Extracting Conditions for Megrim (*Lepidorhombus boscii*) Skin Collagen Affect Functional Properties of the Resultant Gelatin

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ABSTRACT

Various procedures for extracting collagen and/or gelatin from megrim skins were compared on the basis of gelatin functional properties. It was possible to prepare a dry powder of soluble collagen with good viscoelastic and gelling properties, which can be converted into gelatin by dissolving at temperatures above 45ºC**.** Physical properties of gelatins are influenced more by extracting conditions than by imino acid composition. A high-quality, readily-dissolved gelatin was obtained from megrim skins using a pre-treatment of the skins with NaCl and dilute NaOH, then swelling with 0.05 M acetic acid followed by an extraction step in water at 45ºC.

Key words: gelatin, collagen, fish skin, extraction, functional properties.

INTRODUCTION

The volume of gelatin used annually by the food industry worldwide is considerable and growing. Gelatin is not only used for its functional properties, but also to increase protein content. It can enhance the elasticity, consistency, and stability of food products, and it is also used as an outer film to protect against drying, light, and oxygen. The quality of a food grade gelatin therefore depends to a large extent on its rheological properties (essentially gel strength and viscosity), but it is also determined by other characteristics, particularly color, transparency, flavor, and easy dissolution.

Soluble gelatin is obtained industrially from collagen in bones or skins; therefore, the source, age of the animal, and type of collagen, all influence the properties of the gelatins (Johnston-Banks, 1990). Gelatin and collagen are originally the same macromolecule, so that the gelation of gelatin could be described as a partial reformation of the collagen structure (Ledward, 1986). The method of manufacture greatly affects the physicochemical properties of the gelatin. Collagen must be pre-treated to convert it into a form suitable for gelatin extraction, which is normally done with water above 45ºC. The degree of conversion of collagen into gelatin is related to the severity of both the pre-treatment and the extraction processes, which depends on pH, temperature, and extraction time. A mild acid pre-treatment is usually used for collagenous material containing a low concentration of intra- and interchain non-reducible crosslinks, i. e., highly soluble collagens as with fish skins (Ledward, 1986; Norland, 1990; Montero et al., 1990, 1995). The collagen rod is extracted in acid and solubilized without altering its original triple-helix configuration. Subsequent thermal treatment cleaves hydrogen and covalent bonds; this destabilizes the triple helix by means of a helix-to-coil transition, leading to conversion into gelatin (Djabourov et al.,

1993). At 40ºC and above, gelatin is soluble and is assumed to exist as a random coil. However, as reviewed by Ledward (1986), certain regions of the gelatin chains may retain some helical structure, the actual amount of structure depending on the number of pyrrolidine residues (proline and hydroxyproline) in the gelatin .

Although conventional gelatin from mammals has been widely studied, less work has been done on fish gelatin and its extraction procedures (Norland, 1990; Leuenberger, 1991; Grossman and Bergman, 1992; Gudmundsson and Hafsteinsson, 1997). The main difficulties in using fish skin to produce gelatin lies, on the one hand, in the dark color and strong odor of the skin of most fish species, and, on the other hand, in that gelatin gels are less stable, as they melt at a lower temperature. Nonetheless, there is considerable interest in the use of fish skins, for two reasons: utilization of by-products, and, from a socio-cultural standpoint, as an alternative to mammalian gelatin, whose consumption is rejected in some cultures. Several marine species have been examined as a source of raw material for edible gelatin, such as tilapia (Grossman and Bergman, 1992), cod (Gudmundsson and Hafsteinsson, 1997), lumpfish (Osborne et al., 1990), or conger eel and arrow squid (Kim and Cho, 1996), but no information is available for a flat fish such as megrim (*Lepidorhombus boscii*), which frequently is deskinned and increasingly commercialized as fresh or frozen fillets.

The objective of this study was to obtain an edible gelatin from fish skin with good functional properties, comparable to mammalian gelatins. For this purpose various physicochemical collagen and gelatin extraction methods were examined.

MATERIALS & METHODS

Fresh (whitin 18-24 hours in ice after capture) megrim (*Lepidorhombus boscii* (Risso)) and hake (*Merluccius merluccius*, L.) skins were obtained from a local fish shop in

Madrid, and were immediately stored at –20ºC until use. All reagents used were analytical grade.

