Elsevier Editorial System(tm) for Food and Bioproducts Processing
Manuscript Draft

Manuscript Number: FBP-D-16-00486R1

Title: Rheological properties and antioxidant activity of protein gels-like systems made from crayfish concentrate and hydrolysates

Article Type: Full Length Article

Keywords: Antioxidant gels; Crayfish; Gelation; SAOS; WHC

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Abstract: Crayfish protein obtained from a renewable, available, and low-cost raw material, may be regarded as an excellent alternative for the development of innovative food products on the basis of its nutritional value and bioactivity.
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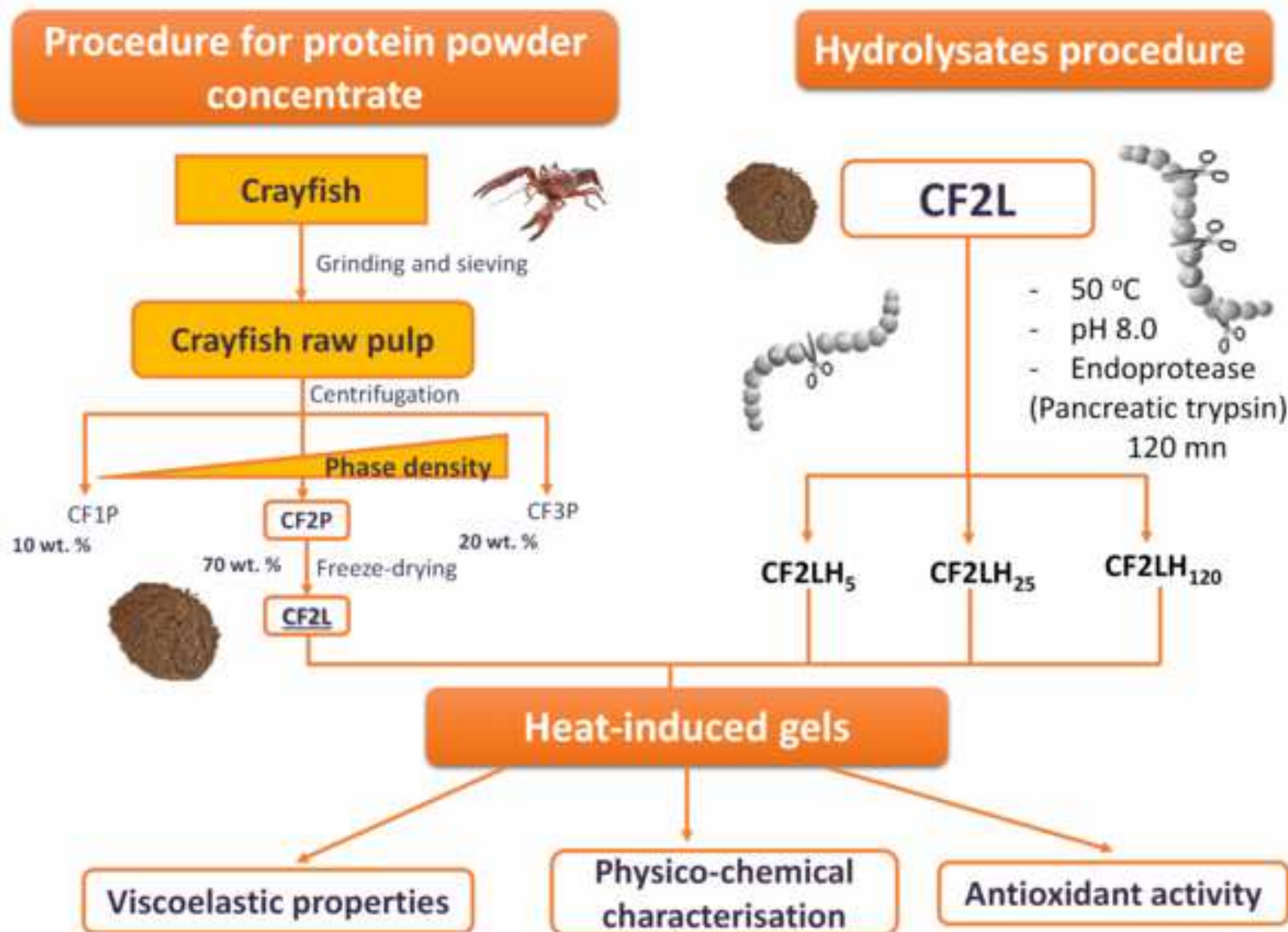
Both the pH and the degree of hydrolysis exerted a strong influence on the gelation behaviour and the properties of the final gels. The results obtained may be explained in terms of the protein interactions involved in the gel formation. A remarkable antioxidant activity against ABTS and DPPH compounds was observed. The effects of pH and the hydrolysis degree on antioxidant activity were only moderate.

Graphical Abstract (for review)



Highlights

- Crayfish protein is an excellent alternative for food products.
- Crayfish-based gels studied display a broad range of gel strengths.
- The pH and the degree of hydrolysis have a strong influence on the gelation.
- Antioxidant activity is highly influenced by the pH and the degree of hydrolysis.

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Rheological properties and antioxidant activity of protein gels-like systems made from crayfish concentrate and hydrolysates

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Abstract

Crayfish protein obtained from a renewable, available, and low-cost raw material, may be regarded as an excellent alternative for the development of innovative food products on the basis of its nutritional value and bioactivity.

Thermal gelation behaviour and antioxidant activity of gels made from non-denatured crayfish protein concentrate or their hydrolysates were studied at three different pH values (2.0, 6.5 and 8.0). Some physicochemical and rheological properties, as well as water holding capacity of the final gel-like systems were also determined.

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1. Introduction

The current growth of population is leading to face the need for finding new sources of high quality protein. In order to incorporate these proteins into food products they should possess some desirable functional properties ie. gelling properties. Moreover, as the interest in food components being beneficial for health is increasing, bioactive properties such as antioxidant properties are also important (Balasundram et al., 2006).

The freshwater crayfish *Procambarus Clarkii* was introduced in the Guadalquivir marshlands (south of Spain) in the early seventies. As a result of abundant food, favourable weather conditions and the lack of predators, the crayfish population has undergone a fast increase over the last decades, leading to its consideration as an invasive species (Geiger et al., 2005). This species is commercialised for human consumption, however an important amount of crayfish is not used for this purpose, producing a big amount of low-value by-product, which is used for animal feed. An alternative to improve the quality, and the value, of these surpluses may be the use of crayfish-meat to produce a non-denatured protein concentrate, which allows storing for later use. This protein concentrate may be used in different types of food products such as gels or emulsions (Romero et al., 2011, 2008). Hydrolysis of this protein system may be a very interesting alternative to improve bioactive properties, since bioactive peptides and protein hydrolysates are called to be valuable functional ingredients (Vastag et al., 2010).

Over the last decade, there have been an increase in the interest of bioactive properties of proteins and hydrolysates to be used in food products (Mine et al., 2010). Although natural protein derivatives usually exhibit less powerful effects than synthetic pharmaceutical drugs, they tend to accumulate on lower levels in the human body, reducing side effects (Li-Chan, 2015). More specifically, meat and fish hydrolysates may offer huge potentials as novel sources of bioactive peptides, since they can display antihypertensive, antioxidant, antimicrobial and antiproliferative effects (Kitts and Weiler, 2003; Wang et al., 2015; Wen-Ch et al., 2003).

Antioxidants as bioactive compounds are quite important in the daily diet of humans, because free radicals are continuously produced during the human metabolism and may induce damages to organic biomolecules that may promote changes in DNA and, as a result, serious health problems (Gey, 1993). In fact, antioxidative activity has been found in numerous proteins, peptides and protein hydrolysates (Elias et al., 2008; Sarmadi and Ismail, 2010).

From a different perspective, techno-functional properties are important for the texture and appearance of food products (Damodaran et al., 2007). Some of these properties such as gelling and emulsifying capacities have been specifically evaluated using crayfish protein isolate (Bengoechea et al., 2008; Romero et al., 2009a, 2009b, 2008). However the bioactive potential of a non-denatured crayfish concentrate compared to different hydrolysates obtained from crayfish has not been analysed earlier.

