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Gel properties of collagens from skins of cod (*Gadus morhua*) and hake (*Merluccius merluccius*) and their modification by the coenhancers magnesium sulphate, glycerol and transglutaminase

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Abstract

The gel properties of two different kinds of fish gelatins prepared from cod (*Gadus morhua*) and hake (*Merluccius merluccius*) and modified by the coenhancers glycerol, salt and microbial transglutaminase, were examined. Gel strength was substantially increased by the addition of coenhancers although results varied, depending on the species. In gelatin from hake (*M. merluccius*) skin, the highest values were obtained with 10 mg/g of transglutaminase, whereas magnesium sulphate was more effective at both concentrations (0.1 and 0.5 M) in gelatin from cod (*G. morhua*) skin. Although, in both gelatins, the addition of any ingredient increased the viscosity modulus (G″), the elastic modulus (G′) was only increased by the addition of glycerol 15% (w/v) and $MgSO_4$ 0.5 M in hake (*M. merluccius*) gelatins; in cod (*G. morhua*) it was increased by all ingredients. The gelling and melting points, very important properties in fish gelatin, showed a notable improvement, the behaviour being different, depending on the species. © 2001 Elsevier Science Ltd. All rights reserved.

Keywords: Fish skin gelatin; Gel; Salt; Transglutaminase; Glycerol; Gel strength viscoelastic properties

1. Introduction

Gelatin is a polypeptide obtained from collagen after several degradation steps. It is used to increase the viscosity of aqueous systems and to form aqueous gels. Traditional sources of gelatin are mainly pig skin and cowhide but, for a number of reasons, such as religious proscription of Judaism and Islam, and diseases such as bovine spongiform encephalopathy (BSE), alternative sources of gelatins are highly demanded. In this sense, fish skin gelatins could be substitutes for mammalian gelatins. Although some fish gelatins are already available commercially, these are not well characterized. Almost all the studies on gelatin refer to mammalian material, and only in recent years have studies on fish gelatins been carried out (Choi & Regenstein, 2000; Gilsenan & Ross-Murphy, 1999, 2000; Leuenberger, 1991; Montero & Gómez-Guillén, 2000; Norland, 1997, 1990; Sarabia, Gómez-Guillén, & Montero, 2000). The behaviour of the gelling process differs depending on the specific amino acid content, which varies from species to species, the state of the protein, its gel-forming ability, the conditions of extraction and the treatment; however, some studies refer to physicochemical characteristics of fish gelatin but there are no relationships with the intrinsic properties of gelatin, and the extraction process (Gilsenan & Ross-Murphy; 2000; Montero & Gómez-Guillén, 2000; Sarabia et al., 2000)

The main differences in properties of mammalian and fish gelatins are that fish gelatins have low gelling and melting temperatures but relatively high viscosities (Leuenberger, 1991). One possible commercial use for these gelatins could be in applications where high viscosity solutions without gel formation is required, but, for many applications, high rheological properties are required by using gelatin-modifying substances. One of the possibilities is to trigger interactions by the addition of solutes, for instance, salts, glycerol and enzymes.

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Electrolytes, in general, have a decisive influence on biophysical properties (swelling, solubility, gelation, viscosity and water-binding capacity) of a protein, and the behaviour will depend on the ionic strength and pH. Lyotropic agents may alter water structure around collagen/gelatin, interrupt internal hydrogen bonds, or interact with internal hydrophobic bonds by direct binding at some sites of the protein chain (Asghar & Henrickson, 1982). The effect of different salts on the rigidity or melting temperature of warm blooded animal gelatins has been known for a long time (Harrington & Von Hippel, 1961). Recently, Sarabia et al. (2000), studied the effect of several salts on the viscoelastic properties of fish skin gelatins from megrim and tilapia. They found that, although salts generally extended the setting time of gelatins, the melting temperatures were increased considerably, mainly by the addition of $MgSO_4$. Also, viscoelastic properties were improved, in suitable conditions of ionic strength and pH, and the difference in behaviour of the gelatins was attributed to differences in amino acid composition.