Cleaning of fish skins

Thawed skins (0.65 kg) were washed with tap water (1:6 w/v) in a Stephan machine (position II) (Model UM5; Stephan und Söhne GmbH & Co., Hameln, Germany) at 5ºC for 10 min, and were rinsed with abundant running tap water. Skins were further cleaned with 0.8 N NaCl (1:6 w/v), again in the Stephan (position I) at 5ºC for 10 min, and again were rinsed with abundant running tap water. This step was repeated three times. Excess water was removed by draining the cleaned skins and manual squeezing. This process was used for all the extraction procedures described below, except MG1 and HG, where washing with NaCl was omitted.

Collagen extraction

Cleaned skins (white megrim side) were stirred with acetic acid 0.5 N (1:6 w/v) in the Stephan (position I) at room temperature for 20 min and then chopped for 2 min in an Omni-mixer (model 17106; Omni-International, Waterbury, CT, USA). The resulting homogenate was filtered in a Büchner funnel with Whatman no. 4 filterpaper (Whatman, Maidenstone, England) and the filtrate air-dried in a convection oven at 40-42ºC until moisture was less than 15%. This collagen sample was called **EC** (total extracted collagen). Soluble collagen (**SC**) was obtained in the same manner as EC but with a centrifugation step at 10,000 x *g* in a Beckman J2-MC centrifuge (rotor JA 14) (Beckman Instruments Inc., Palo Alto, CA, USA) for 45 min, after which the precipitate was removed. Soluble collagen in 0.15 N citric acid (**SCcit**), instead of 0.5 N acetic acid, was obtained following the same procedure described for SC. Freeze-dried

extracted collagen (**LEC**) and soluble collagen (**LSC**) were obtained by freeze-drying instead of air-drying. Heated soluble collagen (**SCh**) and heated extracted collagen (**ECh**) were obtained by a heating step (55ºC for 30 min) prior to air-drying.

Gelatin extraction

Gelatin (**MG1**) was basically obtained following the procedure described by Gudmundsson and Hafsteinsson (1997): washed dark megrim skins were stirred with cold (2ºC) 0.2 N sodium hydroxide (1:6 w/v) for 40 min (repeated three times), and then with 0.2 N sulphuric acid with the same conditions (repeated three times). Samples were drained and rinsed with tap water after each step. Skins were then treated with 0.7% citric acid for 40 min with continuous stirring and after that extracted with distilled water overnight at 45ºC. The mixture with the remains of the skins was then filtered in a Büchner funnel with Whatman no. 4 filter paper and the clear filtrate was then air-dried in a convection oven at 40-42ºC until moisture was less than 15%. Exactly the same extraction procedure was used for hake skin gelatin extraction (**HG**).

A modification of this extraction method was used to the improve physical properties of gelatin (**MG2**): cleaned skins (with 0.8 N NaCl and rinsed with abundant running tap water) were treated with 0.2 N NaOH (1:6 w/v) at 5ºC for 30 min with constant stirring, and again were rinsed with abundant running tap water (repeated three times). Skins were caused to swell with 0.05 N acetic acid (1:10 w/v) at room temperature for 3 hr, rinsed with tap water and then extracted with distilled water overnight at 45ºC. Subsequent processing was as described for MG1.

Gel strength

Gel strength was determined on a 6.67% gel (w/v), formed by dissolving the dry powder in distilled water at 60ºC, and cooling the solution in a refrigerator at 7ºC (maturation temperature) for 16-18 hr. Gel strength at 8-9ºC 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 1mm/s, equipped with a 1.27-cm-diameter flat-faced cylindrical Teflon® plunger. Maximum force (in g), taken when the plunger had penetrated 4 mm into the gelatin gels, are averages of five determinations.