The aim of this research was to evaluate the gel properties and bioactive potentials of gels made from non-denatured crayfish protein concentrate as well as three hydrolysates obtained from it, at three different pH values (2.0, 6.5 and 8.0). To achieve these objectives, some physicochemical properties of the different systems (concentrate and hydrolysates) were determined. Some of the properties of the protein concentrate were

previously determined (Felix et al. 2017), including SDS-PAGE electrophoresis and protein solubility as a function of pH (reporting an isoelectric point around 5). Furthermore, the evolution of rheological measurements of aqueous protein dispersions was monitored over temperature ramp-test in order to follow the gelation process. Further frequency sweep tests, to obtain mechanical spectra, and water holding capacity measurements of the final gel-like systems were carried out. The chemical nature of the different protein interactions was also determined to understand the mechanical response obtained. Finally, the antioxidant activity of the different gels was evaluated against different compounds.

2. Material and methods

2.1 Materials

Crayfish (CF) meat was separated from the shell by grinding and sieving and was kept frozen until use, this first stage was carried out by ALFOCAN (Isla Mayor, Sevilla, Spain). After thawing at 4ºC, CF pulp was homogenized and subjected to centrifugation at 15,000 x g for 15 min, obtaining three different phases: a heavy phase, CF1P (c.a. 20 wt. %), an intermediate phase (CF2P), which mainly consists of water (c.a. 70 wt. %) and a low density phase, CF3P (c.a. 10 wt. %). The CF2P was the selected phase because it is the water soluble protein fraction and it represents the highest protein content. Subsequently, the intermediate phase (CF2) was freeze-dried in order to obtain a protein-rich fraction, denoted by CF2L.

CF2L was subjected to a hydrolysis process using pancreatic trypsin. The enzyme/substrate ratio (E/S) was set at 1:100 (w/w). The pH of the dispersion was kept constant at 8 during the entire period of hydrolysis by the addition of 3 M NaOH. The resulting hydrolysates were heated in boiling water for 10 min to inactivate the enzyme, and centrifuged at 10,000 x g for 15 min. Finally, the supernatant was freeze-dried in

order to obtain a protein-hydrolysate powder. The degree of hydrolysis was determined by formol titration. To obtain the hydrolysis kinetic, the reaction was stopped at different time periods (5, 10, 15, 20, 25, 30, 45, 60, 90 and 120 min), measuring the degree of hydrolysis after freeze-drying of the protein dispersions

All other reagents used 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 Analytical methods

2.2.1 Protein composition.

The protein content of all the systems studied was determined in quadruplicate as % N x 6.25, using a LECO CHNS-932 nitrogen micro analyser (Leco Corporation, St. Joseph, MI, USA). In the same way, lipid, moisture and ash contents were determined according to A.O.A.C. methods (2000).

Separation of sarcoplasmic protein fraction (SPF) and myofibrillar protein fraction (MPF) was performed according to the method of Hashimoto et al. (1979).

Sample (1 g) was homogenized with 10 ml of phosphate buffer A (Na2HPO4; 15.6 mM and KH_2PO_4; 3.5 mM) and centrifuged at 5,000 x g for 15 min at 4°C. The supernatant represents the SPF.

The pellet was homogenized with 10 ml of phosphate buffer B (Na2HPO4, 15.6 mM; KH_2PO_4, 3.5 mM and KCl, 0.45 M) and centrifuged at 5,000 x g for 15 min at 4°C. The supernatant represents the MPF. The protein content was measured using the modified Lowry method (Markwell et al., 1978).

2.2.2 Free and total sulfhydryl groups.

Free and total sulfhydryl groups of all samples were determined using the method developed by Beveridge et al. (1974) and Thannhauser et al. (1984), respectively. 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 buffer. Dispersions were stirred at 25 ºC for 10 min at 500 rpm in a thermomixer and centrifuged at 15,000 x g (10 min, 10 ºC). The supernatant was incubated with Ellman's reagent (5,5'-dithiobis-(2-nitrobenzoic acid), DTNT) (4mg DTNB/mL methanol) and 1 mL NTSB (2-nitro-5-thiosulfobenzoate) was used in the case of the total sulfhydryls. Absorbance at 412 nm was measured in a Genesys-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 Surface hydrophobicity of proteins (H_0).

H_0 of soluble proteins in protein extracts (pH 8.0) was measured according to Kato and Nakai (1980), using the fluorescent probe 1-anilino-8-naphtalene-sulfonate (ANS). Protein extracts were diluted with 0.05 M phosphate buffer (pH 8) to obtain protein concentrations ranging from 5 to 0.005 mg/mL. Then, 40 μL of ANS (8.0 mM in the same buffer) were added to 2 mL of sample. Fluorescence intensity (FI) was measured with a Tucan Infinite 200 PRO Microplate Reader (Tecan Group Ltd, Männedorf, Switzerland), at wavelengths of 365 nm (excitation) and 484 nm (emission). The initial slope of fluorescence intensity versus protein concentration was used as an index of protein hydrophobicity (H_0).

2.2.4 Water imbibing capacity (WIC)

WIC of all protein systems was determined by using a modification of the Baumann apparatus. This device consists on a funnel connected to a horizontal capillary. About 50 mg of sample was dusted on a wetted filter paper which was fastened to a filter

placed on top of the funnel filled with water. The apparatus was kept at 20 ºC. The uptake of water by the sample at equilibrium was read in the graduated capillary and expressed as mL of water imbibed per g of protein concentrate. Determinations were performed at least in triplicate.

2.3 Gel characterisation

2.3.1 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. In fact, all tests were carried out at a stress clearly lower than the critical value for linear viscoelasticity. The gelation process was simulated through heating in situ in the rheometer with three different stages: (i) The first step consisted of a temperature ramp carried out at constant heating rate (5 °C/min) from 20 °C to 90 °C; (ii) After the first step, a isothermal oscillation was performed at 90 °C for 30 min; (iii) Subsequently, a temperature ramp was carried out at constant heating rate (5 °C/min) from 90 °C to 20 °C. All stages were performed at constant frequency (6.28 rad/s). Finally, frequency sweep tests (0.06 - 64 rad/s) were carried out at 20 °C in order to obtain mechanical spectra. The geometry used was cone-plate geometry (50 mm, 2°) and all gels studied were subjected to the same thermorheological history (30 min at room temperature) before performing any rheological test.

2.3.2 Protein interactions

Solubility of all gels in a number of selected solutions was carried out in order to determine ionic bonds, hydrogen bonds, hydrophobic interactions and disulphide bonds according to the method of Careche (1995) and Matsumoto (1980). The selected

solutions were as follows: 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, disulphide 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).

2.3.3 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 × g for 5 min at 5 °C (Queguiner et al., 1989). Water-holding capacity (WHC) was expressed as the percentage of the initial water remaining in the gel after centrifugation.

2.4 Antioxidant activity

2.3.1 DPPH Assay

The 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay was performed as described by Brand-Williams et al. (1995). 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 (PG) and gel solutions at 10 wt. % in methanol were prepared. An aliquot of DPPH* solution (2.9 mL) was well mixed with 0.1 mL of a sample or methanol (blank). After incubation at

room temperature for 20 min, the absorbance at 515 nm was recorded. Water was used as a blank. Results were expressed in propyl gallate equivalents.

2.3.2 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 $ABTS^{\bullet+}$ 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.

2.3.3 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 PG 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% Na_2CO_3 solution (20 g/L) was added. The volume was brought up with deionized water. The absorption at 725 nm was read after incubation at room temperature for 1 h. Water was used as a blank. Results were expressed as equivalent activity of the gel compared to the reference (PG).