Non-electrolytes, such as sugars and glycerol, usually increase the strength of gelatin gels. NMR studies carried out by Naftalian and Symons (1974) indicated that the stabilization of sugars is a structural effect that operates in the order sucrose $>$ D-galactose $\geqslant$ D-glucose, with fructose giving no measurable stabilization. These workers suggested that the stabilization is due to hydrogen bonding which operates in such a way as to involve water in a manner that is not found with sugars or gels alone.

Dextran dialdehydes have already been applied to promote cross-linking in commercial mammalian gelatins and the ability of salts to modify the functional properties of hydrogels has been tested (Schacht, Nobels, Vansteenkiste, Pemeester, Franssen, & Lemahieu, 1993).

Transglutaminase is an enzyme that catalyses an acyl-transfer reaction introducing a covalent cross-link between glutamine and lysine residues. It has been applied to various food proteins to improve functional properties. The reactivity of proteins to microbial transglutaminase depends, not only on the distribution of glutamine and lysine residues, but also on the secondary and tertiary structure of the proteins.

As previously mentioned, the state of the protein, the characteristics of species, the extraction method of the gelatin and, also, the treatment to produce the gels are very significant parameters which have to be taken into account in evaluating the gelatin gel process.

The aim of this study was to examine the gel behaviour of two different kinds of fish skin gelatins — one from cod (*Gadus morhua*) and the other from hake (*Merluccius merluccius*) — in a single system and to determine to what extent their properties are modified by coenhancers such as glycerol, salt and transglutaminase.

2. Materials and methods

Skins from fresh hake (*M. merluccius*) and cod (*G. morhua*) were obtained from the local market, and stored at −20°C until used. All reagents used were of analytical grade. The gelatin extraction procedure consisted of cleaning the fish skins with vigorous stirring in abundant tap water; excess water was removed by draining and squeezing the cleaned skins with a manual press. The gelatins were obtained with a mild acid treatment. The gelatin solution, before being dried, was microfiltered through a 1-μm diameter pore filter (Gelman Science).

Solutions were prepared by mechanical stirring, for 15–20 min, of pre-weighed (6.67% w/v) dry gelatin in water or different solutions (pH 5), depending on the ingredients added, at 60°C. The solutions assayed were: MgSO4 at 0.1 M and 0.5 M and glycerol at 15% (w/v). When the microbial transglutaminase (1 g kg^{-1} transglutaminase + 99 g kg^{-1} maltodextrin; ACTIVA® WM, Ajinomoto Europe Sales Gmbh, Hamburg) was used, it was added at 10 and 15 mg/ml (w/v) to the gelatin water solution. All the samples were cooled in a refrigerator at 7°C (maturation temperature) for 16–18 h overnight before analysis.

2.1. Turbidity

The turbidity of the gelatins was determined by measuring the absorbance at 360 nm, using a Milton Roy Spectronic 20D turbidometer (Milton Roy Co., Rochester, NY, USA), immediately after dissolving the dry gelatin powders in distilled water at 60°C (at 6.67% concentration). The values were recorded as the average of at least three determinations.

