephalopod muscle contains little endogenous transglutaminase activity [9]. The use of a microbial transglutaminase (MTG) isolated from microorganisms of the genus *Streptoverticilium* is another possible means of improving gelation, which has been extensively studied in the last decade [10; 11; 12; 13;14]. The enzyme catalyses an acyl transfer reaction between the γ-carboxamide group of glutamine residues and the ε-amino group of lysine residues of peptide chains [15]. This reaction produces extensive covalent cross-linking, chiefly involving myosin heavy chains (MHC) [13]. Moreover, such covalent bonding has been shown to enhance rubber elastic behaviour in fish gels [14]. The effect of added MTG has been found to be highly dependent on the fish species and the quality of the raw fish [12]. Recent studies have reported that protease inhibitors and microbial transglutaminase can be combined to advantage in fish species with poor gel-forming ability [5; 16].

In a previous study we observed that the addition of different protease inhibitors increased the elastic modulus (G') in the thermal gelation profile of

Loligo vulgaris muscle [17]. The object of the present work was to examine the rheological and chemical properties of squid gels with added protease inhibitors, microbial transglutaminase and a combination of the two, while also evaluating the effect of long-term frozen storage on gelling properties of squid muscle with these ingredients.

MATERIALS AND METHODS

Fresh (from 18 to 24 h in ice after capture) squids (*Loligo vulgaris*) were gutted and skinned. Tentacles were removed and mantles were cut into small pieces (about 3 x 3 cm^2) ready for homogenization. The pieces were placed in polyethylene bags in batches of 400 g, frozen and stored at –50ºC .

NaCl and Na-pyrophosphate (PPi) were supplied by PANREAC (Montplet & Esteban S.A., Barcelona). Iodoacetic acid, phenylmethylsulphonyl fluoride (PMSF) and pepstatin were supplied by Sigma Chem. Co. (St. Louis, MO). A commercial microbial transglutaminase (1 % transglutaminase + 99 % maltodextrin) (ACTIVA® WM, Ajinomoto Europe Sales Gmbh, Hamburg) was used. All other chemicals used were of reagent grade.

Preparation of sample

Squid muscle batches of 400 g were thawed and homogenized in a Stephan machine (Model UM5; Stephan und Söhne GmbH & Co., Hameln, Germany) at 5 ºC for 5 min, with 1% NaCl and 80% moisture.

A mixture of inhibitors was added to squid batter to inhibit all four types of endoproteases, i.e. cysteine, serine, metallo and aspartic proteases. The respective inhibitors were: IAA (0.2 mg/g), PMSF (1 mg/g), PPi (5 mg/g) and pepstatin (0.1 mg/g), in the amounts normally used to produce inhibition. The microbial transglutaminase was added in a proportion of 2% ACTIVA (0.02% TGasa). Different 100 g aliquots of squid muscle sols were properly homogenized in a mortar with the corresponding compounds or mix of compounds. Also, the lot containing the muscle sol without ingredients was homogenized in a mortar for comparison with the others. Before analyses, all squid batters were matured overnight at 7ºC to ensure that the added chemicals were active.

Autolytic activity measurement

The enzyme extracts were prepared by homogenizing 50 g of newly made squid batter (without added inhibitors) with 150 mL of 0.2 M phosphate buffer pH 7.0 in an Omni mixer model 17106 (OMNI International, Waterbury, USA) cooled with crushed ice. Autolytic activity was measured at 25, 35, 40 and 50ºC, according to the method of Morrisey et al [8] as modified by Ayensa et al [18]. The activity was expressed as μmol of tyrosine released per gram of muscle per hour of incubation. Results are averages of at least four determinations.

Preparation of squid gels

The newly made batters were stuffed into stainless steel cylinders (inner diameter 3 cm, height 3 cm) with screw-on lids and rubber gaskets to provide a hermetic seal. At no time during this part of the process did sample temperature exceed 10ºC. Samples were heated at 90ºC by immersion in a water bath for one hour. Immediately after heating, the cylinders were placed in recipients containing ice water for rapid cooling of the gel. They were then stored in a cold room at 4ºC for 16h before analysis.

