Enzyme-assisted extraction of κ/ι-hybrid carrageenan from Mastocarpus stellatus for obtaining bioactive ingredients and their application for edible active films development

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Abstract

Two hydrolysates were obtained from dried *Mastocarpus stellatus* using alcalase. Phenolic content was partially removed from one of them. The phenolic-partially-removed hydrolysate (H) was found to be a potent Angiotensin-I converting enzyme (ACE) inhibitor. However, the phenolic-containing hydrolysate (Hp), showed a higher Folin-reactive substance content and antioxidant capacity (reducing power and radical scavenging capacity). Hp was therefore selected for the development of antioxidant Mastocarpus carrageenan-based films. F-Hp0 (without hydrolysate), F-Hp15 (with 15% hydrolysate) and F-Hp30 (with 30% hydrolysate) films were developed. κ/ι-hybrid carrageenan was the main film constituent and hydrolysate addition resulted in an increased sulfated proportion, higher protein content and higher number of hydrogen bonds. Therefore interactions between carrageenan helices, plasticizer and peptides in the film-forming solution were enhanced, especially in F-Hp15, and consequently the water vapour permeability (*WVP*) of the resulting film decreased. Nevertheless, F-Hp30 considerably improved transparency, UV/Vis light barrier, water resistance and elongation at break (*EAB*). Hp presence increased both puncture force (*F*) and puncture elongation (*E*), but not tensile strength (*TS*) or Young's modulus (*Y*). The addition of an increased concentration of hydrolysate to the films led to a considerable increase in the Folin-reactive substance content and the antioxidant activity, especially the radical scavenging capacity.

Keywords: Seaweeds, Mastocarpus, films, hydrolysate, antioxidant, antihypertensive

1. Introduction

Seaweeds have recently been included in Western diets as food and also as components of functional products because of their richness in polysaccharides, proteins,[1] minerals and vitamins. Moreover, seaweeds are an excellent source of bioactive substances such as sulfated polysaccharides, peptides and polyphenols with biological activities, including antioxidant and antihypertensive properties.[2-5]

In the last decade, new marine bioprocess technologies have allowed the isolation of substances with antioxidant properties or bioactive peptides by enzymatic hydrolysis.[6] Seaweeds have proved to be a good source of peptides and polyphenols.[7-9] Red algae (Rhodophyta) are known to have a high protein content, mainly composed of bioactive phycobiliproteins[10] and other wall proteins that might be more efficiently extracted by an enzyme-assisted treatment.[11,12]

Mastocarpus stellatus is one of the few carrageenophyte species on the Atlantic coast currently harvested for phycocolloid industry purposes, but it is still underutilized.[13]

Commercial carrageenan is commonly extracted at alkaline conditions (pH 7–9) at temperatures near boiling point (80–110 °C) for 3–4 h, providing yields of 20–40%.[13,14] However, high molecular weight carrageenan can also be extracted at mild temperatures (50 °C) for 1–5 h.[15]

κ/ι-hybrid carrageenan has been reported to be the main biopolymer structure extracted from *M. stellatus*,[16] although other components, such as proteins, minerals and polyphenols, are also present in significant amounts.[17] Mastocarpus enzymatic hydrolysis could produce both antihypertensive and antioxidant extracts, as previously reported with another species of the Rhodophyta phylum.[18,19] Protein hydrolysates from different origins have been incorporated in the formulation of protein-based films to improve or confer bioactivity.[20,21]

Mastocarpus extraction could be maximized by first performing an enzymatic hydrolysis at mild temperatures and alkaline conditions followed by carrageenan precipitation and bioactive compound isolation.

Antioxidants have been widely used as food additives both to improve lipid oxidation stability and to extend the product shelf life. The potential use of films as antioxidant releasing packages, capable of improving food preservation, would be a rather interesting application.

The aims of the present study were: (i) to obtain two different potentially bioactive hydrolysates with antioxidant and/or antihypertensive capacities from dried *Mastocarpus stellatus*, and (ii) to develop antioxidant Mastocarpus carrageenan-based films as an active edible food packaging material.

2. Materials and Methods

2.1. Seaweed sampling

Samples of *Mastocarpus stellatus* (M), kindly supplied by Porto-Muiños (Cerceda, A Coruña, Spain), were washed several times with running tap water and air-dried at 50 °C for 24–48 h in a ventilated oven. Seaweed samples were stored in sealed plastic bags at 2–4 °C for 1 week until use.

2.2. Unrefined biopolymer extraction

Dried seaweed was homogenized using an Osterizer blender (Oster, Aravaca, Madrid, Spain) with water in a 1:15 (w:v) proportion and kept for 12 h at 3 ± 2 °C. The seaweed was then filtered and subjected to two consecutive extractions in water at a 1:30 (w:v) proportion, at 91 °C for 2 h during the first step and 1.5 h during the second one. Each extract was centrifuged at 3000 rpm for 5 min (Sorvall Evolution RC Centrifuge, Thermo Fisher Scientific Inc., Landsmeer, The Netherlands) and blended. The supernatant was dried in an oven (FD 240 Binder, Tuttlingen, Germany) at 65.0 ± 0.8 °C and this constituted the Mastocarpus biopolymer extract, which was stored at room temperature.

2.3. Seaweed hydrolysis

Dried seaweed was mixed with 4% distilled water (w/v) and subjected to enzymatic hydrolysis for 3 h, using alcalase 2.4L (EC 3.4.21.14, 2.64 AU/g, Sigma-Aldrich Inc., St. Louis, MO, USA) in optimal conditions for enzymatic activity (pH 8, 50 °C). The enzyme-substrate (seaweed) ratio

was 1:20 (w:w) and the pH of the reaction was kept constant by addition of 1 N NaOH solution to the reaction medium using a pH-stat (TIM 856, Radiometer Analytical, Villeurbanne Cedex, France). The enzyme was inactivated by heating at 90 °C for 10 min. The hydrolysate was centrifuged at 7000 g for 15 min. The supernatant was subjected to two consecutive carrageenan extractions by precipitation with ethanol 1:3 (v/v) at 4 °C for 2 h. The precipitated carrageenan was dried at 65 ± 0.8 °C and weighed in order to evaluate extraction yields. The carrageenan-free liquid phase was centrifuged at 13000 g for 5 min. The supernatant was concentrated by rotary evaporation and was subsequently subjected to five organic extractions with ethyl acetate 1:5 (v/v), to remove most of the polyphenols and other compounds such as pigments. After decanting, the successive aqueous phases were concentrated by rotary evaporation. The concentrate was lyophilized, and this constituted the phenolic-partially-removed hydrolysate (H). The phenolic-containing hydrolysate (Hp) was obtained under the same conditions as described above, with the exception of the removal of polyphenol compounds with ethyl acetate. The Hp hydrolysate was selected for active film development.

2.3.1. Amino acid analysis of hydrolysates

The amino acid composition of the hydrolysates (H, Hp) was determined using a Biochrom 20 amino acid analyser (Pharmacia, Barcelona, Spain) according to the method described by Alemán *et al*.[22] The results were expressed as number of amino acid residues per 1000 residues.

2.3.2. Angiotensin-converting enzyme (ACE) inhibition of hydrolysates

Reversed-phase high performance liquid chromatography (RP-HPLC) was used to determine ACE-inhibitory capacity of the hydrolysates (H, Hp), according to the method described by Alemán *et al*.[22] The IC_{50} value was defined as the concentration of hydrolysate (μg/mL) required to inhibit 50% of ACE activity.

2.4. Film preparation

Three film-forming solutions were prepared to obtain the following films: F-Hp0 (without the addition of hydrolysate), F-Hp15 (with 15% hydrolysate) and F-Hp30 (with 30% hydrolysate).