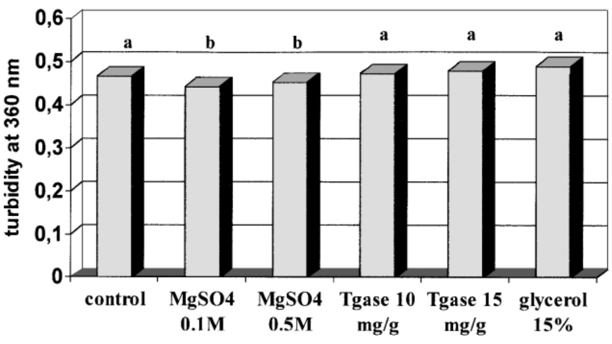
2.2. Gel strength

The gel strength was determined on a 6.67% gel (w/v), formed by dissolving the dry gelatin in distilled water at 60°C, and cooling the solution in a refrigerator at 7°C (maturation temperature) for 16–18 h. The gel strength at 8–9°C was determined on an Instron model 4501 Universal Testing Machine (Instron Co., Canton, MA, USA) with a load cell of 5 kN, cross-head speed 1 mm/s, equipped with a 1.27-cm diameter flat-faced cylindrical Teflon® plunger. The dimensions of the sample were 3.3 cm diameter and 6 cm height. The maximum force (in grams), taken when the plunger had penetrated 4 mm into the gelatin gels, was the average of five determinations.

2.3. Viscoelastic properties

Dynamic studies were performed on a Bohlin CRS-10 rheometer rotary viscometer (Bohlin Instrumments Ltd., Gloucestershire, UK) using a cone-plate geometry

(a) Hake



(b) Cod

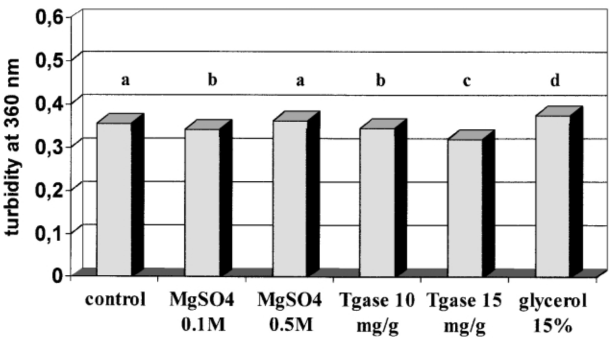


Fig. 1. Turbidity (measured as absorbance at 360 nm) of hake (*Merluccius merlucius*) (a) and cod (*Gadus morhua*) (b) skin gelatins with and without ingredients (tgase 10 and 15 mg/g, $MgSO_4$ 0.1 and 0.5 M and 15% w/v glycerol). Different letters (a,b,c…) indicate significant ($P \leq 0.05$) differences.

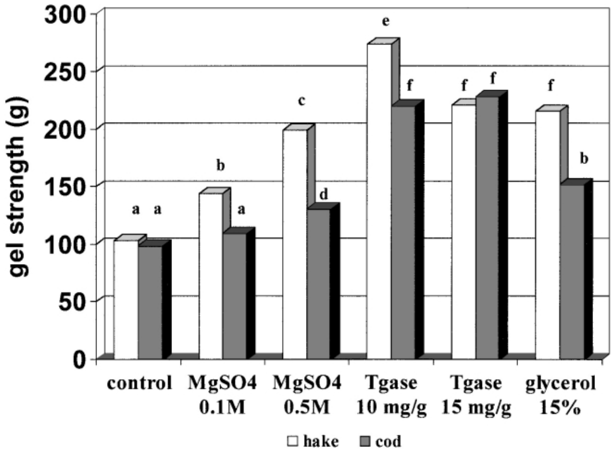


Fig. 2. Gel strength (g) of hake (*Merluccius merlucius*) and cod (*Gadus morhua*) skin gelatins with and without ingredients (tgase 10 and 15 mg/g, $MgSO_4$ 0.1 and 0.5 M and 15% w/v glycerol). Different letters (a,b,c…) indicate significant ($P \leq 0.05$) differences.

(cone angle 4°, gap = 0.15 mm). Temperature ramps were implemented from 5 to 50°C and back 50 to 5°C, and were performed at a scan rate of 0.5°C/min, frequency 1 Hz, and oscillating applied stress of 3.0 Pa. The elastic modulus (G′), viscosity modulus (G″) and the relation between the two, i.e. the phase angle (°), were represented as a function of temperature. The error in the reproducibility of the parameters considered in different determinations of a single sample was 6% or less.

2.4. Statistical analysis

One-way analysis of variance was carried out in some determinations using the Tukey test of computer program SPSS-8 (SPSS Co. Chicago, Illinois). Confidence level was set for $P \leq 0.05$.