2.5 Statistical analysis

At least three replicates of each measurement were carried out. The linear least squares fitting technique was used to correlate the experimental hydrolysis kinetics to Michaelis-Menten model, using the correlation coefficient (R^2) to assess the degree of fitting. 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 Characterisation of protein systems

The determination of SPF and MPF indicates that 35 wt.% of CF2L protein is myofibrilar and 65 wt.% is sarcoplasmic. These results are consistent with the molecular weight observed in a previously reported SDS-PAGE analysis (Felix et al., 2017). Electrophoresis of CF2L showed a light band of myosin and some marked bands that range from 28 to 53 kDa, which were mainly associated to sarcoplasmic proteins.

Figure 1 shows the degree of hydrolysis obtained for a protein dispersion containing 10 wt. % CF2L, by using trypsin enzyme. The kinetics of the hydrolysis is characterised by an initially rapid rate followed by a dramatic decrease in reaction rate. Then, an asymptotical value in the degree of hydrolysis is reached at long time. This is a typical behaviour for enzymatic hydrolysis reactions. Accordingly, it has been fitted to the Michaelis-Menten model (also plotted in Fig. 1), which is a kinetic model widely used for enzyme catalysis. This model describes enzymatic reactions and relates the overall reaction rate to the concentration of a substrate. Thus, a similar evolution was previously found for plant protein such as rice protein and gluten (Kong et al., 2007; Zhao et al., 2012), as well as for fish protein (Kristinsson and Rasco, 2000).

Three different degrees of hydrolysis (after 5, 25 and 120 min) were selected. Table 1 shows the chemical composition and some physicochemical properties of all the systems studied. The protein content of the CF2L system is ca. 80 wt. %, which corresponds to a protein concentrate according to Pearson classification. However, the hydrolysates exhibit lower protein content, which is a clearly consequence of the hydrolysis process. This effect is probably related to the pH adjustment required which

leads to an increase in ash content. The increase in moisture content brought about by protein hydrolysis also reduces the total amount of protein. The CF2L system contains up to 5 wt. % of lipids. This concentration is similar to previous results and could be attributed to a high content of phospholipids (Chalamaiah et al., 2012). However, the relative lipid content in the hydrolysates is reduced as hydrolysis proceeds, as it was in the case of protein content.

As regards sulfhydryl content, there are no significant differences ($p<0.05$) in disulphide bonds for any of the systems studied. The total disulphide bonds in these systems is about twice that of albumen protein concentrate, even though ovalbumin is considered as a protein rich in S-S and –SH groups (Mine et al., 2010). On the other hand, the total sulphide content in crayfish systems is lower than the values reported for legume proteins (Tang, 2008).

A comparison between total disulphide bonds and free sulfhydryl groups shows that the amount of –SH groups is relatively low. Furthermore, a decrease in free –SH was observed over hydrolysis, suggesting that protein aggregation takes place. Since the extraction procedure carried out is rather gentle, this low amount of –SH groups cannot be attributed to protein aggregation or denaturation. This chemical property is probably related to protein conformation, which is retained after the freeze-drying procedure. In general, the amount of free sulfhydryl may be considered acceptable and would suggest that a desirable density of crosslinking could take place during the gelling stage (Buonocore et al., 2003).

Table 1 also shows an increase in protein surface hydrophobicity (H_0) which becomes significant above the lowest degree of hydrolysis studied. This increment obtained for H_0 when a protein is hydrolysed was previously found by Zheng et al. (2015). Protein surface hydrophobicity, in addition to S-S bonds, is important for the

gelation stage. Thus, non-covalent interactions, particularly hydrophobic interactions, are quite important for the aggregation tendency of a protein, in spite of the fact that they are not the driving force. The increase in H_0 after hydrolysis suggests that protein could be unfolded, although protein aggregation is not promoted (Zheng et al., 2015).

Water imbibing capacity (WIC) displays always very low values, compared to other protein systems such as soy or wheat protein concentrates (Linlaud et al., 2009). These results may be related to the high solubility of these protein systems, since the insoluble protein fraction usually contributes to increase WIC, as a consequence of its high denatured state.

3.2 Characterisation of gels

3.2.1 Rheological Characterisation

Figure 2 shows the evolution of SAOS viscoelastic properties (the storage modulus, G' and the loss modulus, G'') at constant protein concentration (12 wt. %) for three different pH values (2.0, 6.5 and 8.0) over the thermal gelation process.

The thermomechanical profiles obtained over the gelation process were classified according to the pH value. In Figure 2A, the gelation for all the systems studied (CF2L, $CF2LH_5$, $CF2LH_{25}$ and $CF2LH_{120}$) at pH 2.0 can be observed. None of them exhibits any gelling potential under the processing conditions studied, since the highest G' achieved is around 1 Pa. Hence, at this pH no strong attractive interactions between protein chains were found, and very weak gel-like products were obtained. In fact, they behave as high viscous liquids that cannot keep their shapes.

Regarding the degree of hydrolyses, only CF2L and $CF2L_5$ exhibit a slight thermal-induced reinforcing potential at pH 2.0, where the system after heating stage is slightly more structured than at the beginning. It seems that the hydrolysis inhibits the cross-

linking and other interaction potentials of CF2L proteins derivatives at this pH (Surendra Babu et al., 2015).

Figure 2B shows the gelation process for all the system studied at pH 6.5. At this pH value the evolution over the thermal cycle is quite different and the gelation profile is more similar to other typical protein gelation processes (Cordobes et al., 2004; Ikeda et al., 1999; Romero et al., 2009b). The behaviour is characterised by a first stage below 45^{0}C where a smooth decrease in G' and G'' takes place. Firstly, an increase in temperature involves an increase in mobility of the protein chains due to thermal agitation, such that electrostatic and hydrogen bonds interactions are typically reduced. As a consequence, the viscoelastic properties of the dispersions decrease. This decrease, which has been previously attributed to the oxidation of sulfhydryl groups, turns to be quite apparent in the early temperature range of 25-50^{0}C (Acton and Dick, 1988). This effect has been also attributed in meat proteins to the denaturation of myosin chains, since α-helices in the tail segment begin to unfold around 30–40 ºC, which seems to yield an initial reduction in consistency (Kim et al., 2005; Romero et al., 2009b; Yoon et al., 2004)

Above 60 ^{0}C, an increase in temperature value involves a marked increase in both modulus (G' and G''). This effect can be a consequence of structural changes of partially denatured proteins either in the form of globular proteins (from the SP fraction) or of helical rod segments of myosin proteins (MP fraction), which promote network formation through sulphide-bonds (Acton and Dick, 1988). Network development proceeds over the isothermal stage of the cycle applied, although showing much slower kinetics.

Finally, at the cooling stage, a slight decrease followed by an increase of both moduli (G' and G'') takes place. Physical interactions (e. g. hydrogen bonds, hydrophobic

interactions…) have been postulated to be able to increase both mechanical moduli. These interactions are important in the stabilization of the protein system (Lanier et al., 2005).

It is worth mentioning that all the systems, except for $CF2LH_{120}$ protein hydrolysate, exhibit similar evolution of the viscoelastic functions over protein cross-linking. The maximum G' value obtained for CF2L, $CF2LH_{5}$ and $CF2LH_{25}$ is around 1,000 Pa, without any significant difference ($p<0.05$) among these systems. In contrast, $CF2LH_{120}$ system cannot achieve the same level of gel strength. Previously, Jin et al. (2014) found that the gel ability is restricted by the degree of protein hydrolysis. Hydrolysis tends to decrease gelation properties, because it reduces the molecular weight, although increases the hydrophobicity of the protein chains (Jin et al., 2014). However, a proper degree of hydrolysis may increase the effective hydrophobicity of certain globular proteins by means of the exposure of buried non-polar residues. On this basis, a high degree of hydrolysis, as in $CF2LH_{120}$, seems to negatively affect to the gel formation efficiency.