Viscoelastic properties

Dynamic viscoelastic 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=150 mm). Cooling and heating from 50 to 5ºC and back to 50ºC were performed at a scan rate of 0.5ºC/min, frequency 1 Hz, and oscillating applied stress 3.0 Pa. Dry powder was dissolved at 45ºC (at 6.67% concentration) just before the start of the test. The melting temperature was taken as the point at which the phase angle peaks immediately after a sharp increase. Setting time (gel onset time) was determined as the time in minutes elapsing between last temperature of maximum phase angle and first temperature of minimum phase angle (gelling point)). In order to allow suitable gelling in all samples studied, reference G'and G'' (Pa) values were taken at 5ºC to compare characteristics at a standard temperature. Results were averages of four measurements. The error in the reproducibility of the parameters considered in different determinations of a single sample was 6% or less.

Apparent viscosity

Apparent viscosity (at 6.67% concentration) was performed in the cone-plate cell of the Bohlin rheometer at 60, 40, and 25ºC at a constant shear stress of 3.0 Pa. Results are averages of five determinations and were expressed as cPa.

Amino acid composition

For the analysis of amino acids, the dry powders were reconstituted with distilled water at 1mg/mL. Approximately 50µg of sample was treated by acid hydrolyisis (HCl 5.6N) at 108ºC for 18h, and injected into a Beckman 6300 analyzer (Beckman Instruments Inc., Palo Alto, CA, USA). Imino acid content was expressed as the number of residues of Pro + Hyp per 1000 residues. Hydrophobic amino acid content was expressed as the sum of residues of Ala + Val + Leu + Iso + Pro + Phe + Met per 1000 residues.

RESULTS & DISCUSSION

Megrim is a flat fish with a light-colored skin on one side. This can be used for extraction of collagen, so that after the drying process a whitish powder is obtained. This collagen dry powder is converted into gelatin by dissolving in water at temperatures above 42ºC. A comparison was made of different procedures based on the extraction of fish gelatin or its precursor, collagen. Fig. 1 shows the dynamic viscoelastic properties of the different materials: modulus of elasticity (G'), modulus of viscosity (G'') and setting time are plotted as a function of melting temperature.

Regarding the collagen extraction procedures, SCcit exhibited the lowest values for melting temperature, G', G'', and setting time. This was mainly attributed to the poor extraction capacity of citric acid at 0.15 N concentration (dilute citric acid is commonly used for pretreatment in gelatin manufacturing) as compared to 0.5 N acetic

acid, which was used in all other collagen extractions. As reported by Norland (1990), it is difficult to dissolve collagen in dilute acid since the molecules are covalently cross-linked into fibrils that may swell, but do not dissolve completely. This means that yield is low and very-high-molecular-weight components remain, which are known to reduce the setting time (Johnston-Banks, 1990). It is this highly covalent cross-linked fraction that is removed by centrifuging the total extracted collagen (EC) to obtain the soluble collagen or procollagen fraction (SC). The latter is transparent and after drying is more readily dissolved than EC.

However, as regards the viscoelastic properties, no great differences were found between the two lots. The reason for this may be that such covalent cross-links are normally located at the end of the chain (telopeptide zone), whereas it is the imino acid-rich regions, distant from the terminal zones, which form the stable junctions that determine the melting point and the G' (Ledward, 1992). Moderate heating at 55ºC, which was carried out to convert the total extracted collagen and the soluble collagen into gelatin prior to drying (ECh and SCh, respectively), clearly reduced G' and G'' in both cases, but there was not the same definite effect on melting temperature. Such heating favors the collagen molecule helix-to-coil transition and helps to obtain a more soluble and transparent product, as reported by Norland (1990). Freeze-drying of the soluble collagen (LSC) and the total extracted collagen (LEC) produced an increase in the viscoelastic properties as compared with the corresponding samples air-dried at 45ºC. A positive effect from freeze-drying has also been reported elsewhere (Gudmundsson and Haffsteinsson, 1997). The air-drying process at such relatively high temperature could have produced partial breakdown of the collagen or, on occasions, partial conversion into gelatin, which is detrimental to physical properties.

With a direct gelatin extraction, both megrim and hake gelatins (MG1 and HG, respectively), pre-treated with diluted citric acid, were colorless, transparent, and odorless, but their melting temperature and G' values were very low compared to collagen extractions in 0.5 N acetic acid. The melting temperature and setting time for HG were slightly higher than for MG1. In megrim gelatin extraction MG2, a mixed procedure was used. This consisted of an alkaline pre-treatment with diluted NaOH (to eliminate the strong fishy odor and raise the pH), pre-extraction of collagen with 0.05 N acetic acid (to efficiently denature the collagen triple helix) and gelatin extraction in heated water at 45ºC (to favor controlled hydrolysis of the opened helices and release of α- and β-chains). This method allows the use of white or dark pigmented skins with around 10% yield (42% on a dry basis), and eliminates the strong odor from the fish skins and the acetic acid, giving a high degree of transparency, and easy dissolution.