Rheological analyses

Dynamic small strain deformation studies were performed on a Bohlin CSR-10 rheometer rotary viscometer (Bohlin Instruments Ltd., Gloucestershire, UK) using a cone-plate geometry (cone angle 4º, gap=1,50 mm). Heating from 7ºC to 80ºC was performed at a scan rate of 1ºC/min, frequency 1 Hz, and target strain 0.02 mm. The elastic modulus (G'; Pa) was plotted as a function of temperature. Results were averages of at least two determinations.

The folding test was carried out according to a 5-point grade system [19]. The puncture test (cross-head speed 10 mm/min; 100-N load cell) and compression-relaxation test (cross-head speed 50 mm/min; 5 kN load cell; compression to 50% of height) were carried out as described by Gómez-Guillén et al [20].

Gel solubility

Two grams of chopped gel was homogenized with 10 mL of an extracting solution composed by 0.8 M NaCl, 8M urea, 2% β-mercaptoethanol and 20mM Tris-HCl (pH 8.0), in an Omni-mixer, Model 17106 homogenizer (OMNI International, Waterbury, CT) for 2 min. The resulting homogenates were stirred at 4-5ºC for 1 h and then centrifuged for 15 min at 20000*g* in a Cryofuge 20-3 centrifuge (Heraeus CHRIST GmbH, Germany). The protein concentration in the supernatant was determined following the method of Lowry et al. [21]. Results are average of three determinations and are expressed as percent protein solubilized with respect to total protein in the gel, which was previously determined by the Kjehldahl method.

SDS-Polyacrylamide gel electrophoresis (SDS-PAGE)

The supernatant was treated with a solution composed of (2.5 % SDS, 5 % mercaptoethanol, 10 mM Tris HCl, 1 mM EDTA and 0.002 % bromophenol blue), adjusted to a final average concentration of 1 mg/mL and then heated for 5 min in a boiling water bath.

Samples were analysed by SDS-PAGE in a SE 250 Mighty Small II Vertical Unit (Pharmacia LKB Biotechnology, Uppsala, Sweden) using 5 % polyacrylamide gels for stacking and 10 % polyacrylamide gels for resolving. Electrophoresis conditions were 20 mA/gel and 250 V. The protein bands were stained with Coomassie Brilliant Blue R-350 (Phast Gel™ Blue R tablets, Pharmacia LKB Biotechnology). As reference for molecular weights, two standard high molecular weight reference kits (Pharmacia LKB Biotechnology) were used. One was composed of thyroglobulin 330 kDa, ferritin half unit 220 kDa, albumin 67 kDa, catalase subunit 60 kDa, lactate dehydrogenase subunit 36 kDa and ferritin subunit 18.5 kDa. The other was composed of myosin 212 kDa, α_2-macroglobulin 170 kDa, β-galactosidase 116 kDa, transferrin 76 kDa and glutaminc dehydrogenase 53 kDa.

Statistical analysis

One-way analysis of variance was carried out. The computer program used was SPSS® (SPSS Inc., Chicago, Ill., U.S.A.). The difference of means between

pairs was resolved by confidence intervals using a Tukey test. The level of significance was set for $P<0.05$.

RESULTS AND DISCUSSION

Given that autolysis of squid muscle may be highly influenced by long-term frozen storage, autolytic activity of squid mantle after 2 days and 5 months of frozen storage was determined at different incubation temperatures, respectively (Fig. 1). Autolytic activity immediately after freezing was considerably greater than after 5 months of frozen storage in the whole range of temperatures assayed. In a previous study we observed that the peak at 40ºC was associated to a strong serine-proteinase activity in this species [17]. From the results shown in the present work, a big part of such activity has been lost as a consequence of frozen storage, and this may influence substantially the thermal gelation properties of squid mantle. Nevertheless, it shoud not be ignored that autolytic activity levels after 5 months of frozen storage still remained at the level described for many fresh fish species [4].