Figure 2C shows the gelation process for the systems studied at pH 8.0. At this pH value the protein hydrolysates exhibit an intermediate behaviour between pH 2.0 and pH 6.5. Thus, the initial decrease in both moduli (G' and G'') is again taking place below 45 ^{0}C. The subsequent increase in gel strength with increasing temperature takes also place. However, in this case, the presence of a net negative charge at this pH entails formation of a gel that is weaker than that obtained near the IEP. For the protein concentrate (without hydrolysis) the gelation process follows a similar pattern to that one observed at pH 6.5. In contrast, the evolution for the hydrolizates is much slower than at pH 6.5 and the temperature-induced gelation is delayed to an extent that depends on the degree of hydrolysis. These results suggest that the growth of aggregates

becomes slower with increasing the hydrolysis degree, resulting in the delay in the gel development. The cooling stage brings about again the contribution of physical interactions (i.e. hydrogen bonds). Once again, the system with the highest degree of hydrolysis ($CF2LH_{120}$) is the one showing the lowest gel ability. Interestingly, an enhancement in the final viscoelastic properties can be observed for $CF2LH_5$ and $CF2LH_{25}$ protein hydrolysates as compared to the original CF2L protein system. At the end of the experiment $CF2LH_5$ is the system with the highest elastic modulus, however the gel system with the lowest tan δ (ratio G''/G') is the $CF2LH_{25}$ protein-based gel, reflecting the highest solid character.

It is worth mentioning, as described above, that protein hydrolysis involves an increase in salt content. In the preparation of dispersions for the gelation process, as the amount of soluble protein was constant, the higher solubility of the hydrolisates partially compensates such increase. As a result, no significant differences in salt content were obtained for the gels prepared from the protein hydrolisates. However, CF2L-based gels contain less salt than those prepared from the hydrolisates (around 1.6 and 2.8%, respectively). In any case, this difference in salt content does not seem to affect the gel properties at pH 2 and 6.5. Thus, only the difference found between the gel prepared from CF2L and CF2LH5 at pH 8.0 could be partially attributed to the increase in salt content.

Figure 3 shows the mechanical spectra by means of frequency sweep tests for all the systems studied after the gelation process, at three different pH values (2.0, 6.5 and 8.0). All the systems show a fairly weak gel-like behaviour, particularly the hydrolysate with the highest degree of hydrolysis.

Mechanical spectra of gels at pH 2.0 can be observed in Fig. 3A. In accordance with previously results for the gelation process, the gel strength is always very low. Results

showing inertial effects at high frequency, as corresponds to the weakest gels ($CF2LH_{25}$ and $CF2LH_{120}$), were discharged.

Figure 3B shows the mechanical spectra for all the gels studied at pH 6.5. In this case, there are significant differences between the high-hydrolysate gel ($CF2LH_{120}$) and the rest of gels. CF2L, $CF2LH_5$ and $CF2LH_{25}$ exhibit high values for both G' and G'' moduli that are nearly parallel, also showing low frequency dependence, which are characteristic features of a gel behaviour (Damodaran et al., 2007).

Finally, Figure 3C confirms the intermediate behaviour between pH 6.5 and 2.0, found for the temperature ramp tests. The above-mentioned enhancement produced by a moderate increase in the degree of hydrolysis is now fairly apparent. Thus, $CF2LH_5$ and $CF2LH_{25}$ exhibit higher values for G' and G'' than those corresponding to the protein dispersion CF2L. In any case, it is also noticeable that both systems display the lowest values for tan δ. This parameter related to the gel stability (Damodaran et al., 2007).

With regards to CF2L system, it seems to have the lowest frequency-dependence, which may indicate an acceptable gel strength, in spite of their relative low G' and G'' values.

Figure 4 shows the evolution of G' at 1Hz (G'_1) for all the protein system studied (CF2L, $CF2LH_5$, $CF2LH_{25}$ and $CF2LH_{120}$), at three different pH values (2.0, 6.5 and 8.0). As can be observed from this graph, both, the pH and the degree of hydrolysis exert a strong influence on the elastic modulus. However, two different behaviours can be found. Thus, whereas at pH 6.5 and 8.0 the hydrolysis induces an increase in G'_1 until reaching a maximum value (for the system $CF2LH_{25}$), at pH 2.0, the hydrolysis always induces a decrease in G'_1. This plot also confirms that gels at pH 6.5 display the highest elastic response, while the weaker gels are obtained at pH 2.0.

3.2.2 Physicochemical characterisation of gels

Figure 5 shows protein solubility values obtained for all the gels studied (CF2L, $CF2LH_5$, $CF2LH_{25}$, $CF2LH_{120}$) at three different pH values (2.0, 6.5 and 8.0) in solutions selected for their capacity to disrupt different kinds of bonds. According to Montero and Gomez-Guillen (1997) these solubility values allow quantifying different interactions among protein chains (ionic bonds, hydrogen bonds, hydrophobic interactions and disulphide bonds). As may be observed, all the specific interactions show generally higher values at pH 6.5 (Fig. 5B) and lower at pH 2.0 (Fig. 5A).

As may be seen in Fig. 5A, hydrophobic interactions are dominant at pH 2.0, in spite of the fact that all the interactions found at this pH are moderate (particularly in comparison to those observed at pH 6.5). These low values put forward the poor ability of crayfish protein derivatives to form heat-induced gels at pH 2.0. These results are consistent with those obtained from rheological measurements that, as previously mentioned, reveal the formation of a weak gel, which is particularly favoured by the absence of strong interactions.

Interestingly, $CF2LH_{25}$ leads to the highest degree of disulphide bonds formation at pH 2.0 and, as a result, to the lowest hydrogen bonds and ionic interactions. These results may explain why this system is the only one that does not show any thermal-induced enhancement in viscoelastic properties over the cooling stage.

Figure 5B shows protein interactions for all the systems studied at pH 6.5, which is relatively close to the isoelectric point (IEP ca. 5). A high level of ionic interactions, coming from the difference in solubility between buffer SB and SA, indicates a relevant participation of ionic bonds in gel formation, which can be related to the absence of repulsive interactions due to the proximity to the isoelectric point at this pH (Gomez-Guillen et al., 1997). Specific disulphide bonds are also relevant at this pH for all systems. This type of specific chemical interactions takes place above 65ºC (Acton and

Dick, 1988), and is related to the increase of both moduli found in temperature ramp tests. Disulphide bonds decrease with the degree of hydrolysis, however these systems are able to form a strong gel (except for $CF2LH_{120}$) since they still maintain a high level of hydrophobic interactions and hydrogen bonds.

In consonance with the viscoelastic properties, $CF2LH_{120}$ system is the gel showing the lowest interactions. It only has noticeable ionic interactions, which may be related with its small protein size. This small size of $CF2LH_{120}$ chains may allow them to reorganise easily in order to facilitate electrostatic interactions.

Finally, Figure 5C shows protein interactions for all systems at pH 8.0. At this pH, protein systems exhibit intermediate interactions between pH 2.0 and pH 6.5. None of the systems studied exhibits remarkable ionic interactions, as pH is far from the IEP, similarly to the behaviour found at pH 2.0. Therefore, repulsive forces seem to inhibit the participation of ionic bonds in gel formation.

Protein interactions can be related to the thermal gelation profile obtained through temperature ramp tests. Hence, CF2L exhibits a moderate increase of both moduli (characterised by a fast initial increased followed by a constant value). This result may be related to the moderate disulphide bonds found at this pH value. On the contrary, $CF2LH_5$ and $CF2LH_{25}$ show a constant increase in both moduli during the heating stage, which is related to those higher values found for disulphide bonds.

Again, $CF2LH_{120}$ is the system that exhibit lowest G' and G'' moduli and higher tan δ (i.e. G''/G'), denoting the formation of a fearly weak gel. It is noticeable that interactions for this system found at pH 8.0 are similar to those found at pH 6.5, this result is in accordance with the moduli obtained at pH 6.5 and 8.0.

3.2.3. Water Holding Capacity

Figure 6 shows the WHC results obtained for all the systems. WHC is a very important property of food gels because the separation of liquid from the gel network can involve physical modifications (e.g. shrinking or alterations in the palatability of the product) due to the moisture reduction (Mao et al., 2001).

