Physicochemical, microstructure and bioactive characterization of gels made from crayfish protein

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Abstract

Crayfish proteins are valuable active ingredients for food products, mainly due to its protein quality and antioxidant activity. A highly soluble crayfish protein concentrate (CF2L) obtained from crayfish surpluses was used to evaluate gelling properties at three different pH values (2.0, 6.5 and 8.0). Thermal gelation processes were monitored by Small Amplitude Oscillatory Shear (SAOS) measurements. Subsequently, gels were characterized by viscoelastic properties, water-holding capacity (WHC) and a selective solubility. All the systems exhibited a gel-like behavior, showing a strong dependence on pH.

Antioxidant measurements were performed by using three different reagents (DPPH, ABTS and Folin-Ciocalteu) and revealed an interesting potential for human food. The pI effect was evaluated, showing a remarkable dependence of ABTS on its value.

The results show that gels made from crayfish surpluses have potential for use in human nutrition, not only based on the physical properties, but also on its protein quality and antioxidant ability.

Keywords: Bioactive properties, Crayfish proteins, Thermal gelation, Viscoelastic properties.
1. Introduction

In the middle of last century, the freshwater red-swamp crayfish (*Procambarus clarkii*) was introduced in the Guadalquivir marshlands. Due to weather conditions, abundant food and the lack of predators, the crayfish population underwent a fast and widespread growth and was soon considered as an invasive species. The use of crayfish-meat surpluses to produce a non-denatured protein concentrate may be a useful method to preserve and utilize the crayfish for later use, from which is possible to produce different food products, e.g. in the form of emulsions or gels (Bengoechea et al. 2008; Romero et al. 2009a).

In the last few years, there have been an interest in the nutritional value of food industry by-products since it is possible to develop food products that are interesting in the health and snack food markets (Glew et al. 2006; Sah et al. 2015). These added-value may be considered on the basis of both their amino-acid composition and bioactive properties such as antioxidant or hypertensive activity (Dey and Dora 2011).

Organic compounds in humans are made up to around 85 wt. % of proteins. Thus, they are an essential part of human diet, not only in infants for growth and development, but also for adults. Protein quality can be determined by the amino
acid composition and especially the content of the 9 essential amino acids (Reeds 2000).

Additionally, in a healthy diet, a sufficient intake of antioxidant compounds is also important, since free radicals are continuously produced as part of the human metabolism. These may induce damages to biomolecules that may promote changes in DNA and, as a consequence, serious health problems (Gey 1993). Antioxidants, present naturally in food, have also been postulated as antiaging agent (Brown 2005). In addition, antioxidant peptides have been found in numerous food products including milk, wheat, potato and fungi (Suetsuna et al. 2000; Sun et al. 2004). More recently, some studies have been focused on antioxidant peptides from fish (Sakanaka et al. 2005; Elias et al. 2008).

Proteins may therefore be used as potential antioxidants (Sa-ard et al. 2014). The antioxidant activity of proteins is attributed to complex interactions amongst their ability to inactivate reactive oxygen species, scavenge free radicals, chelate prooxidative transition metals, reduce hydroperoxides, enzymatically elimination specific oxidants, and alter the physical properties of food systems. As a consequence, proteins are considered unique compared to other food antioxidants, because they can inhibit different steps in lipid oxidation (Elias et al. 2008; Irshad et al. 2013).

Functional properties of crayfish protein isolates such as gel and emulsifying properties have been recently evaluated (Romero et al. 2009a; Romero et al. 2009b; Romero et al. 2011). However, its nutritional value and bioactivity potential have not previously been investigated. The protein quality together with bioactive properties such as antioxidant activity are important for these ingredients to contribute to a healthy diet (FAO 1985).
The overall objective of the present work was to evaluate the gelling properties and bioactive potentials of gels made from non-denatured crayfish protein concentrate (CF2L) at three different pH values (2.0, 6.5 and 8.0). To achieve these objectives, a physical and chemical characterization of CF2L was carried out. Subsequently, heat-induced gelation of CF2L was monitored by Small Amplitude Oscillatory Shear (SAOS) as a function of pH. Further characterization of the final gel was performed by means of WHC and selective solubility in order to evaluate the involved interactions. Finally, antioxidant activity of the different gels was evaluated by three different methods.

