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Preparation and characterization of nanocomposite film from Whitemouth croaker *(Micropogonias furnieri)* protein isolate modified with montmorillonite

[1*]William, R. C. V., [2]Daniela, C. B., [2]Sandriane, P. and [2]Carlos, P.

[1]*Laboratory of Bioengineering, Faculty of Engineering, Federal University of Grande Dourados, Dourados - MS - Brazil*
[2]*Laboratory of Food Technology, School of Chemistry and Food, Federal University of Rio Grande, Rio Grande - RS - Brazil*

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Abstract

The aim of this study was to evaluate films from Whitemouth croaker protein isolate (CPI) with or without addition of montmorillonite (MMT). Results showed that incorporation of organophylic clay montmorillonite decreased the solubility, transparency and water vapor permeability (WVP) (3.82 g mm/m^{-2} day^{-1} KPa^{-1}) when compared with pure CPI film (8.57 g mm/m^{-2} day^{-1} KPa^{-1}). Films with montmorillonite showed increased mechanical properties, tensile strength and elongation percentage at break point (11.4 MPa and 10.9%, respectively), than the pure CPI film (9.8 MPa and 9.3%). The films produced from CPI with MMT showed better properties of WVP, tensile strength, scanning electron microscopy (SEM), structural analysis in Fourier Transform Infrared (FT-IR) spectroscopy and X-ray diffraction (XRD).

Introduction

Biodegradable polymer offer an alternative form of packaging and also a partial solution to the problem of the accumulation of solid wastes composed of synthetic inert polymers (Jayasekara *et al*., 2004; Ferreira *et al*., 2009). In recent years, the interest in using biodegradable materials for food packaging has increased because of consumer awareness of the environmental damage caused by non-biodegradable packaging, thereby reducing plastic wastes (Hoque *et al*., 2010).

Edible films and coatings are prepared from biopolymers and are able to protect food products extending their shelf life. The use of natural blends of protein, polysaccharides and lipids directly obtained from agricultural sources, takes advantage of each component in the original system and appears to be a new opportunity for materials in the area of biodegradable films (Tapia-Blácido *et al*., 2007; Bonilla *et al*., 2012).

Protein and polysaccharide films are generally good barriers against oxygen at low to intermediate relative humidity and have good mechanical properties; however, their barrier against water vapor is poor due to their hydrophilic nature (Kester and Fennema, 1986; Gontard and Guilbert, 1995). Proteins are formed by the condensation polymerization of various combinations of amino acid repeat units; different plant sources produce proteins with different amino acid combinations and thus exhibit different properties. Proteins show properties that are advantageous in the preparation of packaging biomaterials, for example, their ability to form networks, plasticity and elasticity at the films. The film-forming ability of several protein substanceshas been utilized in industrial applications for a longtime (Cuq *et al*., 1998).

Polymer-clay nanocomposites are a class of hybrid materials composed of organic polymer materials and nano-scale clay fillers (Giannelis, 1996; Lagaly, 1999). Montmorillonite (MMT), hectrite, and saponite are frequently used pristine layered silicates, which are combined with polymeric materials to form nanocomposites (Sinha Ray and Okamoto, 2003). MMT is a clay mineral consisting of stacked silicate sheets with a high aspect ratio and a plate-like morphology. This high aspect ratio (ratio of length to thickness) plays an important role for the enhancement of mechanical and physical properties of composite materials. Chemically, MMT consists of two fused silicate tetrahedral sheets sandwiching an edge-shared octahedral sheet of either magnesium or aluminum hydroxide (Giannelis, 1996; Alexandre and Dubois, 2000; Rhim and Ng, 2007).

The objective of this study was to evaluate films from Whitemouth croaker protein isolate (CPI) with or without addition of MMT. The films were evaluated

*Corresponding author.
Email: *williamvega@ufgd.edu.br*
Tel: +55 (67) 3410-2170

in terms of tensile strength, elongation percentage at break point, solubility, transparency and water vapor permeability (WVP), scanning electron microscopy (SEM), structural analysis in Fourier Transform Infrared (FT-IR) spectroscopy and X-ray diffraction (XRD).

Material and Methods

Material

The raw material used was mechanically separated meat (MSM), from co-products of industrialization of Whitemouth croaker *(Micropogonias furnieri)*. The croaker protein isolate (CPI) was obtained according to methodology adapted from Nolsoe and Underland (2009), to solubilize and isolate the protein by the process of pH variation. The organophilic clay utilized was Montmorillonite K10 (MMT) (Sigma-Aldrich) with a particle size of 10 nm. The plasticizer used was glycerol (Vetec, QuímicaFina).