WHC results are rather consistent with those obtained from SAOS measurements and protein interactions assessment. Thus, systems at pH 6.5 exhibit the highest values for WHC, where CF2L is the system which holds water to the largest extent. However, this capacity tends to decline as hydrolysis proceeds, showing the same trend as for WIC. This behaviour can be related to the he fact that CF2L protein is able to develop a gel network that is able to keep water in clusters better than the hydrolysate systems. It has been reported that particulate gels formed at pH near the isoelectric point are characterized by an increased pore size that leads to a decrease in capillary forces and therefore to a higher water loss (Chantrapornchai and McClements, 2002). However, hydrolysis may limit the formation of aggregates. This behaviour can be also observed at pH 8.0. However, at this pH WHC is generally quite lower, which may be related to their lower viscoelastic properties (as a result of lower protein interactions).

As for pH 2.0, the lowest values for WHC are typically obtained, as corresponds to their lower viscoelastic properties and the weaker protein interactions involved in gel formation.

3.2.4 Antioxidant Characterisation

Figure 7 shows the antioxidant activity of the gels made at different pH values (2.0, 6.5 and 8.0), and measured with three different methods: DPPH (Fig 6A), Folin-Ciocalteu (Fig. 7B) and ABTS (Fig. 7C). Results were expressed as mM Eq. of PG. As may be deduced from the different scales of the y-axis, the maximum antioxidant activity was obtained for ABTS, followed by DPPH and FC reagent. Antioxidant

activity against DPPH has been widely used to test the free-radical scavenging ability of different systems, from phenolic compounds (Espinoza et al., 2009) to protein systems (Ryan et al., 2011). No marked differences in the antioxidant activity against DPPH can be noticed. In fact ANOVA test reveals that pH does not yield any significant difference, whereas the degree of hydrolysis leads to a significant minimum at an intermediate degree ($p < 0.02$).

Another agent typically used for assessment of antioxidant activity of proteins and peptides is FC, which is also being used for the determination of protein content and the total phenols in wine Singleton et al. (1999). Antioxidant activity against FC is related to the ability to donate an electron (Kristinova et al., 2009). As may be observed in Fig. 6B, the values found for the antioxidant activity against FC are generally very low, which may be explained because FC reagent is not capable to measure lipophilic antioxidants due to the high affinity of the FC chromophore towards water (Berker et al., 2013). However, according to the ANOVA test performed a significant increase in this antioxidant activity is generally driven by an increase in the degree of hydrolysis and by a reduction in pH (both p values being much lower than 0.001).

ABTS is also frequently used to measure the antioxidant activity in many food products. Thus, ABTS radical cations are reactive towards several antioxidants such as phenolics, thiols and some vitamins (Walker and Everette, 2009). As may be observed, both effects (pH and degree of hydrolysis) exert a strong influence on the antioxidant activity against ABTS. Moreover, results from ANOVA test reveal occurrence of a significant interaction between both variables ($p=0.1$). It should be stressed that antioxidant activity against ABTS, in contrast to the other reagents, shows a maximum value at intermediate degree of hydrolysis. This effect also was found by Kong and Xiong (2006) for potato protein.

4. CONCLUSIONS

SAOS measurements reveal a strong dependence of gelation ability and gel strength on pH. Thus, near the IEP, the absence of net charges facilitates a proper development of gel network structures leading to fairly strong gel-like viscoelastic behaviour, with higher amount of disulphide bonds and enhanced WHC. On the other hand, at pH 2.0, the presence of repulsive interactions among positively charged protein surfaces tend to inhibit formation of S-S bonds, leading to weak gels with lower viscoelastic properties and WHC. At pH 8.0, at which protein surfaces show a negative net charge, an intermediate behaviour is found. Thus, at low hydrolysis degree, S-S bonds are produced in a lower extent as compared to gels formed near the IEP. However, hydrolysis seems to eliminate and event revert this inhibition effect.

The highest antioxidant activity was obtained against ABTS and the lowest when FC was used, since this reagent is specific for phenol compounds. As for the activity against DPPH it was also remarkable but not significantly affected by pH and moderately affected by the degree of hydrolysis. The most remarkable effects observed were a dramatic increase on the activity against ABTS with increasing pH (around one decade) and an apparent maximum in this activity at an intermediate degree of hydrolysis.

To sum up, the CF2L-based gels studied display a broad range of gel strengths, showing a strong dependence on the interactions involved in gel formation, which in turn depends on both the effect of pH and the degree of hydrolysis. However, regardless of their gel strength, their potentials in biofunctional food products may be considered as excellent. An additional advantage is that this gel-family offers a wide range of textures that may be tailored by properly selecting variables such as pH and the degree of hydrolysis.

Acknowledgements

This study was sponsored by the Andalousian Government, (Spain) (project TEP-6134). The authors gratefully acknowledge their financial support. The authors also acknowledge to the Microanalysis Service for providing full access and assistance to the LECO-CHNS-932 equipment. The authors also were supported by a grant from Iceland, Liechtenstein and Norway through the EEA Financial Mechanism. Operated by Universidad Complutense de Madrid

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Figure Captions

Figure 1. Hydrolysis kinetic for CF2L protein concentrate (fitted to Michaelis-Menten model) when pancreatic trypsin is used at 100 (w/w) E/S ratio at 50 ^{0}C and pH 8.0.

Figure 2. Temperature ramp tests performed at constant frequency (0.63 rad/s) for all systems studied (CF2LH, $CF2LH_5$, $CF2LH_{25}$ and $CF2LH_{120}$) at 12 wt. %. Three different pH values were evaluated: 2.0 (A), 6.5 (B) and 8.0 (C). An initial heating step (5 ^{0}C /min from 25 to 90 ^{0}C) was followed by an isothermal step (90 ^{0}C, 30 min) and a final cooling step (rate: 5 ^{0}C /min from 90 to 25ºC).

Figure 3. Evolution of linear viscoelastic properties for CF2L, $CF2LH_5$, $CF2LH_{25}$ and $CF2LH_{120}$ gels as a function of frequency (from 0.06 to 50 rad/s) performed at three different pH values: 2.0 (A), 6.5 (B) and 8.0 (C).

Figure 4. Evolution of G' at 1Hz (G'_1) for CF2L the protein system and their hydrolysates ($CF2LH_5$, $CF2LH_{25}$ and $CF2LH_{120}$), at three different pH values: 2.0, 6.5 and 8.0.

Figure 5. Protein interactions for all systems studied (CF2L, $CF2L_5$, $CF2L_{25}$ and $CF2L_{120}$) at three different pH values: 2.0 (A), 6.5 (B) and 8.0 (C).

Figure 6. Water holding capacity (WHC) for all systems studied (CF2L, $CF2L_5$, $CF2L_{25}$ and $CF2L_{120}$) at three different pH values (2.0, 6.5 and 8.0).

Figure 7. Antioxidant activity for all systems studied (CF2L, $CF2LH_5$, $CF2LH_{25}$ and $CF2LH_{120}$). Three gel pH values were evaluated (2.0, 6.5 and 8.0) behind three different compounds: DPPH (A), FC (B) and ABTS (C). Antioxidant activity was expressed as equivalent activity of PG.

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Table Captions

Table 1. Chemical characterisation of protein concentrate (CF2L) and protein hydrolysates ($CF2LH_5$, $CF2LH_{25}$ and $CF2LH_{120}$).