2. Material and methods

2.1 Materials

Figure 1 shows the process to obtain different protein fractions from crayfish (CF) meat. Initially, CF meat was separated from the shell by grinding and sieving and, subsequently, CF pulp was kept frozen until use. This first stage was carried out by ALFOCAN (Isla Mayor, Sevilla, Spain). After thawing at 4 °C, CF pulp was homogenized and subjected to centrifugation at 15,000 xg for 15 min, obtaining three different phases. A heavy phase (CF1P), which mainly consists of solid materials (some parts of the exoskeleton) and muscle tissue, This phase represents about 10 wt. % of the CF pulp, while its protein content is c.a. 14 wt.%, which constitutes the 8.6 wt.% of the total CF-pulp protein amount. An intermediate phase (CF2P), which mainly consists of water (c.a. 70 wt. % of the CF-pulp) was also obtained. The protein content of this phase is c.a. 80 wt.% and represents the 89.2 wt.% of the total protein amount in the CF-pulp. Eventually, a light phase (CF3P), which mainly consists of lipids (ca. 20 wt. % of the CF-pulp) was obtained.
The protein content of this lipophilic phase is c.a. 27 wt.%, which represents the rest of the protein contributed by the CF-pulp (2.2 wt.%). Moreover, some hydrophobic compounds such as astaxanthin and E vitamin are present in this phase. The CF2P was the selected phase because it is the water soluble protein fraction and shows the highest protein content. In addition, both myofibrillar proteins and sarcoplasmic proteins are present in this phase. Finally, the intermediate phase (CF2P) was freeze-dried in order to obtain a flour-fraction rich in proteins, the protein powder was named CF2L.

The protein content of the CF2L was determined in quadruplicate as % N x 6.25 using a LECO CHNS-932 nitrogen micro analyzer (Leco Corporation, St. Joseph, MI, USA). In the same way, lipid, moisture and ash contents were determined according to A.O.A.C. (1945).

DPPH, ABTS, Folin-ciocalteu, HCl, NaOH reagents were of analytical grade, purchased from Sigma–Aldrich Chemical Company (St. Louis, USA). Milli-Q ultrapure water was used for the preparation of all solutions.

2.2 Protein powder characterisation

2.2.1 Amino acid characterization

CF2L samples were dissolved in 6.0 M hydrochloric acid and were incubated in an oven at 110°C for 24 h. After hydrolysis, pH was adjusted to 7 using 6M NaOH and samples were filtered through a Whatman glass microfibre filter (GF/C). Finally, samples were diluted (1:500) by adding doubly distilled water.

Reverse phase HPLC by precolumn fluorescence derivatization with o-phtaldialdehyde (SIL-9A Auto Injector, LC-9A Liquid Chromatograph, RF-530 Fluorescence HPLC Monitor, all parts from Shimadzu Corporation, Japan) was performed using a NovaPak C18 cartridge (Waters, Milford, MA, USA).
Glycine/arginine and methionine/tryptophan were determined together, as their peaks merged. By this procedure, it is only possible to detect: Alanine (Ala), Aspartic acid (Asp), Glutamic acid (Glu), Histidine (His), Serine (Ser), Glycine (Gly), Arginine (Arg), Threonine (Thr), Tyrosine (Tyr), Methionine (Met), Valine (Val), Phenylalanine (Phe), Isoleucine (Ile), Leucine (Leu) and Lysine (Lys). The other amino acids were not included in the results of this study because they are completely destroyed by acid hydrolysis or cannot be directly determined from acid hydrolyzed samples.