Fig. 2 shows the gelation profiles of squid batters made from frozen mantles stored for 2 days and 5 months respectively, with and without added protease inhibitors and/or microbial transglutaminase (MTG). The batter without ingredients showed very poor gel-forming ability in both cases; however, absolute values of elastic modulus (G') throughout the temperature range were higher after 5 months of frozen storage than immediately after freezing. As shown in Fig. 1, these differences may be due in part to a considerable reduction in proteolytic activity of the muscle induced by frozen storage, with a consequent reduction of proteolytic degradation of the batter during overnight maturation. If this difference in gelation behaviour were dependent solely on the level of proteolytic activity, the result of using protease inhibitors would always be similar regardless of the proteolytic activity in the original muscle. However, the addition of protease inhibitors led to an increase in G' values which was much more pronounced in the newly frozen squid than in the long term frozen squid. Any loss of functionality of the squid muscle through aggregation of myofibrillar proteins during frozen storage was not significant in this case to judge by the considerably high percentage of protein soluble in 5% NaCl

(around 90%) remaining after 5 months frozen stored. Other authors have reported an extremely high level of soluble protein in the muscle of this squid species and resistance of myofibrillar proteins to freeze-induced denaturation [22]. Clearly, then, there must be changes in the protein fraction that do not register as variations in salt soluble protein content, possibly due to denaturation / aggregation of low molecular weight sarcoplasmic proteins, including large numbers of enzymes (mainly proteases). In this connection, Morales [23] reported pronounced differences in the viscosity of homogenates from different cephalopod species unaccompanied by any apparent changes in per cent protein solubility.

In contrast, MTG only increased G' in the case of batters from squid stored for 5 months. In this connection, various studies have shown that the effect of a microbial TGase could be highly dependent on the quality of the raw material [11; 12]. The combined use of protease inhibitors and MTG improved gel formation much more, producing a very sharp increase in G' in the batter from newly frozen squid, and to a lesser extent also in the long term frozen squid. The effect of combined addition of microbial TGase and protease inhibitors has been successfully tested in fish species with low gel forming ability, such as carp, salmon or hairtail [5; 16]. In the case of fresh or newly frozen squid, which have a very high rate of autolytic activity, it seems to be necessary to at least partially inhibit such activity for the MTG to be effective. After a time in frozen storage, where the muscle has lost part of its proteolytic activity, MTG seems to find a more suitable substrate for improvement of gel forming ability. Nevertheless, there are other factors in this raw material, possibly including aggregation of sarcoplasmic proteins, which cause the elastic behaviour of the batter with added MTG and protease inhibitors to be no better than that of equivalent sample made with newly frozen squid at the end of gelation.

Frozen squid stored for 5 months was chosen for further physicochemical study of the gelling process with added protease inhibitors and/or MTG. To this end, the different gels were prepared by heating at 90ºC for one hour. The batter without ingredients did not produce a proper gel and only scored 2 in the folding test, unlike the gels with MTG or with protease inhibitors, which scored 4. The

use of MTG and protease inhibitors together gave the maximum score in the folding test (5).

The results of a puncture test are shown in Fig. 3. Gels made with added MTG registered the highest values of gel strength. The combined addition of MTG and protease inhibitors did not improve gel strength. Analysis of breaking force and breaking deformation showed that breaking force was similar ($P \leq 0.05$) in both gels, but those containing only MTG were more deformable ($P \leq 0.05$). Enhanced rubber elastic behaviour has been reported in gels from Alaska pollack surimi containing microbial transglutaminase [14]. However, in the present case the gel made with inhibitors did not differ significantly ($p \leq 0.05$) from the control (gel with no additives) in terms of breaking force, deformation or gel strength. Nevertheless, the slope (N/mm) of the penetration curves revealed other significant differences among the studied gels, with the gel containing MTG and protease inhibitors attaining the maximum values. According to this parameter, no significant differences were found between the gel with added MTG and the one with protease inhibitors, although both were superior to the control gel. These results were consistent with the folding test scores.

A compression-relaxation test was carried out to gain a better understanding of the rheological properties of the squid gels. Elasticity and hardness are shown in Fig. 4. All the gels with added MTG and/or protease inhibitors exhibited similar elasticity values, which were significantly higher than in the control sample. The three gels with ingredients showed similar recovery capacity after compression, although the gel with MTG scored highest for deformability in the puncture test.

The gel with MTG and inhibitors added together presented significantly ($p \leq 0.05$) more hardness, followed by the gels with MTG or with inhibitors equally. The latter in turn were much harder than the control sample. These results were consistent with the slope values from the puncture test described above, but not with the breaking force or hence the gel strength. This is not surprising, since a number of authors have mooted the possibility of differences

between penetration tests and compression tests, the former measuring the density of the actomyosin network and the latter the overall binding of the gel material [24].