Film-forming solutions (FS) (2% w/v) were prepared from Mastocarpus biopolymer extract by adding hot distilled water (90 °C) and homogenizing with a T25 basic Ultra-Turrax (IKA-Werke GmbH & Co. KG, D-79219 Staufen, Germany) at 17500–21500 rpm for 5 min. A portable pH-meter series 3 Star Orion with an electrode pH ROSS (Thermo Fisher Scientific Inc., Landsmeer, Netherlands) was used for pH measurements (6.4-6.8). Glycerol (Panreac Química S.A., Barcelona, Spain) was added at 10% (w/w) in relation to the seaweed extract content. The film-forming solutions were centrifuged at 3000 rpm for 3 min to remove air bubbles. Hp was then added at 15 and 30% (w/w) in relation to the seaweed extract content, and was magnetically stirred for 5 minutes. The film-forming solutions were cast into petri dishes and dried in an oven (FD 240 Binder, Tuttlingen, Germany) at 35.0 ± 0.8 °C for 21 h. All the films were conditioned at 58.0 ± 0.2% RH and 22 ± 1 °C for 4 days prior to analysis.

2.5. Viscoelastic properties of film-forming solutions (FS)

A dynamic viscoelastic study of the film-forming solutions was carried out on a Bohlin CVO-100 rheometer (Bohlin Instruments Ltd., Gloucestershire, UK) using a cone-plate geometry (cone angle 4°, gap 0.15 mm). A dynamic frequency sweep from 0.1 to 10 Hz took place at auto stress, at a temperature of 10 °C and a target strain of 0.005%. The elastic modulus (G'; Pa) and viscous modulus (G''; Pa) were plotted as functions of the frequency ramp. To characterize the frequency dependence of G' over the limited frequency range, the following power law was used:

$$G' = G_0'\omega^n$$

where G_0' is the energy stored and recovered per cycle of sinusoidal shear deformation at an angular frequency of 1 Hz, ω is the angular frequency and n is the power law exponent, which should exhibit an ideal elastic behaviour near zero in gels. At least two determinations were performed for each sample. The experimental error was less than 6% in all cases.

2.6. Viscosity

A viscosity test for film-forming solutions was performed at 25 °C in the cone-plate cell (cone angle 4°, gap = 150 mm) of the Bohlin rheometer at a constant shear rate of 0.5 s^{-1}. The results are averages of eight determinations and are expressed as Pa·s.

2.7. Thermal properties

Calorimetric analyses of extracts and films were performed using a differential scanning calorimeter (DSC) model TA-Q1000 (TA Instruments, New Castle, DE, USA) previously calibrated by running high-purity indium (melting point, 156.4 °C; melting enthalpy, 28.44 J/g). Samples of around 10–15 mg were tightly encapsulated in aluminium hermetic pans. They were scanned under dry nitrogen purge (50 mL/min) between 5 and 180 °C at a heating rate of 10 °C/min. Peak temperatures (T_{peak}, °C) and enthalpies of conformational changes (ΔH) were measured at least in triplicate, the latter data being normalized to dry matter content (J/g_{dm}) after desiccation of each particular capsule.

2.8. ATR-FTIR spectroscopy

Extract and film infrared spectra between 4000 and 650 cm^{-1} were recorded at least in triplicate using a Perkin Elmer Spectrum 400 Infrared Spectrometer (Perkin–Elmer Inc., Waltham, MA, USA), as described by Ojagh *et al.*[23]

2.9. Antioxidant activities of hydrolysates and films

ABTS radical [2,2′-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid)] scavenging capacity and FRAP (ferric reducing ability of plasma) were used to measure the antioxidant activity of the hydrolysates (H, Hp) and films (F-Hp0, F-Hp15, F-Hp30). Both hydrolysates and films were dissolved in distilled water and shaken until they were totally homogeneous. The film solutions were filtered through Whatman No. 1 paper. The method used for the FRAP and ABTS assays was previously described by Alemán *et al.*[22] Results were expressed as μmoles Fe^{2+} equivalents/g for FRAP and mg Vitamin C Equivalent Antioxidant Capacity (VCEAC)/g) for ABTS, based on standard curves of $FeSO_4 7H_2O$ and vitamin C, respectively. All determinations were performed at least in triplicate.

2.10. Folin-reactive substances content of hydrolysates and films

Total Folin-reactive substances content was determined according to a modified method by Slinkard and Singleton with the Folin–Ciocalteu reagent.[24] An aliquot of 10 μL of sample was mixed with 750 μL of distilled water and oxidized with 50 μL of Folin–Ciocalteu reagent. The

reaction was neutralized with 150 µL of sodium carbonate solution and incubated for 2 h at room temperature. The absorbance of the resulting blue colour was measured at 765 nm (UV-1601, model CPS-240, Shimadzu, Kyoto, Japan). Results were expressed as mg gallic acid (GA) equivalent/g of sample. All determinations were performed at least in triplicate.

2.11. Film determinations

2.11.1. Thickness

The thickness was measured using a micrometer (MDC-25M, Mitutoyo, Kanagawa, Japan), averaging the values of 6–8 random locations in 15 films for each treatment as described by Pérez-Mateos *et al*.[25]

2.11.2. Moisture content

The moisture content was determined at least in triplicate by drying samples of around 0.5 g at 105°C for 24 h, according to A.O.A.C. (Association of Official Analytical Chemists, 1995).[26] Water content was expressed as a percentage of the total weight.

2.11.3. Protein content

The protein content was determined by a LECO FP-2000 nitrogen/protein analyser (Leco Corp., St. Joseph, MI, USA), according to Dumas (A.O.A.C., 2005) [27] and using a nitrogen-to-protein conversion factor of 6.25.

2.11.4. Light absorption and transparency

The light barrier properties and transparency of the films were calculated at least in triplicate using a UV-1601 spectrophotometer (model CPS-240, Shimadzu, Kyoto, Japan) at selected wavelengths from 250 to 800 nm following the method described by Pérez-Mateos *et al*.[25]
The films were cut into a rectangle piece and directly placed in the spectrophotometer test cell, using an empty test cell as the reference. Transparency was calculated by the equation Transparency = $-\log(T_{600}/x)$, where T600 is the light transmission (T) at 600 nm, and x is the film thickness (mm).

193 **2.11.5. Colour**

194 The colour parameters lightness (*L**), redness (*a**), and yellowness (*b**) were measured
195 following the method described by Blanco-Pascual *et a*l.[28]8

196 **2.11.6. Water vapour permeability (*WVP*)**

197 The water vapour permeability (WVP) was determined at least in triplicate following the method
198 described by Sobral *et al.*[29] A round portion of film was cut out and mounted on a plastic cup
199 (permeation area = 15.90 cm^2) containing silica gel, and the cups were then placed in desiccators
200 with distilled water. Weights were taken every hour for seven hours at 22°C. Water vapor
201 permeability was calculated from the equation $WVP = w.x.t^{-1}\cdot.A^{-1}\cdot.\Delta P^{-1}$, where *w* was weight gain
202 (g), *x* was film thickness (mm), *t* was time of gain (h), *A* was permeation area, and *ΔP* was the
203 change in the partial atmospheric vapor pressure with the silica gel and pure water (2642 Pa at
204 22°C). Results were expressed as $g.mm.\cdot h^{-1}.cm^{-2}.Pa^{-1}$. All measurements were carried out in
205 triplicate.

206 **2.11.7. Water solubility**

207 Film circumferences of 40 mm in diameter were placed in plastic containers with 50 mL distilled
208 water and placed at 22 ºC for 24 h. The solution was then filtered through Whatman # 1 filter
209 paper to recover the remaining undissolved film, which was desiccated at 105 ºC for 24 h. Film
210 solubility *FS* (%) was calculated using the expression $[(W_o - W_f)/W_o] \times 100$, where W_o was the
211 initial weight of the film expressed as dry matter and W_f was the weight of the undissolved
212 desiccated film residue. All tests were carried out at least in triplicate.