Table 1

Table 1

	CF2L	CF2LH$_{5}$	CF2LH$_{25}$	CF2LH$_{120}$
Protein Content (wt. %)	78.6 ± 0.5 (a)	72.6 ± 0.4 (b)	70. 8 ± 0.3 (c)	70.0 ± 0.2 (c)
Moisture (wt. %)	6.8 ± 0.1 (a)	7.33 ± 0.1 (b)	8.56 ± 0.6 (c)	8.50 ± 0.1 (c)
Lipid content (wt. %)	5.1 ± 0.3 6 (a)	3.8 ± 0.3 (b)	1.5 ± 0.1 (c)	3.4 ± 0.2 (b)
Ashes content(wt. %)	9.5 ± 0.6 (a)	16.4 ± 0.8 (b)	18.1 ± 0.2 (c)	17.6 ± 0.5 (b)
Free Sulfhydryl (µmol/g protein)	18.4 ± 0.6 (a)	8.9 ± 0.7 (b)	9.6 ± 0.5 (b)	10.1 ± 0.6 (b)
Disulphide bonds (µmol/g protein)	2240 ± 40 (a)	2175 ± 50 (a)	2380 ± 60 (a)	2150 ± 50 (a)
Surface hydrophobicity (H_0)	12.7 ± 0.3 (a)	12.7 ± 0.8 (a)	26.5 ± 1.2 (b)	21.1 ± 1.1 (b)
WIC (mL/g)	0.6 ± 0.1 (a)	0.3 ± 0.1 (b)	0.4 ± 0.1 (a,b)	0.4 ± 0.1 (a,b)
Degree of Hydrolysis (%)	0	11 ± 1 (a)	31 ± 1 (b)	45 ± 2 (c)

Figure1
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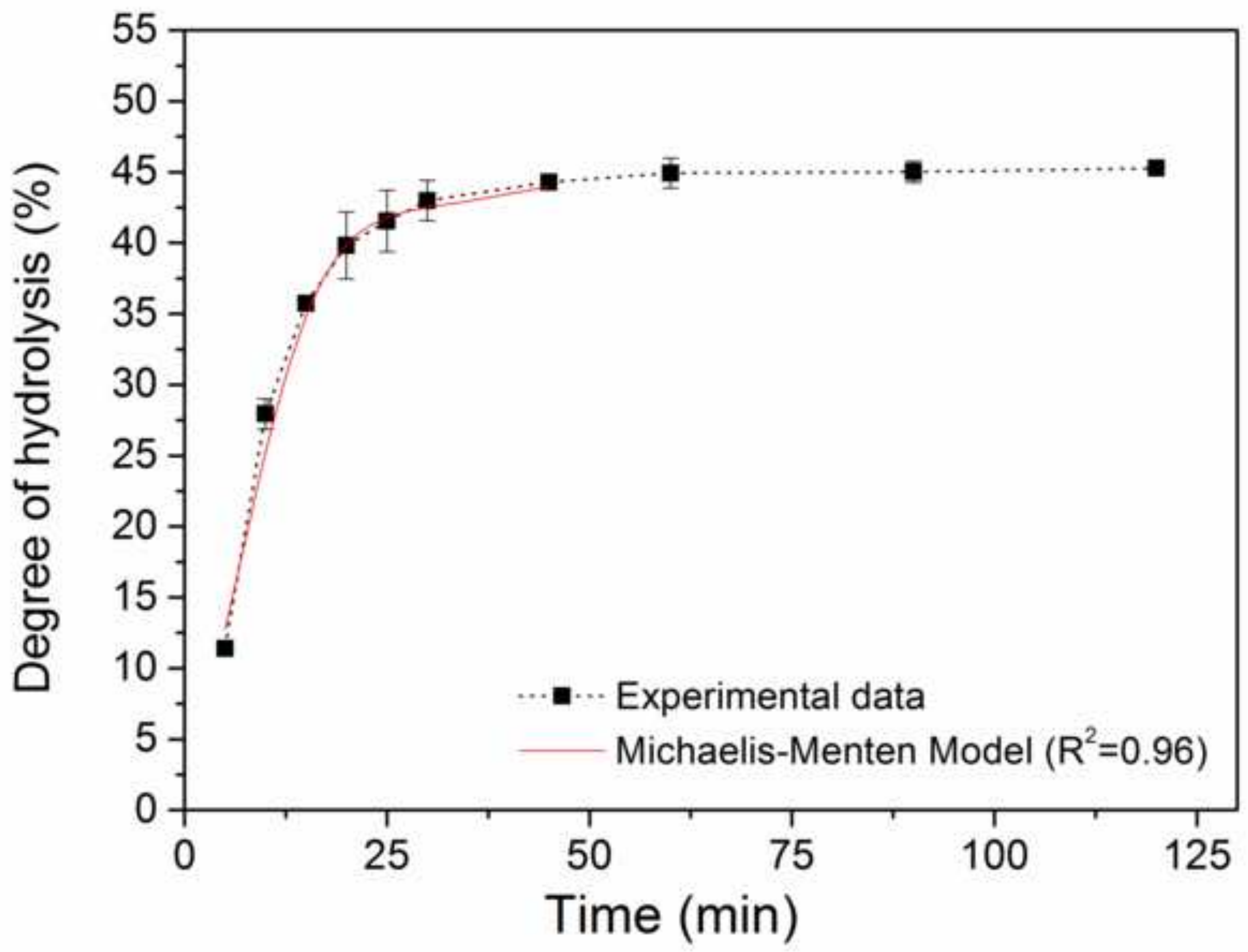

Figure2

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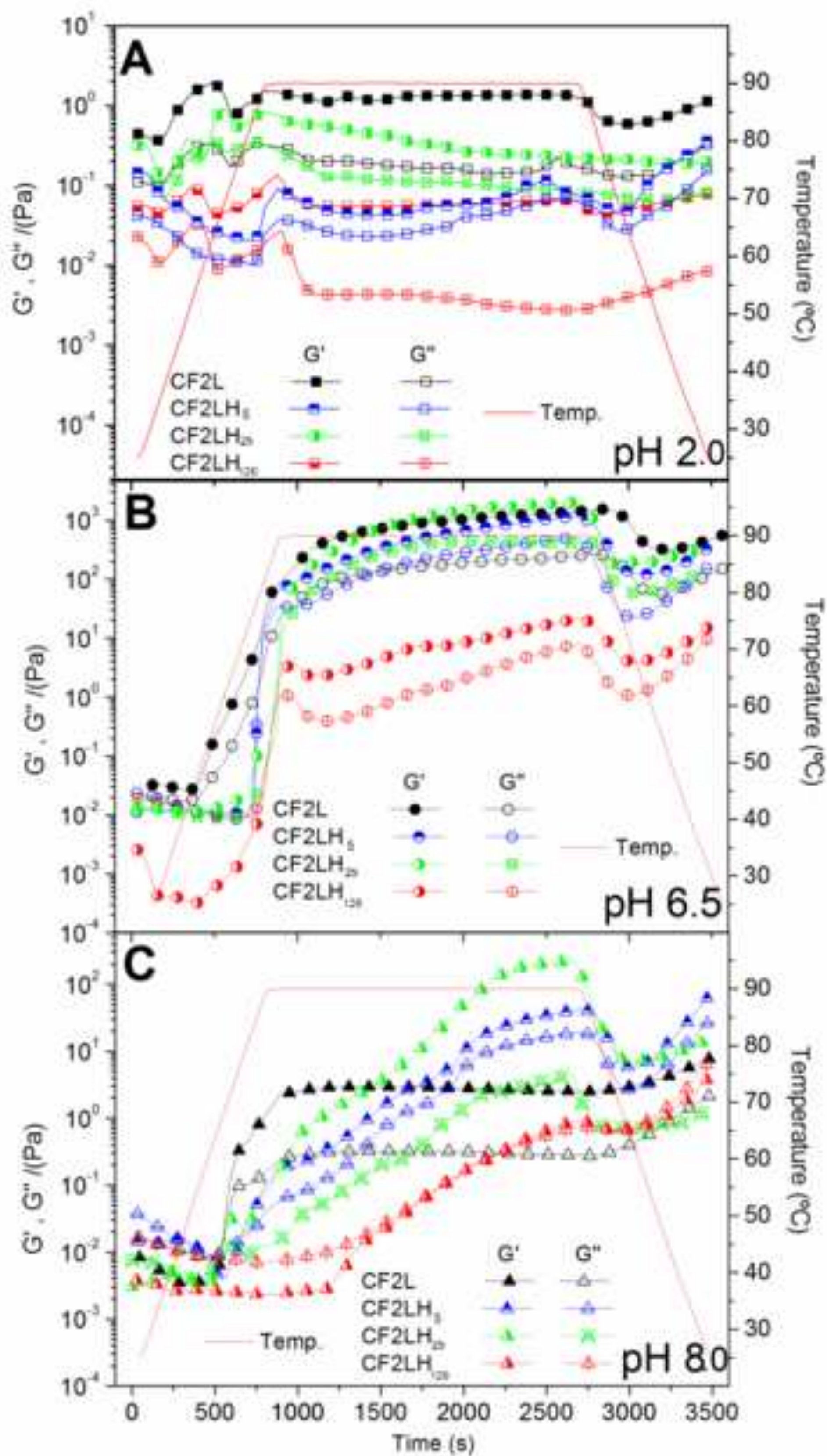