The formation of covalent bonds mediated by TGase activity and the degree of involvement of the principal myofibrillar proteins in these bonds was studied by solubilizing the gels with 0.8M NaCl, 8M urea and 2% mercaptoethanol to cleave strong interactions such as disulphide bonds or hydrophobic interactions. In this way it is assumed that the amount of protein remaining insoluble will be highly aggregated by means of covalent bonds other than S-S bonds, which have been largely attributed to TGase activity [25; 13].

Fig. 5 shows the electrophoretic profiles (SDS-PAGE) of the protein fraction solubilized in the given conditions from the different gels studied, together with the percentage of protein solubilized with respect to total protein content in each case. Both the gels containing microbial transglutaminase, that is with and without protease inhibitors, contained a significantly smaller percentage of soluble protein than the gels without MTG. This confirms that a high degree of protein aggregation is mediated by TGase activity. The presence of protease inhibitors slightly but significantly ($P \leq 0.05$) reduced the amount of soluble protein in these conditions.

The electrophoretic profiles of the solubilized protein show a series of bands in the gel without ingredients corresponding to the principal myofibrillar proteins, i.e., myosin heavy chains (MHC), paramyosin, actin and proteins of lower molecular weight, including tropomyosin, troponins, etc. The solubilization of these myofibrillar proteins was also observed in previous experiments with gels from other squid species, *Dosidicus gigas* [26]. There are also considerable numbers of other bands corresponding to peptides of molecular weights between 180 and 65 kDa, which could be products of proteolytic degradation of the MHC [27]. In the soluble protein fraction from the gel with added protease inhibitors, the 180 kDa peptide is less strongly marked than in the gel without protease inhibitors (control), while the other degradation products are practically imperceptible. This confirms that the added substances inhibited proteolysis.

With regard to the gels made with added MTG with or without protease inhibitors, there was practically no trace of MHC and paramyosin bands or their degradation products, which suggests that they were part of the insoluble protein fraction in the precipitate. The polymerization of the MHC and its consequent disappearance from the soluble protein profile has been attributed largely to covalent crosslinking mediated by TGase activity in many fish gel studies [13]. Although the percentage of soluble protein was similar in gels with MTG and with MTG-IN, there were small qualitative differences, such as a greater presence of polymers in the sample application zone, and of paramyosin and tropomyosin/troponins in the soluble protein from the gel with MTG-IN. Given that 8M urea and 2% mercaptoethanol were used as solubilizing agents, the greater presence of these proteins in the gel, especially the high molecular weight polymers, indicates more formation of disulphide bonds and hydrophobic interactions than in the gel containing only MTG, where covalent bonds other than S-S were prevalent.

Conclusions

The poor gel forming ability of squid muscle as a consequence of a high rate of proteolysis could be substantially improved by the combined use of protease inhibitors and microbial transglutaminase (MTG). When the squid muscle had been in frozen storage for a time, there was a considerable decrease in autolytic activity, possibly due to denaturation and aggregation of sarcoplasmic proteins (mainly enzymes). This caused a slight reduction of gel forming ability when inhibitors and MTG were added together, but it improved the effect of the MTG when this was added alone. In formed gels, the addition of both protease inhibitors and MTG considerably improved the elasticity of the gel; however, gels with MTG alone had significantly higher breaking deformation than other gels, and also presented a greater prevalence of covalent cross-linking where mainly MHC and paramyosin were involved. The combination of protease inhibitors and MTG led to the hardest gels, which were characterized by a slightly higher prevalence of hydrophobic interactions and disulphide bonds than in the gel with MTG alone.

REFERENCES

1.- Gómez-Guillén MC, Solas T, Borderías J, Montero P (1996) Z Lebensm Unters Forsch 202: 215-220.

2.- Gómez-Guillén MC, Montero P (1997) Z Lebensm Unters Forsch 204: 379-384.

3.- Nagashima Y, Ebina H, Nagai T, Tanaka M, Taguchi T (1992) J Food Sci 57: 916-917/922.

4.- Hurtado JL, Borderías J, Montero, P, An H (1999) J Food Biochem 23: 469-483.

5.- Ni S, Nozawa H, Seki N (1999) Fish Sci 65: 606-612.