213 **2.11.8. Water resistance**

214 Films were fixed onto the opening of calibrated cells (area 15.90 cm^2) and the cells placed in
215 desiccators at 22°C and exposed over distilled water. Distilled water (5 mL) was poured over
216 the film surface. The film deformation due to the water effect, the time when the water started to
217 leak and the time when the film broke were annotated. All tests were carried out at least in
218 triplicate.

2.11.9. Mechanical properties

Tensile and puncture tests were run using a texture analyzer TA.XT plus TA-XT2 (Texture Technologies Corp., Scarsdale, NY, USA) (58% RH and 22°C) controlled by the Texture Exponent Software (Texture Technologies and by Stable Micro Systems, Ltd., Scarsdale, NY, USA), using a 5 kg load cell. Tensile test: At least three probes were cut rectangular (100 mm x 20 mm), leaving initial grips separation (l_0) of 60mm and using cross-head speed of 100 mm/min. The tensile strength (*TS,* MPa) (break force/initial cross-sectional area) and elongation at break $[(l_{break} - l_0)/l_0] \times 100$, (*EAB,* %), were determined from the stress *vs* strain curves at the breaking point, and the elastic modulus or Young's modulus (*Y,* MPa) calculated as the slope of the linear initial portion (elastic response zone) of the curve $(l_{break} - l_0)/l_0$. Puncture test: Films of 100X100 mm were fixed in a 35 mm diameter cell and punctured to breaking point with a round-ended stainless steel plunger (5 mm) at a cross-head speed of 100 mm/min, for breaking force (*F*, N), and breaking deformation (*D*, %) data according to Sobral *et al*.[29] which were carried out at least in triplicate at room temperature and keeping the samples at 58% RH until the text performance.

2.12. Statistical analysis

Statistical tests were performed using the SPSS computer programme (SPSS Statistical Software Inc., Chicago, Illinois, USA) for one-way analysis of variance. The variance homogeneity was evaluated using the Levene test, or the Brown-Forsythe when variance conditions were not fulfilled. Paired comparisons were made using the Bonferroni test or the Tamhane test (depending on variance homogeneity), with the significance of the difference set at $P \leq 0.05$.

3. Results and Discussion

3.1. Extraction yield of seaweed hydrolysis

Carrageenan extraction yield was 28.65% (dry weight basis) and hydrolysate yields were 19.04% for H and 39.17% for Hp (dry weight basis); therefore total seaweed extraction yield by H and Hp hydrolysis was 47.69 and 67.82%, respectively. While H extraction resulted in a

246 similar yield to another previously reported alcalase red seaweed hydrolysis, Hp was much
247 higher than almost all the protease extracts tested.[18]

248 Although carrageenan extraction is normally performed at high temperatures (80 °C),[14] good
249 extraction yields were obtained in the present using milder temperatures (50 °C), thereby
250 demonstrating that these conditions could be suitable for extraction. Montolalu *et al.* indicated
251 that the carrageenan obtained in extractions at 50 °C for long times (5 h) showed good gelling
252 properties, being better than those obtained with shorter times.[15] Therefore, enzymatic
253 hydrolysis would allow concomitant extraction of bioactive compounds (hydrolysate) and
254 carrageenan, improving the total yield and adding value to the seaweed extraction.

255 **3.2. Mastocarpus protein hydrolysates**

256 **3.2.1. Protein content and amino acid composition**

257 The protein content of marine algae varies greatly within species. Reports have shown that, in
258 general, red seaweeds contain high levels of proteins.[30] The dried *Mastocarpus stellatus*
259 contained 15.02 ± 0.53% of protein. Protein content was concentrated in the hydrolysates up to
260 37.86 ± 1.07% for H and 31.32 ± 0.96% for Hp.

261 The amino acid composition of H and Hp, expressed as residues per 1000 total amino acid
262 residues, is shown in Table 1. As expected, a similar amino acid profile was observed in both
263 hydrolysates. Both H and Hp showed high contents of Ser, Gly, Ala, Asp and Glu, and relatively
264 high contents of Leu, Thr, Val, Pro and Phe. The sum of the aspartic and glutamic acid contents
265 was 192 residues/1000 residues and 198 residues/1000 residues for H and Hp, respectively.
266 The high acidic amino acid content is typical of red seaweeds.[19] Nevertheless, some differences
267 between the hydrolysates were noteworthy. Some amino acids (Ser, Thr, Arg, His) were
268 concentrated in the more purified hydrolysate (H), owing to the removal of other amino acids,
269 mainly hydrophobic residues (Ala, Val, Ile, Leu, Pro, Met). These hydrophobic amino acids
270 might have been extracted during the ethyl acetate extraction, suggesting that some of them
271 could be linked to the polyphenols extracted.

272 **3.2.2. ACE-inhibitory capacity**

Angiotensin-I converting enzyme (ACE) plays an important role in the regulation of blood pressure and hypertension, because it catalyses the conversion of inactive angiotensin-I into angiotensin-II, a potent vasoconstrictor, and inactivates bradykinin, a potent vasodilator.[31] The amount of Mastocarpus hydrolysate required to inhibit 50% of the ACE activity (IC_{50}) is shown in Table 2.

Both H and Hp showed a high ACE-inhibitory capacity. The phenolic-partially-removed hydrolysate (H) showed considerably higher ACE-inhibitory capacity (IC_{50} of 91 µg/mL) than the phenolic-containing hydrolysate (Hp), probably owing to H's higher peptide concentration (7%). Furthermore, some peptides of Hp might be interacting with polyphenols, therefore being less available for ACE binding. However, Jeon reported that, among seven flavourzyme enzymatic digestions of brown seaweed, the hydrolysate with the highest polyphenol content showed the highest ACE-inhibitory capacity.[32] Moreover, some polyphenolic compounds have been shown to exert ACE-inhibitory activity.[33] ACE-inhibitory capacity might also be influenced by small differences in the amino acid compositions of the hydrolysates. Peptide ACE-inhibitory activity could be strongly influenced by the presence of hydrophobic (aromatic or branched side chains) amino acid residues at the C-terminal positions.[31,34] The hydrophilic–hydrophobic partitioning in the sequence is also a critical factor in the inhibitory activity.[35] ACE inhibition is also highly dependent on the molecular weight of peptides, those that are very short and have low molecular weight being more active.[36]

The IC_{50} of the ACE-inhibitory capacity of the H hydrolysate was 17.5 times lower than the IC_{50} value of the alcalase hydrolysate derived from red algae *Porphyra yezoensis*.[37] The ACE-inhibitory capacity of algae hydrolysates has been reported in other works.[34,38,39] Although, the use of a different method and its associated modifications to test ACE-inhibitory capacity makes direct comparison of IC_{50} values difficult,[22] H could be considered as a potent ACE-inhibitory hydrolysate.