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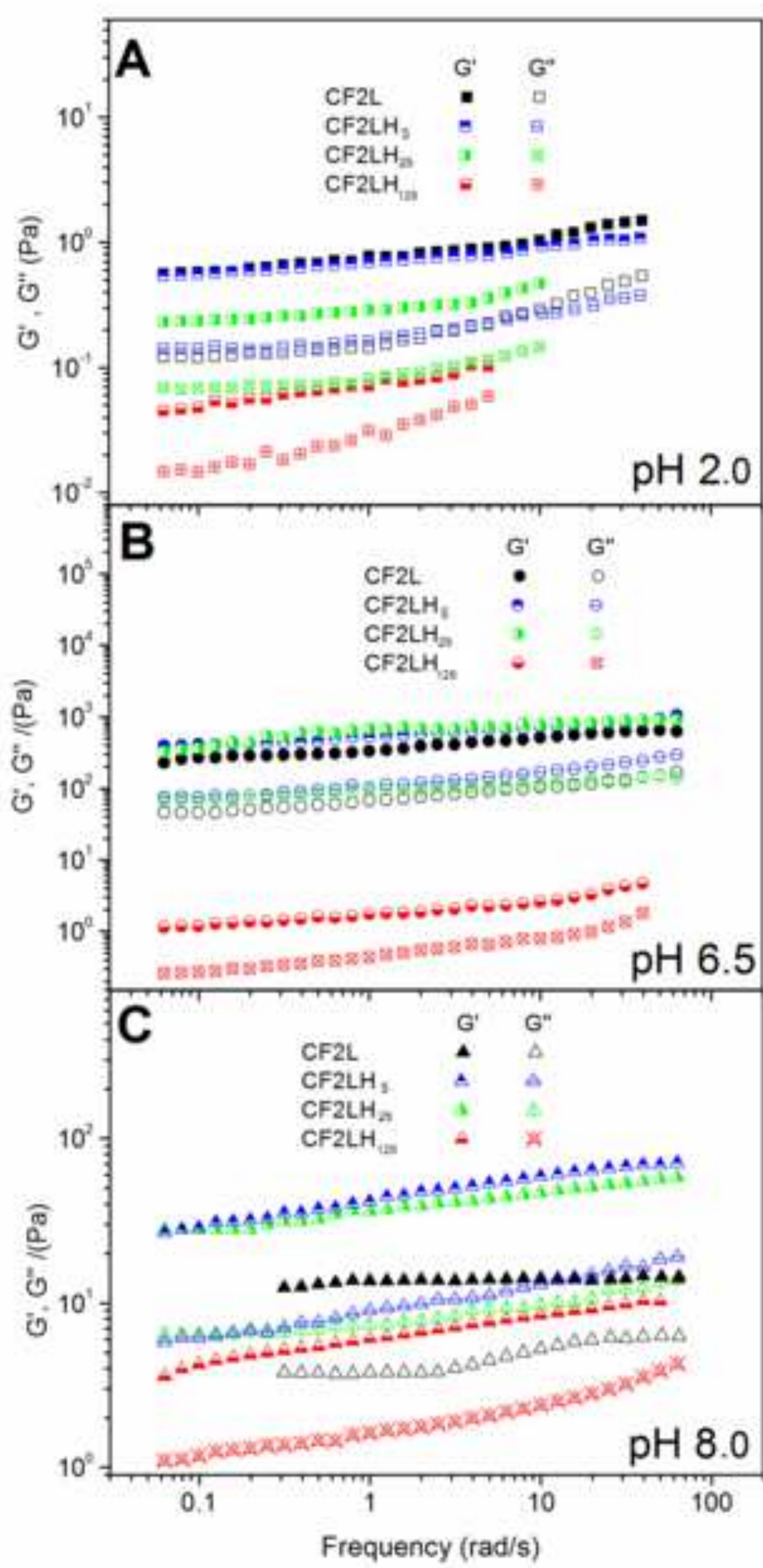

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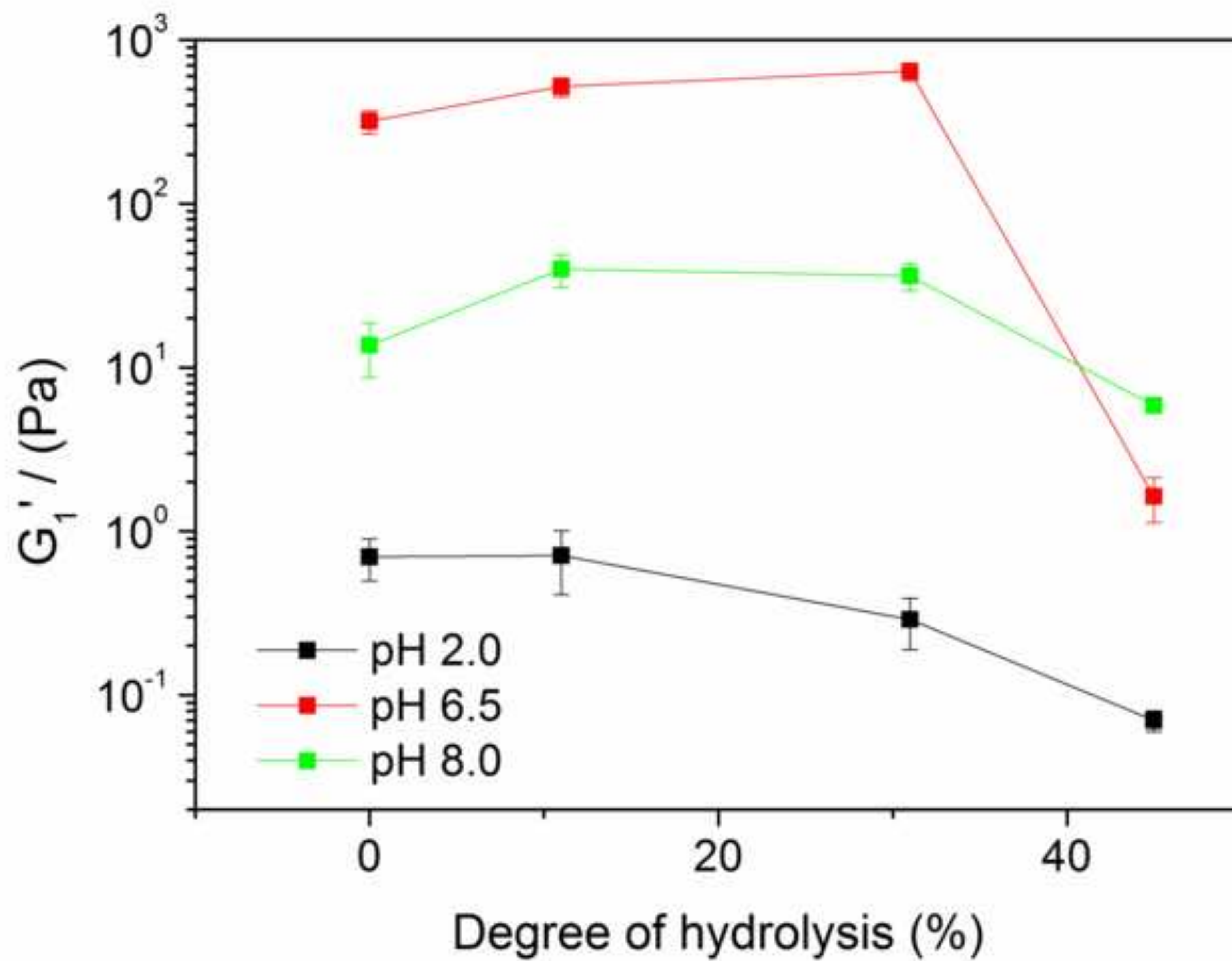