2.2.2 Free and total sulphhydrlys.

Free and total sulphhydryl groups of CF2L samples were determined using the method developed by Beveridge et al. (1974). Samples were suspended (10 mg/mL) in 0.086 mol/L Tris-HCl – 0.09 mol/L glycine – 4 mmol/L EDTA – 8 mol/L urea – pH 8.0 buffer. Dispersions were stirred at 25°C for 10 min at 500 rpm in a thermomixer and centrifuged at 15,000 xg (10 min, 10°C). The supernatant was incubated with Ellman’s reagent (DTNB) (4mg DTNB/mL methanol) and 1 mL of 2-nitro-5-thiosulfobenzoate (NTSB) was used in the case of the total sulphhydrlys. Absorbance at 412 nm was measured in a Genesis-20 spectrophotometer (Thermo Scientific, USA). The molar extinction coefficient of NTB (13,600 L·mol⁻¹·cm⁻¹) was used. Protein concentration of extracts was determined by the modified Lowry method (Markwell et al. 1978).

2.2.3 Protein solubility

Protein solubility was determined as a function of pH in the range 2 to 10. Aqueous dispersions at 1mg/mL were prepared and pH of different aliquots was adjusted with 6 N NaOH and 6 N HCl at alkaline and acid pH values, respectively. Samples were homogenized and centrifuged for 15 min at 15,000 × g. The total
protein content (protein + peptides) was determined in quadruplicate by a modified Lowry method (Markwell et al. 1978). Percentages of soluble protein (calculated as protein content of supernatant x 100/weight of CF2L) were plotted.

2.2.4 SDS-PAGE Electrophoresis

CF2L composition was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Continuous and stacking gels of 10 and 3.5% of acrylamide, respectively, were prepared. A buffer containing 2 M Tris-base, containing 0.15% SDS pH 8.8 was used for the separating gel. A running buffer consisted of 0.027 M Tris-base, 0.38 M glycine pH 8.3 with the addition of 0.15% SDS, was utilized. Coomassie Brilliant Blue was used as colorant agent, and β-MercaptoEthanol was used in the sample buffer. Precision Plus Protein standards (Bio-Rad-Calibration kit, Richmond, CA, USA) containing ten protein bands were used: 10 kDa, 15 kDa, 20 kDa, 25 kDa, 37 kDa, 50 kDa, 75 kDa, 100 kDa, 150 kDa, and 250 kDa.

2.3. Gelation process

Aqueous dispersion of 12 wt. % protein concentration were subjected to thermal gelation in three different stages: (i) The first step was carried out at a constant heating rate (5 °C/min) from 20 °C to 90 °C; (ii) After the first step, an isothermal step was performed at 90 °C for 30 min; (iii) Subsequently, a cooling stage was carried out at a constant cooling rate (5 °C/min) from 90 °C to 20 °C.

Gels were performed at three different pH values: 2.0, 6.5 and 8.0.

2.4 Gel characterisation

2.4.1 Viscoelastic measurements of gels

Small Amplitude Oscillatory Shear (SAOS) measurements were performed in a controlled-stress rheometer (Kinexus Ultra +) from Malvern Instruments.
(Malvern, Worcestershire, United Kingdom). In a preliminary experiment, stress sweep tests were performed in order to establish the linear viscoelasticity range. The gelation process was simulated through in situ heating in the rheometer. All stages were performed at constant frequency and gap (6.28 rad/s⁻¹). Finally, frequency sweep tests (0.06 - 64 rad/s⁻¹) at 20°C were carried out in order to obtain the mechanical spectra of the final gels. All rheological measurements were carried out at constant gap (1mm).