MG2 had a higher melting temperature and G' value than any of the collagen samples. The lime pre-treatment may produce a reduced molecular size through slight hydrolysis of the polar regions. Stabilization of the poly-L-proline helix is more likely to occur primarily by intermolecular rather than intramolecular associations, which thus increases rheological properties (Ledward, 1986). The setting time was reduced, and was similar to MG1. This is attributed to the relative similarity of the two procedures, given that the main extraction was done by heating in water overnight, favoring the helix-to-coil transition typical of normal gelatin extraction (Djabourov et al., 1993).

The other cases mainly involved collagen, although by dissolving the powder at 45ºC some of it was successfully converted to gelatin. The acid pre-treatment is the main point of difference between the two extraction processes: MG1 was treated with citric acid for 40 min, and MG2 with acetic acid for a longer time (3 hr). Both, type of acid used and time of swelling, appeared to be decisive for the physical properties.

As shown in Fig. 2, the viscosity of LSC at 25ºC was much greater than the rest of the samples. This is because LSC was freeze-dried, pure SC in which the temperature had at no point exceeded 7-8ºC, and which largely retained its original triple helix. The viscosity of LEC at 25ºC was considerably lower than LSC, probably due to the presence of the highly cross-linked insoluble collagen fraction. It is precisely because of the lack of this fraction in the SC sample that benefits more than EC by freeze-drying as compared to air-drying.

However, when SC was heated at 55ºC prior to drying (SCh), viscosity was greatly reduced, whereas this did not occur in the case of total extracted collagen. This indicates that, for the production of gelatin, it is better to retain the insoluble fraction of collagen. The viscosity of LSC decreased sharply at 40ºC and 60ºC. At such high temperatures the SC becomes denatured, losing its triple helix to become gelatin. At 60ºC, LEC had the highest viscosity of all, a fact attributed to a higher proportion of cross-linked collagen that is not susceptible to thermal hydrolysis. Gelatins MG1, HG, and MG2 were the least viscous at all experimental temperatures, the rest consisting mainly of collagen. These gelatins were subjected to an alkaline pre-treatment, and the combination of this with the heating for extraction almost certainly reduced the number of inter-chain covalent linkages, leading to reduced viscosity (Johnston-Banks, 1990).

Gelatin MG2 registered the highest gel strength (Fig. 3), even though it had the lowest viscosity at 60ºC. This is not surprising given that when gel strength is measured at a temperature below 10ºC, the many short-chain peptides present in such low-viscosity gelatins tend to strengthen the gel (Johnston-Banks, 1990).

Values were significantly higher in freeze-dried collagen than in the corresponding air-dried collagen samples. The effect of heating at 55ºC, to convert collagen into gelatin prior to drying, was only apparent in the SC samples, where there was a clear fall in SCh with respect to SC. This confirms that the insoluble fraction of collagen should not be removed to produce gelatin.

The MG1 extraction procedure applied to hake skin (HG) produced a gelatin with lower gel strength, even though it had the highest melting temperature. There may be inter-species differences in amino acid composition which would influence the gel properties. The stability of collagen and gelatin has been reported to be proportional to the total content in pyrrolidine imino acids, given that it is the Pro+Hyp rich zones of the molecules that are most likely to be involved in the formation of junction zones stabilized by hydrogen bonding (Ledward, 1986; Johnston-Banks, 1990). Fig. 4 shows the proportion of imino acids (Pro + Hyp) and hydrophobic amino acids in samples produced by the most representative extraction procedures. Imino acid content (Fig. 4a) did not differ in the various megrim extractions and was slightly higher than in the hake gelatin (HG). The hake gelatin contained more hydrophobic amino acids than the megrim gelatin (MG1) when extracted in the same conditions (Fig. 4b). At the same time, gel strength and viscosity at 25ºC in HG were lower than in MG1, and melting temperature and setting time were slightly higher.