3. Results and discussion

Characterization of the transparency was carried out by determining the turbidity, as a measure of absorbance at 360 nm, of the different gelatins in solution at 60°C (Fig. 1) Cod (*G. morhua*) gelatin showed lower turbidity than hake (*M. merluccius*) gelatin. The addition of any of the ingredients, at the concentrations specified, slightly modified the turbidity of gelatin solutions ($P \leq 0.05$), which is very convenient for maintaining the quality of gelatins.

The gel strengths of hake (*M. merluccius*) and cod (*G. morhua*) skin gelatins, after maturation overnight at 7–8°C, are shown in Fig. 2. Both fish skin gelatins have similar gel strength ($P \leq 0.05$). The addition of any ingredient resulted in a considerable increase in gel strength, although the behaviour was different depending on species. In hake skin gelatin, the highest values were with 10 mg/g of transglutaminase, but in cod the values were significantly lower ($P \leq 0.05$) and similar for both concentrations of enzyme added. Magnesium sulphate was more effective at both concentrations in hake than in cod. The glycerol increased the gel strength two-fold in hake gelatin and one and a half times in cod gelatin. Given that the same extraction processes were used, it could be assumed that the different behaviours between the species is due to intrinsic characteristics, such as protein chain composition (molecular weight distribution) and amino acid composition. In previous studies, it was observed that cod gelatin had considerably lower hydroxyproline and alanine contents than hake. The pyrolidine rings of imino acids (hydroxyproline and proline) were considered to be determinant for the stability of the collagen helix although the hydrogen bonds through the hydroxy groups also contributed to the intermolecular stability of the helix (Gilsenan & Ross-Murphy, 1999). In molecular weight distribution, the main differences were that cod gelatin presented a considerable amount of very high molecular size aggregates. Hake gelatin did not show these aggregates but had more α-chains and less β-chains. Probably the lower degree of aggregation of hake gelatin allowed greater interaction of saline ions and glycerol with the positively-charged centres of the protein chains than in