2.4.2 Protein interactions

Solubility of CF2L gels in a number of selected solutions was used to determine ionic bond, hydrogen bond, hydrophobic interaction and disulfide bond according to the method of Gomez-Guillen et al. (1997). The selected solutions were: 0.05 mol/L NaCl (SA), 0.6 mol/L NaCl (SB), 0.6 mol/L NaCl + 1.5 mol/L urea (SC), 0.6 mol/L NaCl + 8 mol/L urea (SD) and 0.6 mol/L NaCl + 8 mol/L urea + 0.5 mol/L β-mercaptoethanol (SE) solutions. Quantification of ionic bonds was obtained from the difference between protein solubilized in SB and protein solubilized in SA; hydrogen bonds were quantified by the difference between protein solubilized in SC and protein solubilized in SB; hydrophobic interactions were obtained from the difference between protein solubilized in SD and protein solubilized in SC and, finally, disulfide bonds were expressed as the difference between protein solubilized in SE and protein solubilized in SD. The protein concentration was determined with a modified Lowry method (Markwell et al. 1978).

2.4.3 Water-holding capacity of gels

Each gel (0.3–1.3 g) was equilibrated at room temperature and placed on a nylon membrane (5.0 mm pores, Micronsep, New York, N.Y., U.S.A.) maintained in the middle position of a centrifuge tube. Water loss was determined by weighing
before and after centrifugation at $120 \times g$ for 5 min at 5°C. Water-holding capacity (WHC) was expressed as the percentage of the initial water remaining in the gel after centrifugation.

2.5 Bio-active properties

2.5.1 DPPH Assay

The 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay was performed as described by Brand-Williams et al. (1995) with some modifications. Briefly, the day before analysis, 0.1 mM methanolic DPPH* working solution was prepared and kept on a magnetic stirrer overnight at 4°C. A series of 0-750 μM methanolic working solutions of Propyl Gallate and gel solutions at 10 wt. % in methanol were prepared. An aliquot of DPPH* solution (2.9 mL) was mixed well with 0.1 mL of a sample or methanol (blank). After 20 min of incubation at room temperature, the absorbance at 515 nm was recorded. Results were expressed in propyl gallate (PG) equivalents.

2.5.2 ABTS Assay.

The 2,2’-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) assay was performed as described by Nenadis et al. (2004) with a few modifications: ethanol was replaced with methanol and the amount of sample added to the ABTS* solution was 200 μL. For the analysis, a series of 0-55 μM working solutions of Propyl Gallate and gels at 1 wt. % were prepared from stock gels. To compare the antioxidant activities, the absolute values for each antioxidant and each assay were recalculated into Propyl Gallate equivalents.

2.5.3 Folin-Ciocalteu (FC) Assay

The FC assay was performed as described by Singleton et al. (1999) with some modifications. Briefly, a series of 0-4 mM working solutions of Propyl Gallate and
suitable gel solutions were prepared. Deionized water (10 mL), antioxidant solution (1 mL), and 2.0 M Folin-Ciocalteu phenol reagent (1 mL) were transferred to a 20 mL volumetric flask. The reaction mixture was mixed by shaking, and after 3 min, 2 mL of 20% Na₂CO₃ solution (20 g/L) was added. The volume was brought up with deionized water. The absorption at 725 nm was read after 1 h of incubation at room temperature. Water was used as a blank. Results were expressed as equivalent activity of the gel compared to the reference (Propyl Gallate).

2.6 Statistical analysis

At least three replicates of each measurement were carried out. Statistical analyses were performed using t-test and one-way analysis of variance (ANOVA, p<0.05) by means of the statistical package SPSS 18. Uncertainty was determined as standard deviation.

3. Results and discussion

3.1 Protein Characterization

The chemical composition of the CF2L fraction reveals a 78.6 ± 2 wt. % of protein content, the 6.8 ± 0.1 wt. % of moisture, 5.1 ± 0.3 wt. % of lipids and finally, 9.5 ± 0.6 wt. % of ashes. The protein content is high (ca. 80 wt. %) although, according to Pearson classification (1983), it cannot be considered as a protein isolate because the protein content is less than 90 wt. % on a dry basis. The lipid content was up to 5 wt. % despite the centrifugation stage included in the process. These results are similar to previous results and could be attributed to a high content of phospholipids (Chalamaiah, Kumar, Hemalatha, & Jyothirmayi, 2012). Results also
show that the powder has a low moisture content and an ash content typical for these fractions (Romero et al. 2009a; Felix et al. 2014).