3.2.3. Folin-reactive substances and antioxidant activity

Folin-reactive substances, ferric reducing power and ABTS radical scavenging ability of the hydrolysates are shown in Table 3. Both hydrolysates presented a noticeable amount of Folin-

301 reactive substances. As was expected, considering the method of hydrolysate preparation, the
302 Folin-reactive substances content was higher in Hp (phenolic-containing hydrolysate) than in H
303 (phenolic-partially-removed hydrolysate). Some polyphenols, however, might not have been
304 fully separated with ethyl acetate in the H hydrolysate. Moreover, although the Folin–Ciocalteu
305 assay is a widely used method to determine total phenolic content, additional substances can
306 react with the Folin reagent, including sugars and proteins, and should be taken into account.[40]

307 The hydrolysis process would allow an improved extraction of phenolic compounds as well as
308 the release of low molecular weight peptides,[18] which contribute to enhance the antioxidant
309 properties. Hp showed higher antioxidant activity than H (1.4 times higher for reducing power
310 and 2.7 times higher for ABTS radical scavenging), probably owing to a greater presence of
311 phenolic compounds in Hp. The positive correlation between the polyphenolic content of algae
312 and their antioxidant activity has been well documented.[41-45]
313 On the other hand, the hydrolysate peptide fraction can also contribute to antioxidant activity. It
314 is well known that biological activities of protein hydrolysates are related to the amino acid
315 composition, sequence, molecular weight and peptide configuration. For example,
316 phosphorylated serine and threonine are known to bind metals,[46] being more hydrophilic and
317 reactive because of their hydroxyl group. Amino acids with non-polar aliphatic groups, such as
318 alanine, leucine or proline, have high reactivity to hydrophobic PUFA radicals, while hydrogen
319 donors such as aspartic and glutamic acids are able to quench unpaired electrons or radicals by
320 supporting protons.[47] The abundance of these amino acids in the peptide sequences of
321 hydrolysates could also be responsible for their antioxidant activity. As previously mentioned,
322 the hydrophobic amino acid content was higher in Hp than in H, which might also have
323 contributed to the higher antioxidant capacity (ABTS and FRAP) of Hp compared with H.

324 Various studies have been carried out to evaluate the antioxidant potential of marine algae
325 hydrolysates.[7,8,18,48-50] However, to our knowledge, no reference has been made in previous
326 studies to the antioxidant or ACE-inhibitory activity of Mastocarpus hydrolysates.
327 Given the Folin-reactive substances content and antioxidant activity results of the hydrolysates,
328 both hydrolysates could be considered potential antioxidants. However, because of its greater
329 potential, Hp was selected to develop active Mastocarpus films with antioxidant activity.

3.3. Development of active films

Increasing concentrations of hydrolysate (Hp) were added to Mastocarpus biopolymer film-forming solutions. Concentrations above 30% produced sticky, unmanageable films. For this reason, 30% was chosen as the maximum hydrolysate concentration that could be used for film development. A concentration of 15% hydrolysate was also chosen in order to maintain a balance between the film's physico-chemical properties and the active properties that could be provided by the hydrolysates.

3.3.1 FTIR-ATR

Infrared spectra of F-Hp0, F-Hp15 and F-Hp30 films and Hp freeze-dried *Mastocarpus stellatus* hydrolysate were measured (Figure 1). Hp was analysed to assess its possible contribution to film structure. All film spectra showed a band at approximately 845 cm^{-1} (C-O-S vibration), assigned to D-galactose-4-sulfate (present in both κ- and ι-carrageenan), and a strong band at 924 cm^{-1}, which indicated the presence of 3,6-anhydro-D-galactose, a typical feature of κ-carrageenan.[51,52] The concomitant presence of κ- and ι-carrageenan features strongly suggested a greater extraction of κ/ι-hybrid carrageenan, as previously reported with *M. stellatus*.[14,53] F-Hp0 and F-Hp15 spectra had similar band intensities, while F-Hp30 had the above-mentioned bands considerably reduced, attributed to the reduced carrageenan amount in this film formulation in comparison with Hp, which had a much lower IR intensity at these wave numbers. Although high temperature (80–90 °C) is adequate for suitable carrageenan extraction, a certain amount of biopolymer might have been extracted during the hydrolysis carried out at 50 °C. In this regard, Montolalu *et al*. reported an appreciable extraction yield of high molecular weight carrageenan at 50 °C in *Kappaphycus alvarezii*.[15] The strong band at 1037 cm^{-1} in Hp confirmed the predominantly polysaccharide nature of the hydrolysate. Moreover, the greater absorption in the Hp IR-spectrum at wave numbers between 1100 and 1150 cm^{-1} as compared to the films also suggested an increased proportion of shorter polysaccharide chains.[54] A small band at approximately 803 cm^{-1} in the film spectra indicated the presence of two sulfate ester groups on the anhydro-D-galactose residues (sulfation on C2), characteristic and distinctive of ι-carrageenan.[51,55] This feature, which was not found in the hydrolysate, was most prominent in F-Hp0, and became smaller with an increasing Hp amount

in the film formulation (F-Hp0>F-Hp15>F-Hp30). Despite the presence of ι-carrageenan, the *M. stellatus* film spectra were quite similar to κ-carrageenan standards, as previously shown by Gómez-Ordóñez and Rupérez.[52] No evidence of a broad band between 820 and 830 cm^{-1} was found, indicating the absence of highly sulfated λ-carrageenan. The second derivative spectra of the films revealed trace evidence at 871 cm^{-1} of μ-carrageenan (κ-carrageenan precursor) and ν-carrageenan (ι-carrageenan precursor), which was not observable in Hp (data not shown).

The strong absorption bands at ~1216–1217 cm^{-1} (S = O), assigned to the presence of ester sulfate groups, were noticeably lower in F-Hp30, coinciding with the highest proportion of added hydrolysate. The 1217/924 cm^{-1} ratio, as a measure of relative total sulfate groups with respect to κ-carrageenan content, was much higher in Hp (2.6) than in any of the films studied (0.88 in F-Hp0, 0.91 in F-Hp15 and 0.94 in F-Hp30), suggesting an additional source of sulfated compounds in Hp, probably phycobiliproteins with sulfur-containing amino acids.[56] In this respect, Dumay *et al.* observed that enzymatic digestion was an effective treatment for phycoerythrin extraction.[57] Accordingly, the 1217/924 cm^{-1} ratio became higher as a result of the increase in the hydrolysate amount in the film formulation.

The IR-spectrum of Hp also revealed strong bands at 3277 cm^{-1}, 2929 cm^{-1} and 1600 cm^{-1} and a small shoulder at 1518 cm^{-1}, which could be assigned, respectively, to amide A, amide B, amide I and amide II of constituent proteins, most likely phycoerythrin and phycocyanin.[58] Comparison of the films showed that as the added hydrolysate percentage increased the amide I amplitude became more evident, and it exhibited a wave number down-shift to 1631 cm^{-1} in F-Hp30 as compared to 1639 cm^{-1} in F-Hp0 and F-Hp15, which denoted more hydrogen bonding in F-Hp30, attributed to the higher proportion of shorter peptides. In addition, the reduced band intensity of the ester sulfate group in F-Hp30 and the slight frequency up-shift from 1216.1 cm^{-1} in F-Hp0 and F-Hp15 to 1217.5 in F-Hp30 could be indicative of appreciable carrageenan-peptide interactions in the film with the highest amount of added hydrolysate.

3.3.2. DSC

386 DSC thermograms of the freeze-dried *Mastocarpus stellatus* hydrolysate, Hp, and the F-Hp0, F-
387 Hp15 and F-Hp30 films are shown in Figure 2. The hydrolysate showed two main endothermic
388 peak temperatures, T_{peak} (°C), at 86.62 ± 6.22 and 130.16 ± 0.63, and corresponding ΔH (J/g_{dm})
389 values of 3.51 ± 0.72 and 0.48 ± 0.05, respectively, which might correspond to phycoerythrin
390 and phycocyanin fragments.[59] Temperatures were higher than those where protein normally
391 features, probably because hydrolysis might shift maximal peak temperature towards higher
392 temperatures.[60-62] Low enthalpies also suggested the presence of hydrolysis products stabilized
393 by different amounts of hydrogen bonds and hydrophobic interactions. Another endothermic
394 transition in Hp with T_{peak} (°C) at 50.75 ± 0.66 and ΔH (J/g_{dm}) 0.39 ± 0.05 was evidence of the κ-
395 carrageenan constituent.[60]

396 DSC thermograms of F-Hp0, F-Hp15 and F-Hp30 are shown in Figure 2B. Slight endothermic
397 transitions were hardly observable, with T_{peak} values (°C) of 57.73 ± 0.20 in F-Hp0, 61.07 ± 2.14
398 in F-Hp15 and 64.65 ± 1.86 in F-Hp30, and ΔH (J/g_{dm}) of 0.41 ± 0.02, 0.19 ± 0.01 and 0.11 ±
399 0.04 respectively, indicative of the helix-to-coil transition suffered by the κ/ι-carrageenan as a
400 result of the breakage of weak physical cross-links.[63] Thermal transitions, however, were not as
401 sharp as those in a pure carrageenan curve.[64,65] The increased protein content in films with
402 added hydrolysate might promote carrageenan-peptide interactions,[66] which could explain the
403 increase in T_{peak} temperatures. However, the enthalpy reduction suggested a hydrolysate-
404 induced plasticizing effect in the films as a result of increasing the free water and chain mobility.