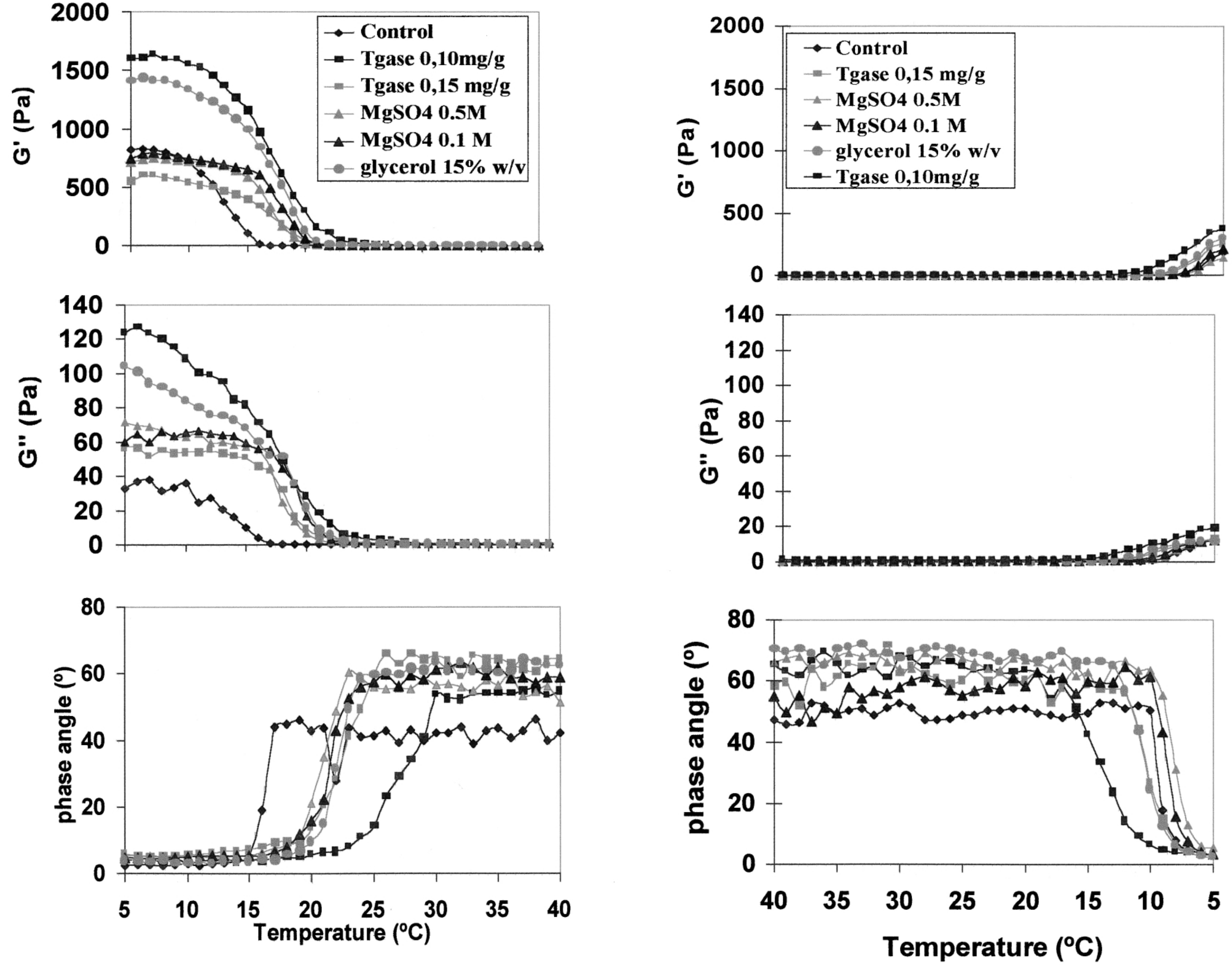


Fig. 3. Elastic modulus (G′), viscous modulus (G″) and phase angle of hake (*Merluccius merlucius*) skin gelatin with and without addition of ingredients (tgase 10 and 15 mg/g, $MgSO_4$ 0.1 and 0.5 M and 15% w/v glycerol).

the bigger aggregates of cod gelatins. Choi and Regenstein (2000) observed that sodium chloride decreased the gel strength of several commercial gelatins from different sources and attributed this effect to the fact that sodium chloride is capable of breaking both hydrophobic and hydrogen bonds, which prevents stabilization of the gel junction zones, either directly by preventing hydrogen-bond formation and/or by modifying the structure of the liquid water in the vicinity of these sites (Finch, Gardener, Ledward, & Menashi, 1974). These same authors observed that an increase in sucrose content in the gel solution resulted in a slight increase in gel strength. Naftalian and Symons (1974) suggested that this increase of gel strength is due to the fact that sucrose stabilizes hydrogen bonding.

With respect to the addition of transglutaminase, it is well known that this enzyme catalyses an acyl-transfer reaction, introducing a covalent cross-link between glutamine and lysine residues. In these gelatins this type of covalent bond is likely to be formed as can be observed in Fig. 2. Obviously, the different behaviours of the gelatins from the two species could be attributed to the distribution of reactive residues and to the secondary and tertiary structures of the two gelatins. In previous work it was observed that the quantity of α-helix in hake skin gelatin was higher than in cod skin gelatin, and, given that the percentage of alanine is lower in gelatin from cod, it probably contributed to form fewer hydrophobic regions in this gelatin (Gómez-Guillén, Turnay, Fernández-Diaz, Olmo, Lizarbe, & Montero, 2001).