Glutamic acid was the most abundant amino acid found (Figure 2), followed by alanine and aspartic acid (without considering glycine and arginine which appear merged into a single peak). The proportion of essential/total amino acids in CF2L was around 0.75. As not all amino acids were detected by the method used, this value is not accurate. However, since the value is well above 33.9% recommended by FAO (1985), the nutritional value can be regarded as high.

The CF2L protein concentrate contains a significant amount of the essential amino acids (adding up 43 wt. % of total amino acid content). All essential amino acids are present except Tryptophan (Trp), which could not be determined by the analytical procedure used. In CF2L fraction, Met is one of the least abundant amino acid in the sample and Cys could not be determined through the performed HPLC analysis. Therefore, it is not possible to predict the crosslinking potential, based on cysteine content, however this will be discussed later, based on sulfhydryl content.

Nevertheless, the second limiting amino acid in the maintenance requirement after the sulphur amino acids, Thr, can be found in a significantly in this protein concentrate. These results are consistent with results from other authors, who have found similar results for crab (Vilasoa-Martinez et al. 2007) and crayfish (Cremades et al. 2003).

Lysine has received attention as the likely limiting amino acid in cereals. CF2L protein concentrate is an excellent lysine source since lysine is the fifth most abundant amino acid.

In general high protein solubility values were obtained for CF2L (Fig. 3), showing similar solubility profile to that one previously obtained for the spray-
dried crayfish pulp (Chalamama et al. 2012). Minimum solubility was found at pH around 5, which is most probably related to the proximity to the isoelectric point (IEP). Based on these results, three pH values were selected in order to study the gel formation, the first at acidic pH (pH 2.0), the second close to neutral pH (pH 6.5) and the last at alkaline pH (pH 8.0).

The amount of free sulphydryl groups is 18.4 ± 0.6 μmol/g protein while the total disulphide bonds reaches values up to 2290 ± 20 μmol/g protein. Note that the determination has been carried out at pH 8, where about 1/3 of the cystein residues that have not formed a disulfide bridges are present as S-S, and 2/3 as S-H. In addition, gels were performed at pH 2.5 and 6.5, where the amount of S-H and S-S may change. S-S and S-H at pH 8 are about twice the sulphide content of albumen protein concentrate (Felix et al. 2014). However, the amount of S-S bonds is lower than the value reported for legume proteins (Tang et al. 2012), where it was said that it must be related to the presence of Cys, up to 3 wt. %. Moreover, these data show that the amount of –SH groups is very low compared to the total sulphide content. These results suggest that a high amount of S-S bonds is naturally present in CF protein since little crosslinking formation is expected during protein manipulation as freeze drying was used to obtain the protein concentrate. However, the relatively high content of free sulphhydrals obtained from CF2L as compared to other proteins would also yield a higher density of crosslinking by forming disulphide bonds.

Fig. 3B shows the SDS-PAGE protein pattern of CF2L protein concentrate constituted by numerous bands spread out over the whole gel in a range of molecular weights from 10 kDa to 250 kDa. A first light band may be appreciated at 205 kD. This band has been reported to correspond to myosin heavy chain
protein (Suzuki, 1981). The main bands are those between 28 and 53 kDa, which may correspond to actin and tropomyosin (Cremades et al., 2001). However, albumins (from the sarcoplasmic fraction) are also typically found in this region (Ikemoto, Bhatnagar, Nagy, & Gergely, 1972). The presence of several bands of relative low-molecular weight is related to the addition of β-mercaptoethanol to the sample buffer. Thus, the bands found at 23 and 18 kDa may come from the disruption of myosin, while the band at 14 kDa can be caused by the disruption of the profilin from the actin (Schutt, Myslik, Rozycki, Goonesekere, & Lindberg, 1993). Other water-soluble low-molecular-weight proteins (e.g. myoglobin and enzymes) may be also located in this region (Suzuki, 1981). These results indicate that CF2L is a complex combination of sarcoplasmic and myofibrillar-soluble proteins.