Of the megrim samples, LEC (cold-extracted) had a higher proportion of hydrophobic amino acids than MG1, ECh, or SCh. Imino acid and hydrophobic amino acid composition, therefore, influenced the physical properties of gelatins from different species; however, within the same species, the extraction conditions influenced these properties through changes in hydrophobic amino acid composition rather than imino acid.

CONCLUSIONS

A high-quality, readily-dissolved gelatin was obtained from megrim skins using a pre-treatment of the skins with NaCl and diluted NaOH, then swelling with acetic acid followed by an extraction step in water at 45ºC. It is possible to prepare a dry powder of soluble collagen with good viscoelastic and gelling properties, which can be converted into gelatin by dissolving at temperatures above 45ºC. Moreover, elimination of the highly cross-linked insoluble fraction of collagen heightens the benefit of freeze-drying as opposed to air-drying. However, to manufacture the best gelatin, the insoluble collagen fraction should not be removed.

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LEGEND TO FIGURES

Fig. 1.- Elastic modulus (a) and viscous modulus (b), measured at 5ºC, and setting time (c) plotted as functions of melting temperature of both gelatin and collagen samples.

SCcit: soluble collagen extracted in 0.15 N citric acid; SC: soluble collagen extracted in 0.5 N acetic acid; EC: total extracted collagen in 0.5 N acetic acid; SCh: soluble collagen extracted in 0.5 N acetic acid and heated at 55ºC; ECh: total extracted collagen in 0.5 N acetic acid and heated at 55ºC; LSC: lyophilized soluble collagen extracted in 0.5 N acetic acid; LEC: lyophilized total extracted collagen in 0.5 N acetic acid; MG1: gelatin pretreated with citric acid 0.7% for 40 min; HG: gelatin from hake skin pretreated with 0.7% citric acid for 40 min; MG2: gelatin pretreated with 0.05 N acetic acid for 3 hr.

Fig. 2.- Apparent viscosity measured at 25, 40, and 60ºC of gelatin and collagen samples.

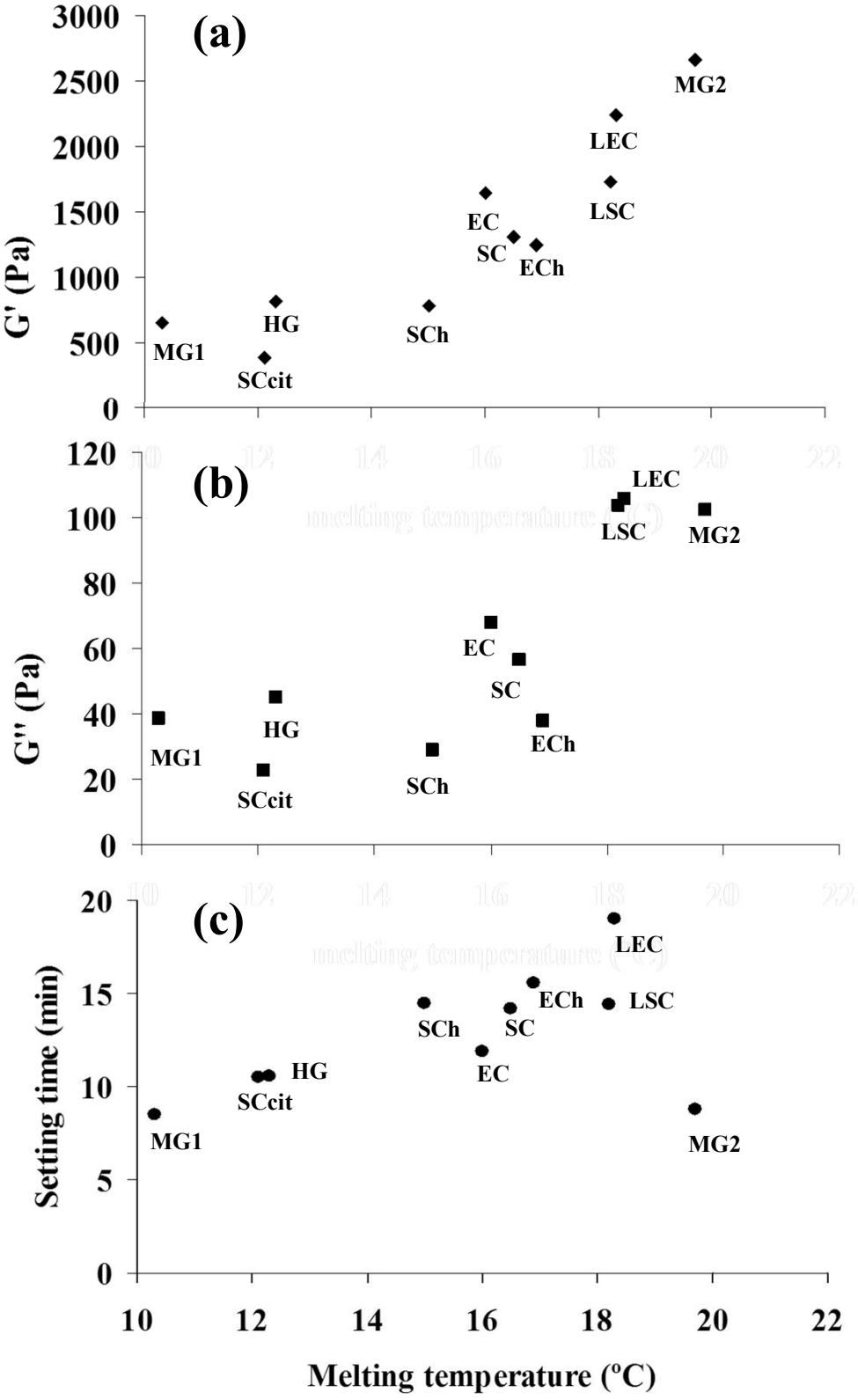
Sample identification as described in Fig. 1. Bars represent standard error.