405 **3.3.3. Rheology**

406 Figure 3 shows the mechanical spectra of the film-forming solutions at 10 °C, in terms of elastic
407 modulus (*G′*) and viscous modulus (*G″*) as a function of angular frequency. The F-Hp0 solution,
408 which had the lowest G′ values, was the only one with a crossover point where $G' = G''$. At
409 frequencies below 2 Hz, the F-Hp0 solution was characterized by a dominant viscous behaviour
410 ($G' < G''$), which turned into a gel-like behaviour at higher frequencies; thus it could be classified
411 as a concentrated solution constituting an entanglement network. In the absence of KCl,
412 solutions of κ-carrageenan cooled down to 9 °C have been shown to adopt helical structures,
413 which did not aggregate to form self-supporting gels.[67]

In contrast, the F-Hp15 and F-Hp30 solutions showed a typical gel-like behaviour denoted by *G′* > *G″* values within the whole frequency range, as previously reported in other studies on carrageenan.[68] The *G′* values were successfully modelled according to the power law (r^2~0.99) in all three cases. The hydrolysate addition to the film-forming solution at 15% concentration caused a remarkable increase in *G′*, much higher than with 30%. The rheological behaviour of the F-Hp15 solution showed lower frequency dependence of *G′* than the F-Hp30 and F-Hp0 solutions, as deduced from the lowest power law exponent value (n′) (0.63 in F-Hp0, 0.40 in F-Hp15, 0.52 in F-Hp30). Hp concentrations higher than 30% conferred sticky, unmanageable properties to the films. All these findings suggest that the hydrolysate added at the appropriate concentration led to stronger gels with increased structural stability, probably due to hydrolysate components (mainly peptides and phenolic compounds) favouring aggregation of carrageenan helices to form a three-dimensional network. Interactions between carrageenan and proteins have previously been shown to produce much stronger gels than single carrageenan gels.[66] Similarly, the ability of polyphenols to interact with polysaccharides forming complexes has been well documented.[69] Nevertheless, the higher hydrolysate amount in the F-Hp30 solution considerably reduced the gel-forming capacity with respect to the F-Hp15 solution, with the helical aggregates probably having more difficulty in being created as a result of a carrageenan-dilution effect.[70]

The apparent viscosity of the film-forming solutions, measured at 25 °C and shear rate of 0.5 s^{-1}, was considerably higher in the F-Hp15 solution (14.89 ± 0.53 Pa·s) than in the F-Hp0 and F-Hp30 solutions (3.47 ± 0.01 Pa·s and 4.72 ± 0.15 Pa·s, respectively), strongly suggesting effective interactions at the right concentration between carrageenan and other compounds naturally present in the hydrolysate, presumably peptides and phenolic compounds.

3.3.4. Light barrier properties

Colour parameters, *L** (lightness), *a** (reddish/greenish) and *b** (yellowish/bluish), are shown in Table 4. All the films were quite similar, having low lightness (28–29) and slightly greenish and yellowish tendencies. The F-Hp30 film exhibited the highest ($P \leq 0.05$) lightness and greenish colouration, and lowest ($P \leq 0.05$) yellowish tendency. Changes in *L*a*b** values, however, did not correlate with increasing amounts of added hydrolysate in the film, which could be due to a

443 different degree of interactions between protein pigments and carrageenan. Comparing these
444 results with previously developed commercial κ-carrageenan films, the present *M. stellatus* films
445 presented considerably lower lightness and more red tendency, owing to the concomitant
446 extraction of non-carrageenan compounds.[71-73]

447 In general, the films exhibited low light transmission in the UV range (250–300 nm) (0–1.12%)
448 (Figure 4), as compared to commercial κ-carrageenan films,[74] with F-Hp0 providing the least
449 efficient UV barrier. Two absorption peaks were defined in all the films in the ranges 400–450
450 nm and 600–700 nm, which might be associated with the presence of pigments, such as
451 carotenoids and chlorophyll, which absorb at 400–450 (violet-blue-green colours), and
452 phycoerythrin and phycocyanin at 600 nm (red colour).[75] In the visible range, the light
453 transmission was significantly ($P \leq 0.05$) lower in F-Hp30, especially in the wavelength range
454 between 350 and 700 nm, which might be largely due to the increase in thickness associated
455 with the hydrolysate addition, as Table 5 shows. The hydrolysate contained small molecules
456 (mainly peptides and oligosaccharides) that might have interfered in carrageenan helix
457 aggregation during the film drying process. This interference might have caused a plasticizing
458 effect with an increase in free volume that would have resulted in thicker films.

459 **3.3.5. Physico-chemical properties**

460 Slight variations in moisture content were observed among the three film formulations (Table 5),
461 with F-Hp30 showing slightly higher values, which could be related to its increased thickness.
462 The protein content in the films increased significantly ($P \leq 0.05$) with the addition of increasing
463 amounts of hydrolysate (Table 5).

464 **3.3.5.1. Water barrier**

465 No significant differences in film water solubility were found in *M. stellatus* films with either 15 or
466 30% added hydrolysate (Table 5). A similar finding was reported earlier in gelatin films
467 incorporating different percentages of gelatin hydrolysate.[76] Although the solubility values were
468 not high, the films totally lost their original structure, becoming a very viscous solution with
469 gelling tendency at low temperatures. Solubility was similar to previous results obtained in
470 commercial carrageenan films.[73]

No significant (P≤0.05) differences were found between F-Hp0 and F-Hp30 water vapour permeability (*WVP*) (Table 5). In contrast, F-Hp15 had the lowest permeability, probably owing to effective carrageenan-protein interactions, as previously commented. Despite the greater thickness of F-Hp30, *WVP* was not reduced by adding 30% hydrolysate. The extra protein and plasticizer effect caused by Hp addition may have resulted in a less dense network.[77] The present films were more water vapour permeable than previously reported commercial carrageenan films.[71-73,78,79]

Water resistance test results are shown in Figure 5. Noticeable differences among samples were observed after 10 minutes. Although every film elongated up to 2 cm until breakage, F-Hp0 showed a faster elongation speed (3.2 cm/h) than F-Hp15 and F-Hp30 (~2.9 cm/h). The hydrolysate addition led to a significantly higher breakage resistance in F-Hp30 in comparison with F-Hp0 and F-Hp15, probably related to the higher carrageenan peptide interactions. No water leaking before film breakage could be detected in any sample.