3.2 Gel Characterization

3.2.1 Rheological Characterization

Figure 4A shows the evolution of SAOS viscoelastic properties (the storage modulus, $G'$ and the loss modulus, $G''$) of CFL2 dispersions at constant protein concentration (12 wt.%) for three different pH values (2.0, 6.5 and 8.0) during the thermal gelation process. Figure 4B shows the mechanical spectra ($G'$ and $G''$ as a function of frequency) obtained for each sample after thermal processing.

The SAOS profiles for all the pH values show a different evolution depending on the stage of the thermal cycle applied (Fig. 4A):

(i) The first heating stage, performed at constant heating rate, begins with a smooth decrease in $G'$ and $G''$, taking place at temperature lower than 45°C. Over these first several hundreds of seconds, the increase in temperature leads to an increase in mobility of the protein chains, due
to thermal agitation, where physical interactions (i.e. hydrogen bonds) are reduced. However, protein aggregation has also been reported at temperatures below 50°C (Ramos et al. 2014). This behavior has previously been found for crayfish protein and was attributed to the aggregation of the globular head regions of myofibrillar protein molecules (Romero et al. 2009b). This aggregation depends on the oxidation of sulphhydryl groups, which shows considerable reduction in the early temperature range of 30-50°C (Acton and Dick 1988; Sano et al. 1994). Other authors have also found a minimum in \( G' \) in this temperature region. The increase in \( G' \) with increasing temperature above the minimum was attributed to the formation of a three dimensional protein network, which is favored by the denaturation of myosin chains, since \( \alpha \)-helices in the tail segment begin to unfold around 30-40°C (Kim et al. 2005; Romero et al. 2009b). Moreover, in this temperature range, \( G' \) is above \( G'' \) at pH 2.0 and 6.5, whereas \( G' \) is below \( G'' \) at pH 8.0, which suggests that physical interactions are initially weaker at this pH.

Subsequently, in all systems, above 60°C a strong increase in both moduli (\( G' \) and \( G'' \)) takes place as the temperature increases. This effect may be related to structural changes of the helical rod segments of myosin proteins which promote the network formation through sulphide-bonds of these protein segments (Acton and Dick 1988).

(ii) During the second heating stage, performed at constant temperature, no apparent increase in the moduli could be noticed, neither at pH 2.0 nor at pH 8.0, whereas \( G' \) and \( G'' \) still undergo a moderate increase at
pH 6.5. These results indicate that formation of disulphide-bonds of the helical rod segments of myosin is counterbalanced at pH 2.0 and 8.0 by electrostatic repulsion between protein chains, whereas it is favored at pH 6.5, at which the protein surface charges are much weaker due to its proximity to the IEP.

(iii) Finally, during the third stage, performed at constant cooling rate, an increase in both viscoelastic moduli occurs, although it may be preceded by a smooth decrease in viscoelastic properties. During this cooling stage, physical interactions (i.e. hydrogen bonding) are responsible for this increase in mechanical moduli. These interactions may be important in the stabilization of the protein system. In addition, hydrogen bonds may also contribute to immobilization of water into the hydrogel network (Lanier et al. 2004).

The frequency sweep tests (Figure 4B) show that all the systems exhibit gel-like behavior, where $G'$ is higher than $G''$. The evolution of linear viscoelastic properties for CF2L gels with increasing pH is quite similar to that found for the thermal processing.

Two different gel strength behaviors were observed: At pH 6.5, the system exhibits an almost parallel evolution over the whole frequency range, which denotes the formation of a strong gel (showing the lowest tan $\delta$ values, around 0.2 in the whole/total interval). This behavior may be attributed to the absence of net charge observed at this pH value (shown in Figure 3A), which means that at this pH value, electrostatic repulsions could not be taken into account as a destabilizing phenomenon. On the contrary, at low pH (pH 2.0), the CF2L-gel behavior is closer to that of a typical weak gel, where tan $\delta$ values is not constant over the frequency
interval studied, increasing its value at high frequency (from 0.2 to 0.45). This low
gel-strength exhibited may be related to the remarkable increase of the relative
amount of peptide fraction found in Figure 3A at pH 2.0, which may be a
consequence of acid hydrolysis. Finally, although the gel at pH 8.0 also exhibits
strong gel behavior, tan δ at high frequency rise up to 0.36, exhibiting an
intermediate gel-like structure between those found at pH 6.5 and 2.0.