Fig. 3.- Gel strength of gels made of gelatin and collagen samples, matured at 7ºC for 16-18 hr.

Sample identification as described in Fig. 1. Bars represent standard error.

Fig. 4.- Imino acid (a) and hydrophobic amino acid (b) composition of several gelatin and collagen samples

SCh: soluble collagen extracted in 0.5 N acetic acid and heated at 55ºC; ECh: total extracted collagen in 0.5 N acetic acid and heated at 55ºC; LEC: lyophilized total extracted collagen in 0.5 N acetic acid; MG1: gelatin pretreated with citric acid 0.7% for 40 min; HG: gelatin from hake skin pretreated with 0.7% citric acid for 40 min.


(a)
G' (Pa)
3000
2500
2000
1500
1000
500
0
MG2
LEC
LSC
EC
SC
ECh
HG
SCh
MG1
SCcit
(b)
G'' (Pa)
120
100
80
60
40
20
0
LEC
LSC
MG2
EC
SC
ECh
HG
MG1
SCh
SCcit
(c)
Setting time (min)
20
15
10
5
0
LEC
ECh
LSC
SCh
SC
EC
HG
SCcit
MG1
MG2
10
12
14
16
18
20
22
Melting temperature (°C)

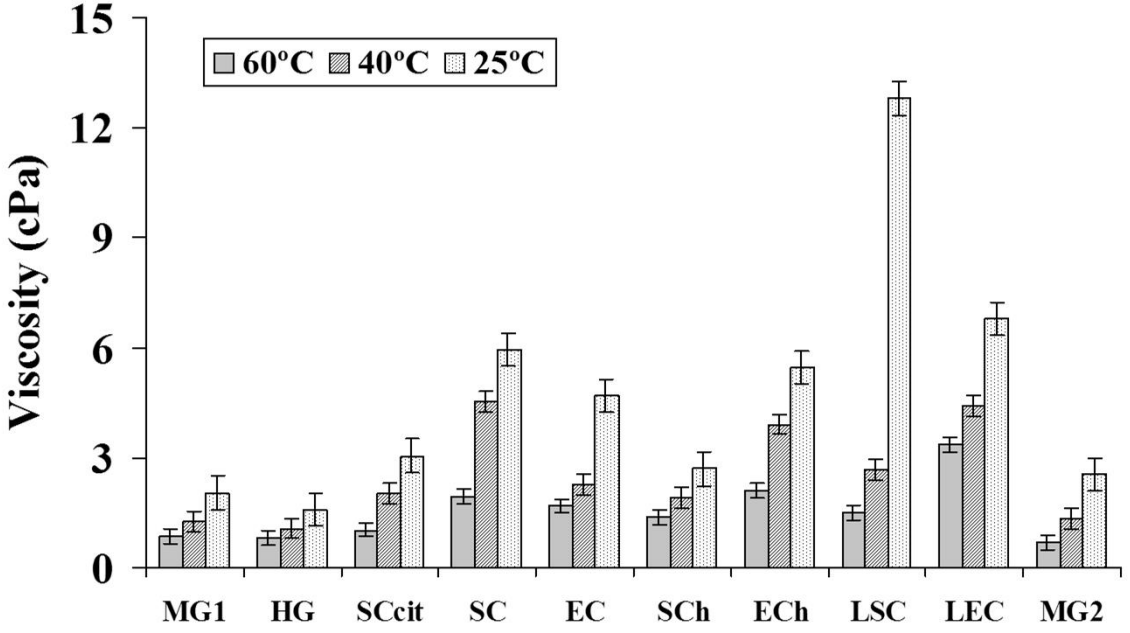

60°C
40°C
25°C
Viscosity (cPa)
15
12
9
6
3
0
MG1
HG
SCcit
SC
EC
SCh
ECh
LSC
LEC
MG2

Fig. 2

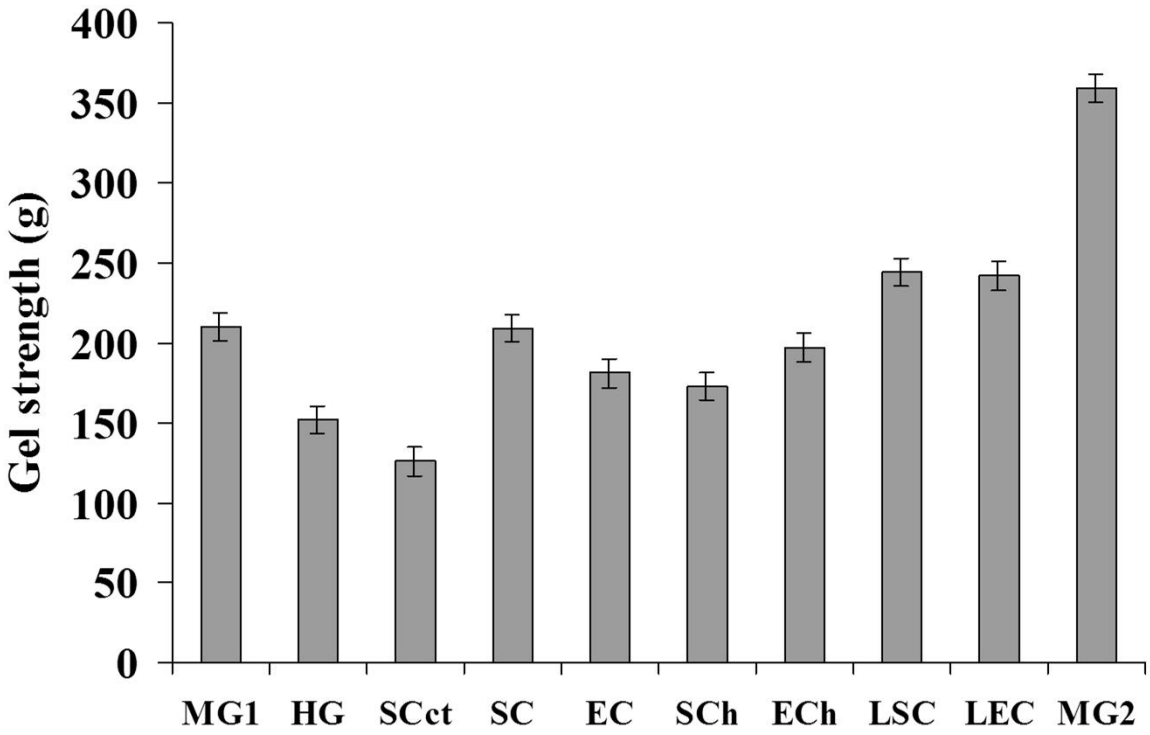

Gel strength (g)
400
350
300
250
200
150
100
50
0
MG1
HG
SCct
SC
EC
SCh
ECh
LSC
LEC
MG2