3.3.5.2. Mechanical properties

F-Hp0 had the significantly highest (P≤0.05) tensile strength (*TS*) (Table 5), which was lowest in F-Hp30. The opposite behaviour was found regarding the elongation at break (*EAB*) values, confirming the hydrolysate-induced plasticizing effect in the film. The *TS* and *EAB* values in the three *M. stellatus* films studied were, respectively, higher and lower than the results reported with commercial κ-carrageenan or ι-carrageenan films,[73,79] suggesting a reinforcement effect caused by the presence of other non-carrageenan components. As far as Young's modulus (*Y*) is concerned (Table 5), the highest stiffness also corresponded to F-Hp0 (P≤0.05), decreasing with increasing amount of Hp. The small molecules (mainly peptides and oligosaccharides) that form part of the hydrolysate have been proved to act as film plasticizers by preventing carrageenan helix associations and increasing the molecular mobility of polymer chains, which in the case of F-Hp30 was favoured by the increased water plasticizing effect. Similarly, Salgado *et al*. also observed a reduction in *TS* and *Y* and an increase in *EAB* in protein films with added hydrolysate, which, in view of the lack of film moisture increase, was attributed to interferences in protein cross-linking.[21]

There were no significant differences in puncture force (*F*) between F-Hp0 and F-Hp15 (Table 5), whereas F-Hp30 had higher values (P≤0.05). Puncture deformation (*D*) was also significantly higher (P≤0.05) in F-Hp30, with no differences between F-Hp0 and F-Hp15. It is worth noting the different information provided by both type of mechanical tests. The high plasticizing effect exerted by the hydrolysate in F-Hp30, mostly by preventing carrageenan helix associations, was the main factor determining the film tensile properties; however, this film showed higher resistance to perforation, which could be attributed to some carrageenan-protein interactions. Tensile strength gives more general information about the film resistance to traction, while puncture test focuses on the resistance to perforation in a specific matrix area.

3.3.6. Antioxidant activity and Folin-reactive substances of the films

Folin reactive substances, ferric reducing power and ABTS radical scavenging capacity of the films are shown in Table 3. Films without algae hydrolysates (F-Hp0) contained Folin reactive substances and exhibited some antioxidant activity measured by both FRAP and ABTS assays. The incorporation of increasing concentrations of Hp significantly increased the Folin-reactive substances content (3-fold increase in F-Hp30), as well as FRAP and ABTS values (3- and 9-fold increase, respectively, in F-Hp30).

The antioxidant properties of squid gelatin films and sunflower films were also improved by the addition of hydrolysates from squid gelatin and bovine plasma, respectively, but the antioxidant activity increase reported was much lower than in the present work.[21,76]

4. CONCLUSION

Enzyme-assisted *Mastocarpus stellatus* hydrolysis could be a complementary way to extract bioactive components in addition to carrageenan. The more purified peptide hydrolysate (phenolic content partially removed) showed the highest ACE-inhibitory capacity; while keeping peptides and polyphenols together resulted in a more antioxidant hydrolysate, which was suitable as active ingredient for antioxidant film development. The addition of the hydrolysate resulted in a more plasticized film by reducing the tensile strength and increasing the elongation at break, however, the film was more resistant to perforation. In view of the results obtained, these films might be used as a complementary material for active packaging development.

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668

669 **Table 1.** Amino acid composition of Mastocarpus hydrolysates (H, Hp)

Amino acid	Number of residues / 1000 residues	
	H	Hp
Asp	102	105
Thr	73	66
Ser	153	92
Glu	90	93
Gly	112	116
Ala	104	120
Cys	5	7
Val	47	65
Met	16	19
Ile	34	40
Leu	72	88
Tyr	28	28
Phe	43	43
His	12	6
Lys	29	26
Arg	27	23
Pro	48	57
Hyp	0	0
Hyl	5	5

670

671

672 **Table 2.** ACE-inhibitory capacity of Mastocarpus hydrolysates

	IC_{50}* (µg/mL)
H	91.62 ± 2.44 a
Hp	148.32 ± 3.16 b

677 Different letters (a, b) indicate significant differences ($p \leq 0.05$).

678 * IC_{50}: concentration (µg/mL) required to inhibit 50% of ACE activity.
679

680 **Table 3.** Antioxidant activity and Folin reactive substances of hydrolysates and films

Sample	ABTS (mg Vit C eq/g)	FRAP (µmol Fe/g)	Folin reactive substances (mg/g)
H	35.95 ± 1.59 a	84.52 ± 1.38 a	36.02 ± 3.26 a
Hp	93.26 ± 2.55 b	106.19 ± 1.05 b	75.61 ± 0.56 b
F-Hp0	3.07 ± 0.18 a	4.54 ± 0.08 a	7.33 ± 0.34 a
F-Hp15	17.56 ± 0.90 b	11.77 ± 0.38 b	15.97 ± 1.46 b
F-Hp30	27.51 ± 0.83 c	13.75 ± 0.06 c	22.17 ± 0.36 c

681 Results are the mean ± standard deviation. One-way ANOVA: Different letters indicate
682 significant differences among the different hydrolysates (H) or different films (F) ($P \leq 0.05$).

683

684 **Table 4.** L^*, a^*, b^* and Transparency ($-\log(T_{600}/x)$) of F-Hp0, F-Hp15 and F-Hp30

Film	*L**	*a**	*b**	Transparency
F-Hp0	28.65 ± 0. 29 a	-0.57 ± 0.03 a	4.57 ± 0. 11 a	7.14 ± 0.24 a
F-Hp15	27.99 ± 0.08 b	-0.30 ± 0.05 b	4.72 ± 0. 04 b	6.61 ± 0.30 ab
F-Hp30	29.25 ± 0.05 c	-0.70 ± 0.03 c	4.36 ± 0.04 c	6.12 ± 0.12 b

685 Results are the mean ± standard deviation. One-way ANOVA: Different letters indicate
686 significant differences among the different films (P≤0.05).

687

688 **Table 5.** Thickness, moisture, protein content, film solubility, water vapour permeability (*WVP*),
689 tensile strength (*TS*), elongation at break (*EAB*), Young's modulus (*Y*), puncture force (*F*) and
690 puncture deformation (*D*) of F-Hp0, F-Hp15 and F-Hp30

	F-Hp0	F-Hp15	F-Hp30
Thickness (µm)	51.82 ± 3.34 a	56.05 ± 3.65 a	68.36 ± 4.04 b
Moisture (%)	13.46 ± 0.28 a	12. 13 ± 0. 14 b	14.59 ± 2.75 ab
Protein content (%)	8.90 ± 0.06 a	10.98 ± 0.21 b	13.05 ± 0.24 c
Film solubility (%)	20.97 ± 4.5 a	25.77 ± 3.64 a	22.16 ± 2.95 a
WVP ($x10^{-8}$ g m^{-1} s^{-1} Pa^{-1})	3. 78 ± 0. 17 a	3. 20 ± 0. 12 b	4.04 ± 0. 30 a
TS (MPa)	59.94 ± 2.27 a	51.37 ± 3.75 b	41.63 ± 2.95 c
EAB (%)	0.95 ± 0.11 a	1.59 ± 0.09 b	2.47 ± 0.24 c
Y (MPa)	1797 ± 61 a	1347 ± 74 b	1054 ± 45 c
F (N)	23.47 ± 1.08 a	26.36 ± 2.14 b	30.38 ± 2.27 b
D (%)	7.73 ± 0.47 a	8.61 ± 0.53 a	12.24 ± 1 b

691 Results are the mean ± standard deviation. One-way ANOVA: Different letters indicate
692 significant differences among the different films (P≤0.05).