The gel structure has been evaluated by quantifying the interactions among
different protein chains present in gels (Figure 5A) and the water holding capacity
(WHC) for each gel for three different pH values (Figure 5B).

Interactions depend strongly on pH values (Fig. 5A). Ionic bonds, hydrogen
bonds, hydrophobic interactions and disulphide bonds are highly involved in the
formation of the gel network (Cofrades et al. 1996; Gomez-Guillen et al. 1997).
Thus, at acid pH, interactions are low and, as a result, the gel is weak. These results
are consistent with the above mentioned viscoelastic properties (low G’ and G”
values).

At pH close to the IEP (pH 6.5), ionic interactions are the most important in
absolute terms, which suggests that weak interactive forces are active. This effect
is probably related to the absence of repulsive electrostatic interactions as a
consequence of the proximity to the IEP. However, it does not suffice to explain the
gel strength, taking place at this pH. The remarkable gel strength found in the
rheological study is probably attributed to disulphide bonds which are the main
responsible for the gel strength (Lanier et al. 2004).

Finally, at alkaline pH (pH 8), hydrophobic interactions are the most important
interactions, in spite of the large number of disulphide bonds, compared to pH 2.0.
These high values for hydrophobic interactions were usually attributed as the most
important bond in surimi gels (Gomez-Guillen et al. 1997). The heating process might induce unfolding of native proteins and exposure of protected polar groups and, as a result, hydrophobic protein-protein interaction (Lanier et al. 2004).

All these results indicate that disulphide bonds show the greatest effect on gel strength. However, formation of disulphide bonds seems to be counterbalanced by repulsive interactions taking place as a result of charged protein surfaces (far from the IEP). Ionic interactions, being particularly relevant at the IEP, and low repulsive electrostatic interactions, thus contribute to facilitate formation of S-S bonds. Hydrophobic interactions, typically enhanced over thermal heating, as well as hydrogen bonds, play a role at the cooling stage, both contributing to reinforce the gel network structures. Thus, G’ and G” profiles undergo a noticeable increase upon cooling at pH 8, where hydrophobic interactions are relevant.

In addition, the results from WHC tests are in agreement with those obtained from linear viscoelasticity and interactions measurements, all of them reflecting different aspects of gel strength. In this sense, the gel with the highest value of WHC is the gel made at pH 6.5. This corresponds to the gel showing higher viscoelastic moduli (higher G’ and G” and lower tan δ) and higher amount of disulphide bonds.

3.2.2. Antioxidant Activity

Figure 6 shows the antioxidant activity of the gels formed at different pH values compared to the reference (propyl gallate, PG) measured according to three different methods: DPPH, Folin–Ciocalteu and ABTS. For the DPPH method, all the gels show a similar radical scavenging activity at around 310 mM of PG equivalents and it is not pH-dependent. This antioxidant properties will be not influenced on the pH of the gel performed, what is very interesting. Most of the studies on DPPH
scavenging activity have been focused on different vegetable oils, grapes and wines (Arranz et al. 2008; Espinoza et al. 2009; Spatafora et al. 2013), even some researchers have found this property for proteins such as egg yolk and fish proteins, which can scavenge free radicals (Sakanaka et al. 2005; Elias et al. 2008; Kristinova et al. 2009). Recently some studies have been based on fish proteins and hydrolysates (Kristinova et al. 2009), however the pH influence was not evaluate with proteins.