In spite of an increase of gel strength with all ingredients, in hake skin gelatin it was only with $MgSO_4$ (0.5 M) and glycerol (15%), that the elasticity modulus (G′) was higher than the control (hake gelatin without ingredients; Fig. 3). However, the viscosity modulus (G″) was lower in the control. These increases in viscosity could be attributed to an unfolding process produced by interactions of ingredients with the molecule of gelatin. The most important effect concerned the melting point, which was considerably, more evident when $MgSO_4$ 0.5 M was added. On the subsequent cooling ramp, the renaturation was not so high, probably because there is a necessary maturation time for the gel set in order to recover the effectiveness in viscoelastic properties (G′ and G″). The highest gelling point was in hake gelatin containing $MgSO_4$ (0.5 M). Gelling points of gelatins with $MgSO_4$ (0.1 M) and glycerol were only slightly higher than the control.

The viscoelastic properties of cod skin gelatins, after the addition of each ingredient, were notably different from hake (Fig. 4). This could be attributed to the different intrinsic properties of the gelatins. With cod gelatin, any kind of ingredient improved the elasticity modulus (G′), the most effective being the addition of transglutaminase at both concentrations. However the addition of salts showed similar values to hake gelatins. In cod skin gelatin, the viscosity modulus (G″) was increased several times with almost all the ingredients when compared to cod gelatin alone. The gel-sol transition temperature increased progressively with the addition of enzyme at both concentrations and $MgSO_4$ at low concentration (0.1 M); the highest melting point was achieved with glycerol. In previous work, we observed that the effect of transglutaminase depended on both the manner and the amount of its addition. Amounts higher than 15 mg/g meant notable increases in melting and gelling points and produced a very elastic gel with lower gel strength, whereas lower quantities of enzyme meant an increase in gel strength and lower increase in the other properties. Sarabia et al. (2000) studied the effects of diverse salts in megrim and tilapia gelatins and also observed a notable increase in the melting temperature with respect to gelatin without added salt when sulphates and sodium phosphate were added, suggesting that these salts are more likely to interact with the surrounding water and produce greater screening of electrostatic interactions. As a result, they open the protein chains more and thus increase the likelihood of a suitable formation of useful strong junctions which, as reviewed by Ledward (1992), dictate the melting point. Slade and Levine (1987) studying mammalian gelatin, reported that salts destabilise gelatin structure, probably as a direct competition for water to hydrate. Sarabia et al. (2000) also observed, in the case of megrim gelatin that lower ionic strength (0.1 M instead 0.5 M) of $MgSO_4$ would appear to be more suitable for promoting useful junctions by correct protein unfolding without distorting the subsequent assembly of chains into collagen-like helical rods. In tilapia gelatin this effect was not evident. This difference in the behaviour of $MgSO_4$ in both fish gelatins — megrim and tilapia — according to pH and concentration, was attributed directly to differences in the conformation of the protein molecules of the two species and other intrinsic properties, such as isoelectric point and amino acid composition. As commented above, the highest viscoelastic properties attained by hake gelatin without ingredients added, compared to cod gelatin, and the effects of salts in each could be explained in terms of the amount of triple helical structure, which is highly dependent on the amount of pyrolidine residues. Also the molecular weight and/or aggregation of protein chains contributed to the different behaviours of the gelatins. Together with the amount of pyrolidine residues, the other relevant difference in amino acid composition is the lower alanine content in cod compared with hake, then the non-polar region is lower in molecules of cod protein (Gómez-Guillén et al., 2001).

In summary, it is possible to improve several functional properties of fish gelatins by the addition of $MgSO_4$ at 0.1 and 0.5 M levels, glycerol (15%w/v) and