6.-Tsukamasa Y, Miyake Y, Ando M, Makinodan Y (2000) Nippon Suisan Gakkaishi 66: 719-725.

7.- Yamashita M, Henmi H, Ueda T, Obara M, Taro T, Nishioka F, Konagaya S (1996) Nippon Suisan Gakkaishi 62: 934-938.

8.- Morrissey MT, Wu JW, Lin D, An H (1993) J Food Sci 58: 1050-1054.

9.- Ayensa MG (1997) PhD Thesis. Universidad Complutense. Madrid.

10.- Sakamoto H, Kumazawa Y, Toiguchi S, Seguro K, Soeda T, Motoki M (1995) J Food Sci 60:300-304.

11.- Seguro K, Kumazawa Y, Ohtsuka T, Toiguchi S, Motoki M (1995) J Food Sci 60:305- 311.

12.- Asagami T, Ogiwara M, Wakameda A, Noguchi SF (1995) Fish Sci 61:267-272.

13.- Lee HG, Lanier TC, Hamann DD, Knopp JA (1997) J Food Sci 62:20-24.

14.- Lee HG, Lanier TC, Hamann DD (1997) J Food Sci 62:25-28/32.

15.- Folk JE (1983) Adv Enzymol 54:1-57.

16.- Jiang ST, Hsieh JF, Ho ML, Chung YC (2000) J Food Sci 65: 241-245.

17.- Gómez-Guillén MC, Hurtado JL, Montero P (2001) J Food Sci (accepted for publication).

18.- Ayensa MG, An H, Gómez-Guillén MC, Montero P, Borderías AJ (1999) Food Sci Tech Int 5: 391-396.

19.- Suzuki T (1981) Fish and krill protein: processing technology. Applied Science Publishing. London.

20.- Gómez-Guillén MC, Borderías AJ, Montero P (1996) J Agric Food Chem 44:746-750.

21.- Lowry OH, Rosebrough NJ, Farr AL, Randall RJ (1951) J Biol Chem 193: 265-275.

22.- Moral M, Tejada M, Borderías AJ (1981) Refrig Sci Technol 4:325-332.

23.- Morales J (1997) PhD Thesis. Universidad Complutense. Madrid.

24.- Lee CM, Chung KH (1989) J Texture Stud 20:363-377.

25.- Kamath GG, Lanier TC, Foegeding EA, Hamann DD (1992) J Food Biochem 16: 151-172.

26.- Gómez-Guillén MC, Montero P, Solas MT, Borderías AJ (1998) J Agric Food Chem 46:3440-3446.

27.- Niwa E, Suzuki S, Nowsad A, Kanoh S (1993) Nippon Suisan Gakkaishi 59: 1013-1016.

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LEGEND TO FIGURES

Fig. 1. Autolytic activity of 2 days and 5 months frozen stored squid mantle, at different incubation temperatures.

Fig. 2. Changes in elastic modulus (G') during thermal treatment at 1ºC/min of batters made from 2 days (a) and 5 months (b) frozen stored squid mantle.
CO= squid batter with no additives; TG= with microbial transglutaminase; IN= with protease inhibitors; TG-IN= with microbial transglutaminase and protease inhibitors.

Fig. 3. Gel strength, breaking force, breaking deformation and slope, measured by a puncture test, of gels made from 5 months frozen stored squid mantle.
CO= squid gel with no additives; TG= with microbial transglutaminase; IN= with protease inhibitors; TG-IN= with microbial transglutaminase and protease inhibitors.
Different letters (a,b,c...) indicate significant ($P \leq 0.05$) differences.

Fig. 4. Elasticity and hardness, measured by a stress-relaxation test, of gels made from 5 months frozen stored squid mantle.
CO= squid gel with no additives; TG= with microbial transglutaminase; IN= with protease inhibitors; TG-IN= with microbial transglutaminase and protease inhibitors.
Different letters (a,b,c...) indicate significant ($P \leq 0.05$) differences.

Fig. 5. Percentage and electrophoretic profile of protein solubilised in 0.8 M NaCl, 8M urea, 2% β-mercaptoethanol from gels made with 5 months frozen stored squid mantle.
CO= squid gel with no additives; TG= with microbial transglutaminase; IN= with protease inhibitors; TG-IN= with microbial transglutaminase and protease inhibitors.