693

694

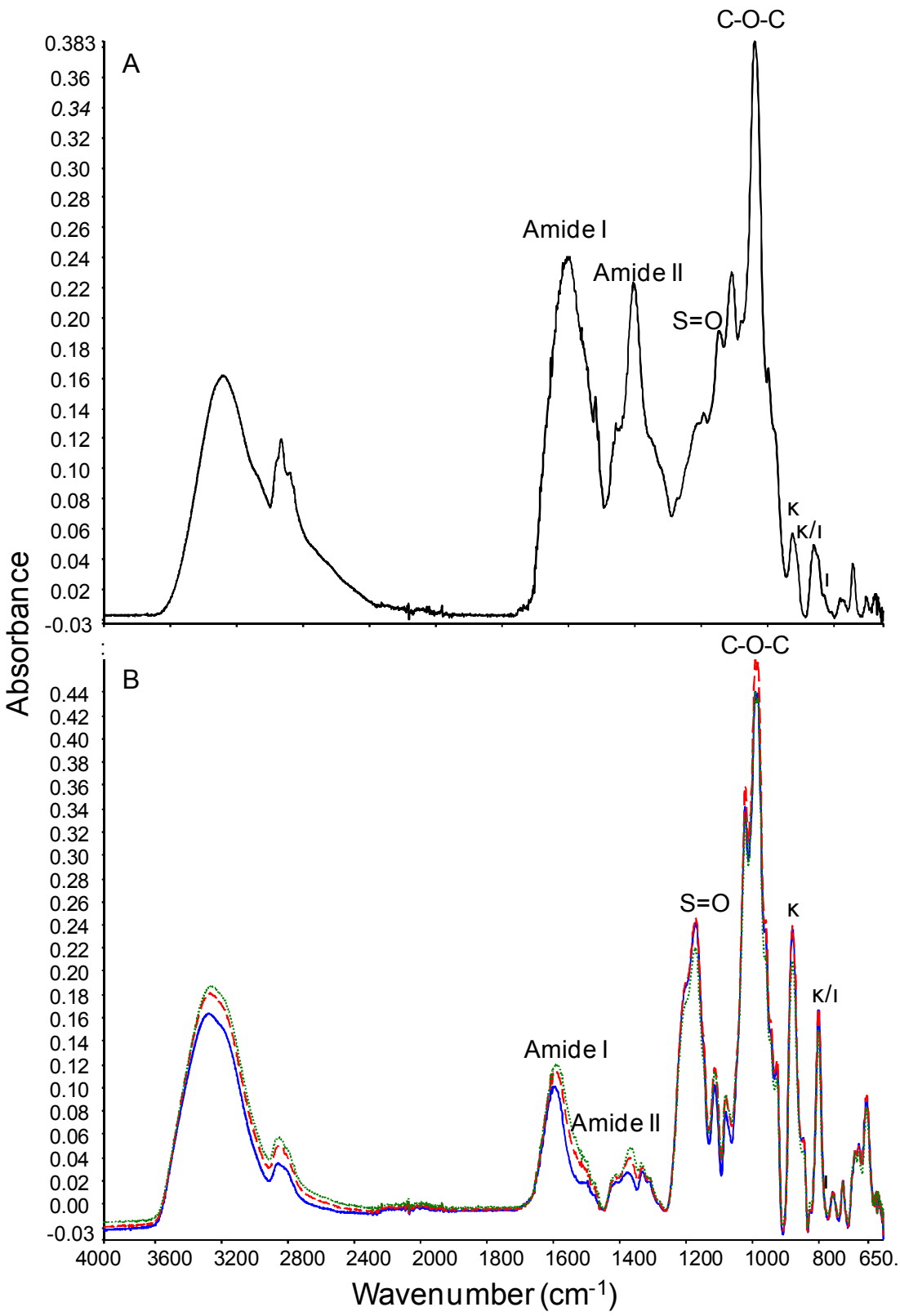
A
B
C-O-C
Amide I
Amide II
S=O
κ
κ/ι
ι
Absorbance
Wavenumber (cm-1)
0.383
0.36
0.34
0.32
0.30
0.28
0.26
0.24
0.22
0.20
0.18
0.16
0.14
0.12
0.10
0.08
0.06
0.04
0.02
-0.03
0.44
0.42
0.40
0.38
0.36
0.34
0.32
0.30
0.28
0.26
0.24
0.22
0.20
0.18
0.16
0.14
0.12
0.10
0.08
0.06
0.04
0.02
0.00
-0.03
4000
3600
3200
2800
2400
2000
1800
1600
1400
1200
1000
800
650.

Fig. 1

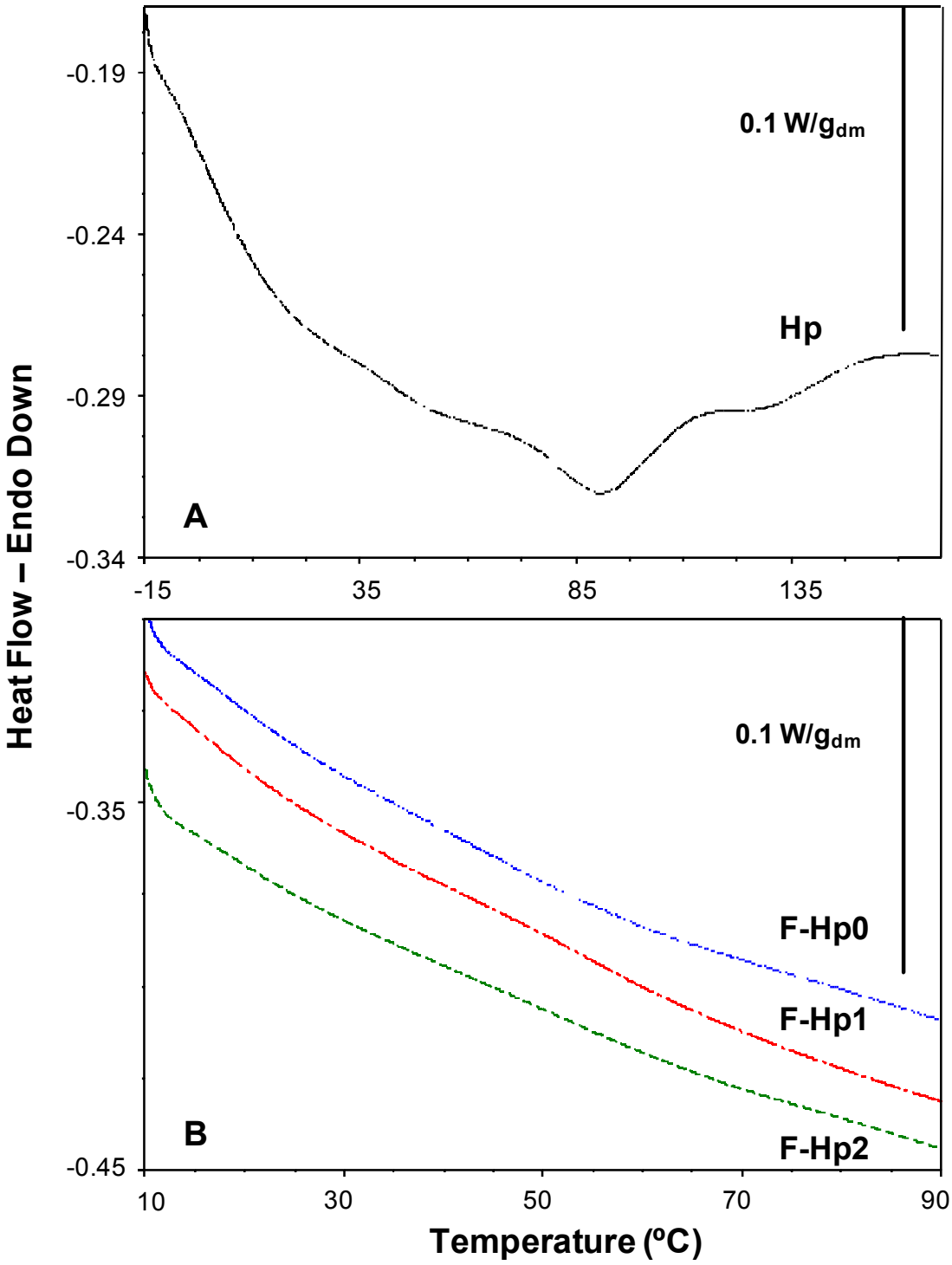
Heat Flow – Endo Down
-0.19
-0.24
-0.29
-0.34
-15
35
85
135
0.1 W/g$_{dm}$
Hp
A
-0.35
-0.45
10
30
50
70
90
0.1 W/g$_{dm}$
F-Hp0
F-Hp1
F-Hp2
B
Temperature (ºC)