Figure5

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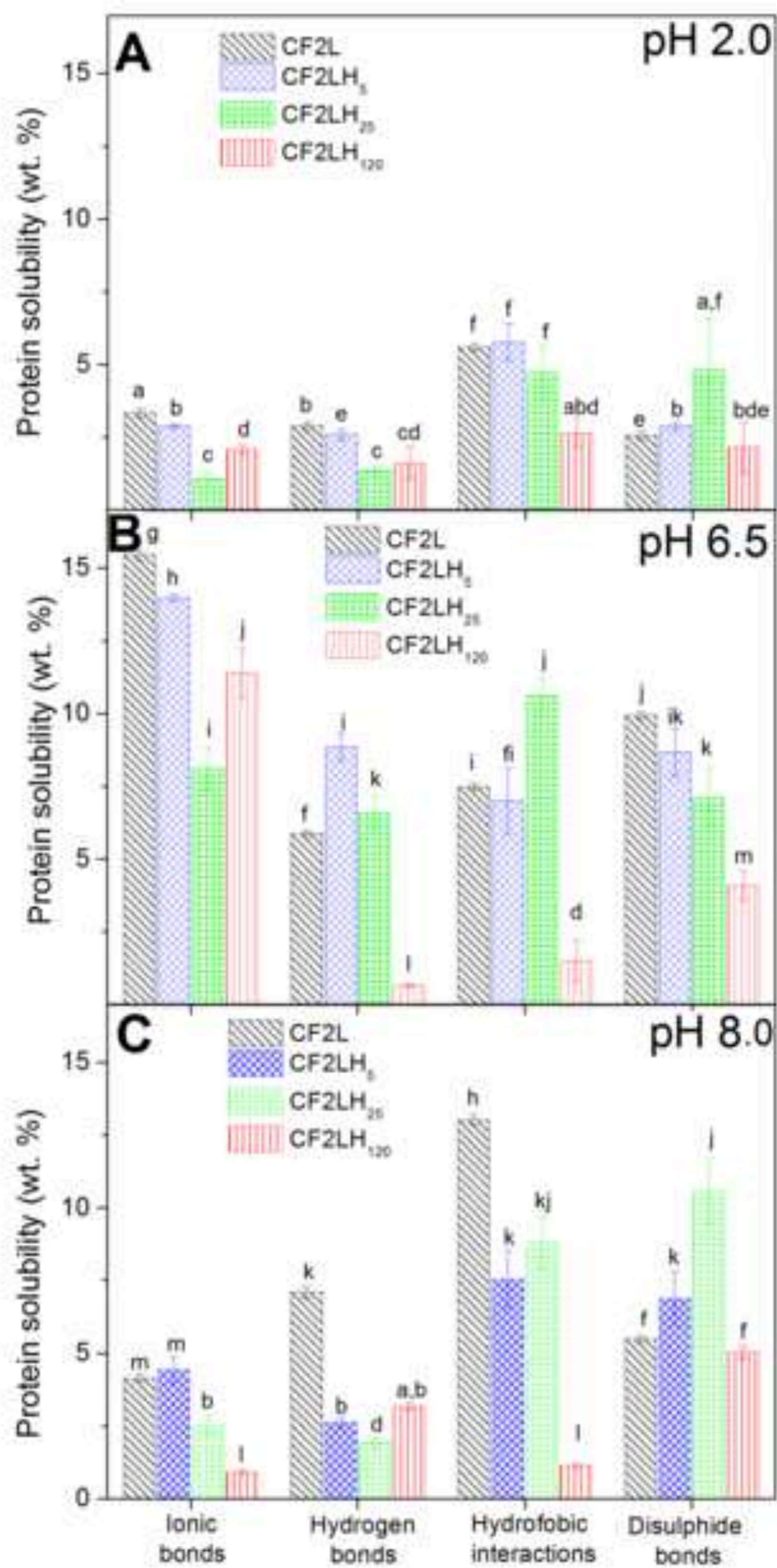

Figure6

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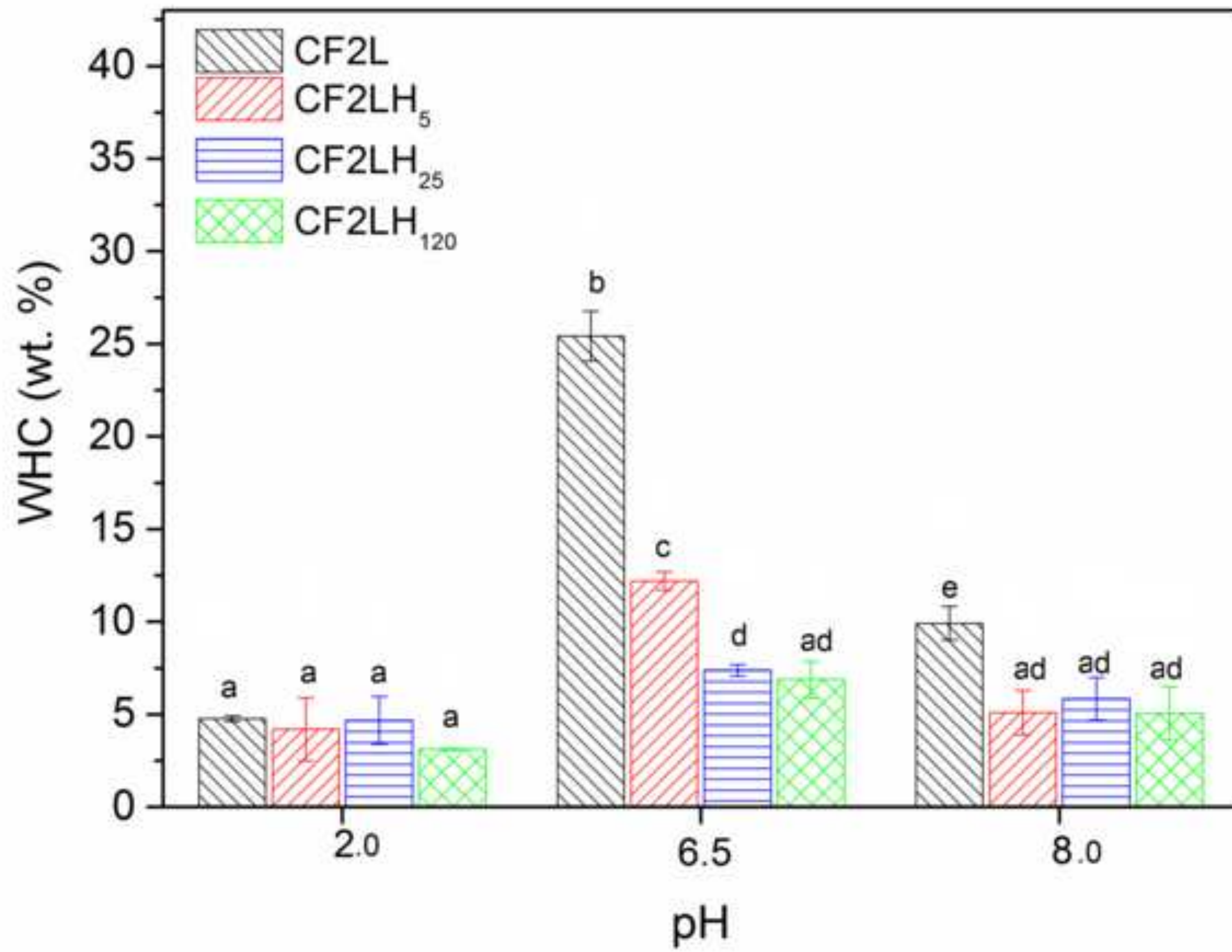

Figure7
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