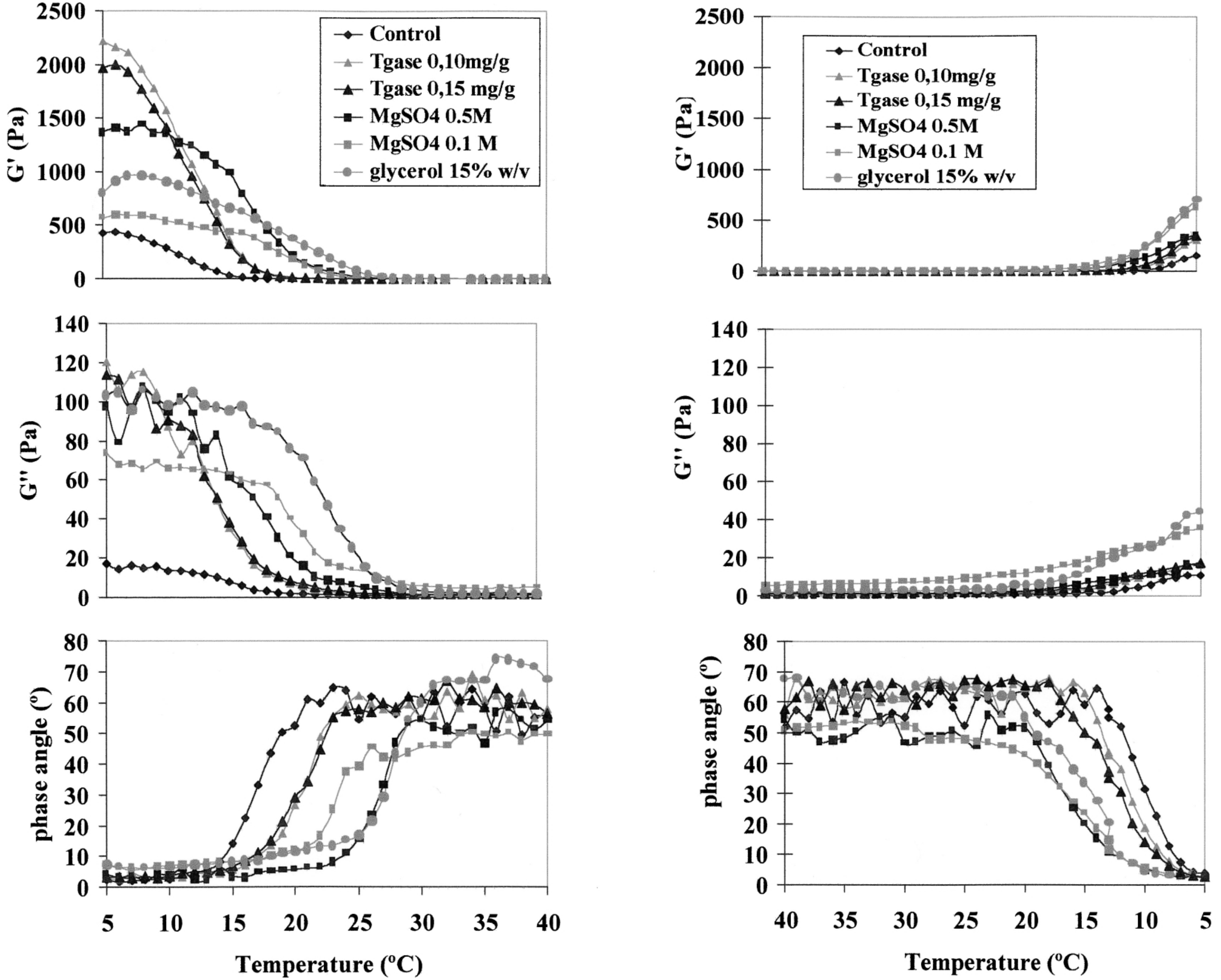


Fig. 4. Elastic modulus (G'), viscous modulus (G'') and phase angle of cod (*Gadus morhua*) skin gelatin with and without addition of ingredients (tgase 10 and 15 mg/g, $MgSO_4$ 0.1 and 0.5 M and 15% w/v glycerol).

transglutaminase at 10 and 15 mg/g levels, without modification of the quality, colour or transparency. Depending on the intrinsic properties of the molecules of protein, the behaviour of complexes between gelatin and ingredients will be different, making it possible to have a great variety of characteristics in gelatin, which could enlarge the field of industrial applications of gelatin.

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