Fig. 2

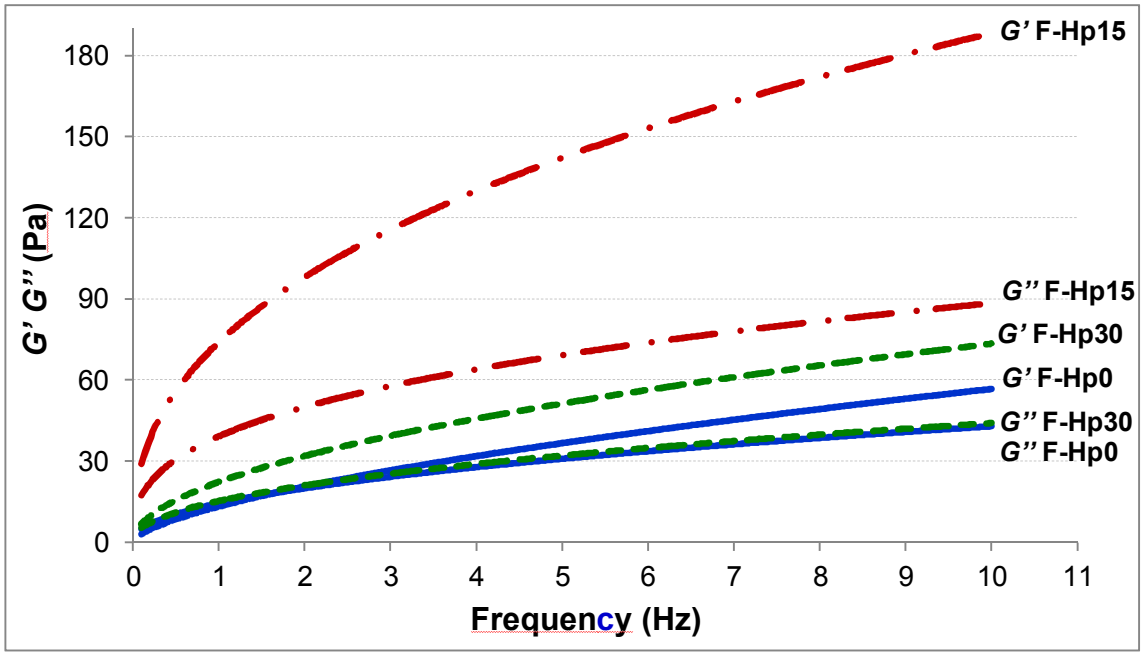
G' F-Hp15
G'' F-Hp15
G' F-Hp30
G' F-Hp0
G'' F-Hp30
G'' F-Hp0
G' G'' (Pa)
Frequency (Hz)
0
30
60
90
120
150
180
0
1
2
3
4
5
6
7
8
9
10
11

Fig. 3

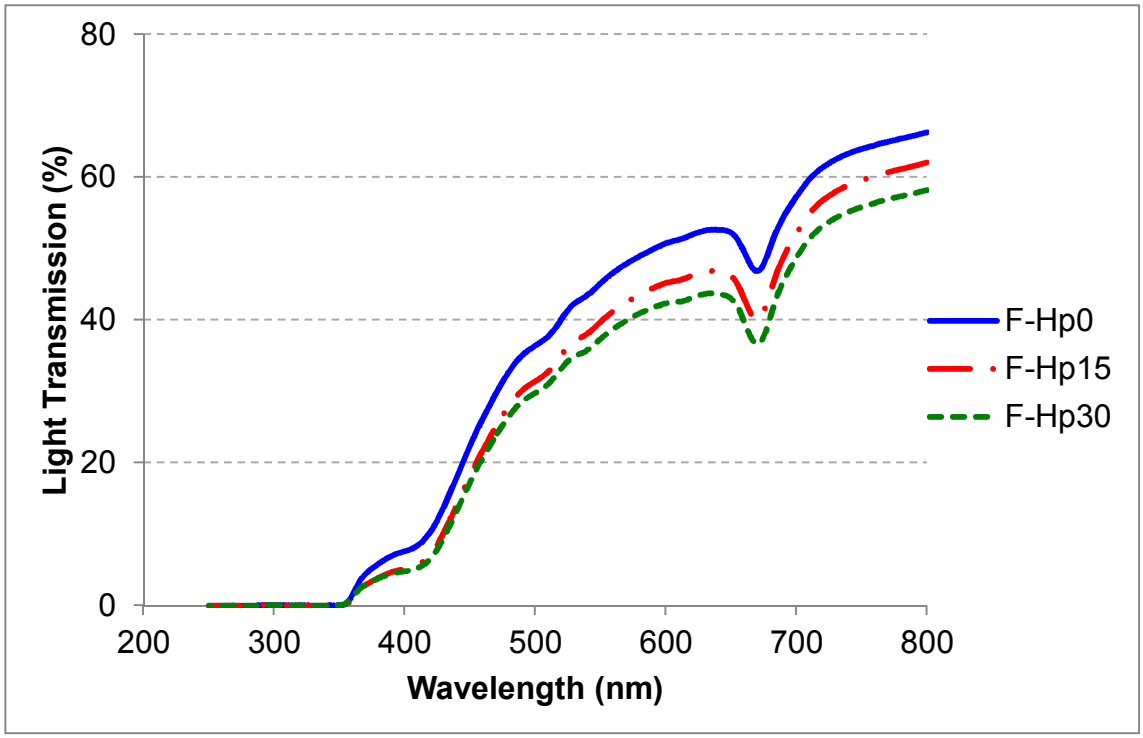
80
60
40
20
0
Light Transmission (%)
200
300
400
500
600
700
800
Wavelength (nm)
F-Hp0
F-Hp15
F-Hp30

Fig. 4

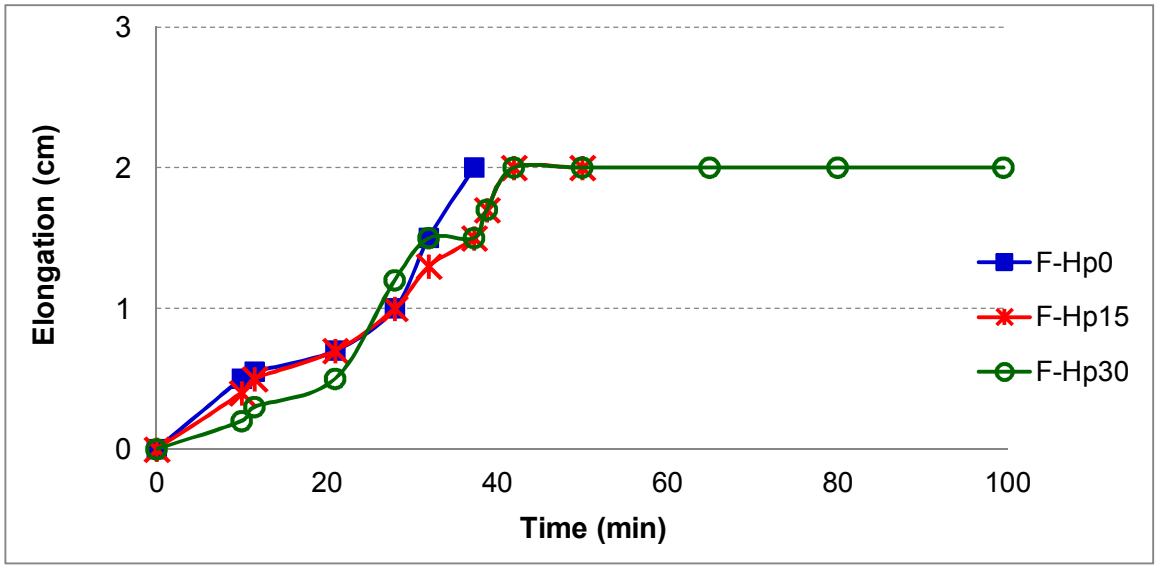

Fig. 5