Figure 6 shows that these gels have a high ABTS antioxidant activity. In contrast to DPPH, the pH has a large effect, the activity varied between 500 and 5500 mM PG equivalents. This strong dependence on pH cannot be justified on the basis of protein interactions, since the trend followed by the ABTS assay is not in accordance to the gel interactions and rheology measurements. Thus, whereas gels exhibit a higher ABTS activity when an increase in pH values takes place, structural characterization showed that stronger interactions (and as a consequence higher gel strength) occurred at pH 6.5. This pH effect is also contradictory for other authors, Floch et al. (2007) found the highest activity in soil at low pH values. However, Lemanska et al. (2001), found the same pH effect for hydroxyflavones, this means that the ABTS assay is strongly dependent on the nature of the compound studied.

As may be observed in Fig 6 all gels show a FC antioxidant activity with a slight increase if the pH value rises from 2.0 to 8.0. This antioxidant activity is quite different from the ABTS radical cation assay, which also was sensitive to phenol compounds. This difference may be explained because the FC reagent is not capable to measure lipophilic antioxidants due to the high affinity of the FC chromophore towards water (Huang et al. 2005).
Besides the different behavior of protein gels, many studies have been reported showing this effect that depends on the active agent and the type of assay (DDPH, ABTS, FC) (Wootton-Beard et al. 2011).

4. CONCLUSIONS

The CF2L protein concentrate corresponds to a highly soluble fraction from crayfish surpluses. CF2L exhibits a significant amount of the essential amino acids with excellent nutritional importance that makes this product as a potential food ingredient (i.e. gelation).

SAOS measurement shows a high dependence of the gel strength on pH. Thus, near the IEP, the absence of net charges leads to a gel with strong viscoelastic properties (showing the highest values for $G'$ and $G''$), with higher amount of disulphide bonds and enhanced WHC. However at pH 8.0 and 2.0, hydrophobic interactions are more relevant, leading to a gel with lower viscoelastic properties and WHC. This fact is related to the presence of repulsive interactions among charged protein surfaces, which inhibit the development of S-S bonds. Gels at pH 2.0 need special attention since a weak gel is obtained, probably due to the protein hydrolysis with a large increase in the relative amount of the peptide fraction.

Finally, antioxidant activity was found using three different compounds. The highest value found (expressed as mEq of PG) was for ABTS assay at pH 8.0. However, noticeable antioxidant ability was also found for Folin-Ciocalteu and DPPH reagents, where the dependence on pH is not as strong as the one found for the above mentioned ABTS assay.

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Figure 1

Crayfish (CF)

Grinding and sieving

CF raw pulp

Centrifugation

20 wt. % 70 wt. % 10 wt. %

Phase density

CF1P CF2P CF3P

Freeze-drier

CF2L
Figure captions

**Figure 1.** Diagram of the procedure carried out in order to obtain the CF2L protein concentrate.

**Figure 2.** Amino acid profile of CF2L protein concentrate obtained from HPLC.

**Figure 3.** Protein solubility as a function of pH value (A) and SDS-PAGE of CF2L (B).

**Figure 4.** Temperature ramp tests performed at constant frequency, 6.3 rad/s, and constant heating rate, 5ºC/min, for CF2L dispersions (12 wt. %) at three different pH values (2, 6.5 and 8) followed by a isothermal step (90ºC, 30 min) and a cooling step (rate: 5ºC/min) (A), as well as the evolution of linear viscoelastic properties for CF2L gels as a function of frequency (from 0.06 to 50 rad/s) (B) performed at three different pH values (2, 6.5 and 8).

**Figure 5.** Effect of pH on interactions nature: Ionic bonds, hydrogen bonds, hydrophobic interactions and disulphide bonds (A) and water holding capacity (B) of gels performed at 3 different pH values (2, 6.5 and 8).

**Figure 6.** Antioxidant activity of CF2L gels at three different pH values (2, 6.5 and 8) behind three different compounds: ABTS, DDPH and Folin-Ciocalteu, compared to the activity of the reference compound (Propyl Gallate, PG).