Fig. 3

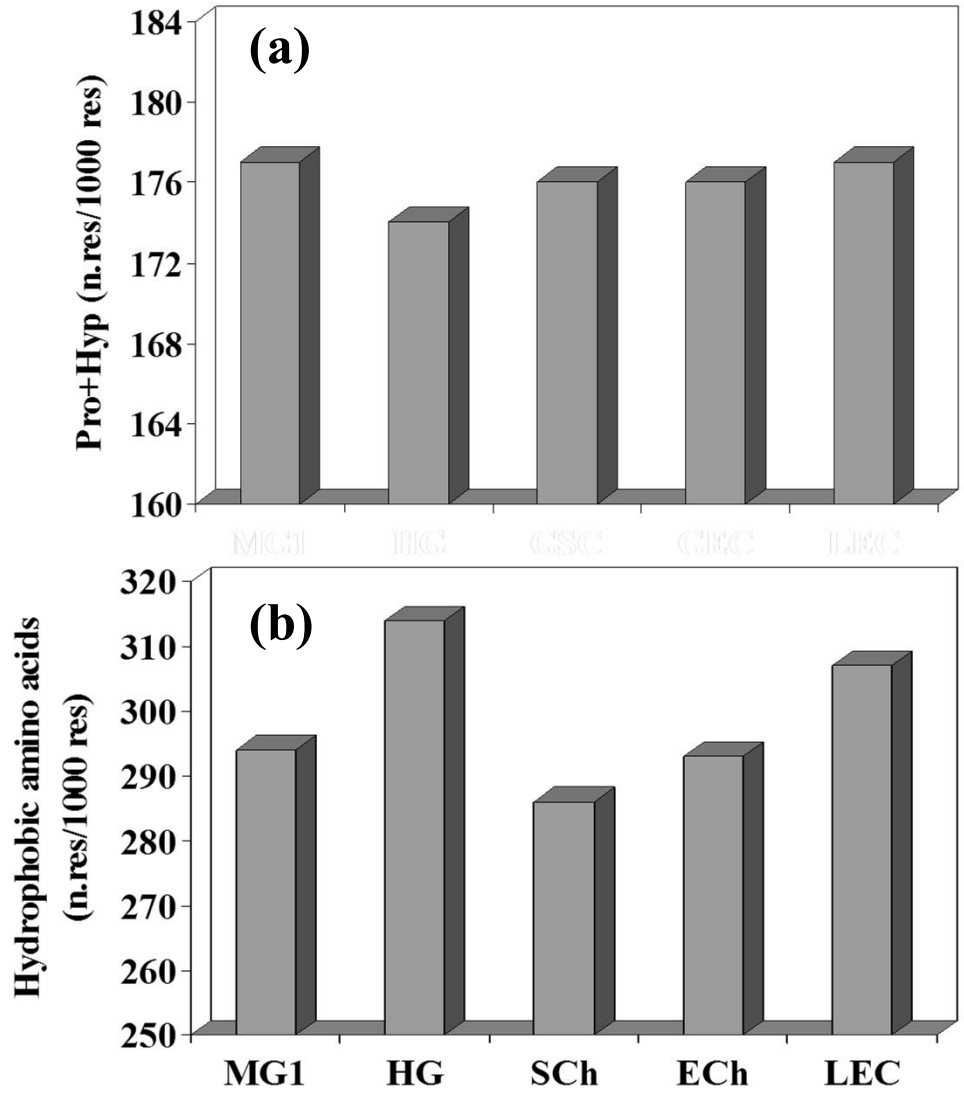

(a)
Pro+Hyp (n.res/1000 res)
184
180
176
172
168
164
160
MG1
HG
GSC
GEC
LEC
(b)
Hydrophobic amino acids
(n.res/1000 res)
320
310
300
290
280
270
260
250
MG1
HG
SCh
ECh
LEC

Fig. 4