Development of the films

The polymeric films were developed by the technique of "casting", where each film solution (100 ml), a CPI (3.5 g/100 ml) dispersion was prepared in distilled water. This aqueous dispersion was maintained with gentle and constant stirring for 20 minutes with a stirring propeller shaft (IKA Model RW 20DZM.n) at 30°C in thermostatic ultrasonic bath (QUIMIS, model 214 D2) for hydration of the CPI. After the hydration, the pH was adjusted to 11.2 with the addition of 1N NaOH (Merck) using pH meter bench (Marconi Model PA 200) while maintaining constant stirring for 10 minutes. MMT (0.5 g/100 ml) was then added and the temperature was raised to 80°C. After complete dissolution of the CPI and MMT, glycerol (30 g glycerol/100 g protein) previously dissolved in distilled water was added to the film solution (80°C) maintaining the pH at 11.2. Subsequently, the film solution was placed in homogenizer (Ultra-Turrax T25 IKA Model) for 2 minutes, then manually spread on Petri dishes with a diameter of 15 cm, standardizing the thickness to 0.120 ± 0.005 mm and subjected to drying in an oven with circulating air (242 QUIMIS 314D) at 40 ± 1°C for 12 h. The same procedure was used to obtain films with no MMT. After drying, the films were stored in desiccators for 48 h at 25 ± 2 °C and relative humidity of 55 ± 2% until the beginning of the analysis. To control the relative humidity (RH), a saturated solution of $CaCl_2$ was used. All assays were performed in triplicate (Cortez-Vega *et al*., 2013).

Film characterization

After the storage period, the film thickness was measured using a digital micrometer (INSIZE model IP54, São Paulo, Brazil) with 0.001 ± 0.0005 mm resolution. The thickness was set as the arithmetic mean of eight random measurements over the area of the film.

Water vapor permeability (WVP) of films was determined gravimetrically at 25°C, using the ASTM standard method E96-95 (ASTM, 1995). Samples of each film in the form of discs (diameter = 70 mm) were fixed with paraffin cell permeation of aluminium, containing anhydrous calcium chloride. These cells were placed in desiccators at 25°C and 75% relative humidity. By increasing the mass of anhydrous calcium chloride (measured in intervals of 24 h for 7 days), it was possible to determine the water vapour transferred through the film, according to Eq. 1.

$$WVP = (Wma \times L)/(t \times A \times \Delta P) \quad (1)$$

where W_{ma} is the weight of absorbed moisture (g); t is the time duration of the test (days); L is the average film thickness (mm); A is the area of the exposed film surface (m^2); and ΔP is the partial vapor pressure difference across the film (Pa).

Tensile strength (TS) and elongation percentage (E) at break point were measured uniaxially by stretching the specimen in one direction using a Texture Analyzer (TA.XTplus, Stable Micro Systems, Surrey, England) according to the ASTM D-882 standard (ASTM, 2001), with a 50 N load cell. Sample films were cut into 25-mm-wide and 100-mm-long strips. The initial grip separation and cross-head speed were set to 50 mm and 50 mm/ min, respectively.

The films were analyzed using FT-IR spectrophotometry (Prestige 21), in a spectral range from 4000 to 400 cm^{-1}. Film samples were examined for surface characteristics using a scanning electron microscope (SEM) (model JSM-5800 LV, Jeol, Tokyo, Japan) operated at 10 kV. Five samples were mounted on a bronze stub and sputter-coated (Sputter coater SPI Module, Santa Clara, CA, USA) with a layer of gold prior to imaging.

For X-ray diffraction a Siemens D500 diffractometer Bragg-Brentano geometry with Cu radiation was used, operated at 40 kV and 17.5 mA, with graphite monochromator for diffracted X-ray beams. Measurements were obtained with steps of 0.05° (2θ), counting time of 5 seconds/step, and at measurement intervals of 2θ from 2-60 degrees.

Table 1. Values of tensile strength, elongation percentage at break point, solubility, transparency and WVP

Films	Tensile strength (MPa)	Elongation at break point (%)	Solubility (%)	Transparency (mm^{-1})	WVP (g mm/m^{-2} day^{-1} kPa^{-1})
CPI	9.8 ± 0.2^{b}	9.3 ± 0.1^{b}	23.4 ± 0.2^{a}	15.3 ± 0.1^{a}	8.57 ± 0.2^{a}
CPI + MMT	11.4 ± 0.1^{a}	10.9 ± 0.3^{a}	18.6 ± 0.3^{b}	13.7 ± 0.1^{b}	3.81 ± 0.4^{b}

Mean values ± standard error (in triplicate) Different superscripts letters in the same column indicate significant differences ($p \leq 0.05$) between the film properties CPI: Whitemouth croaker protein isolate; CPI + MMT: Whitemouth croaker protein isolate and montmorillonite; WVP: water vapor permeability

Statistical analysis

Tukey's multiple comparison tests were used to statistically determine significant differences ($p \leq 0.05$) among averages, using the software Statistic 6.0 (Statsoft, Tulsa, OK, USA).

Results and Discussion

All films showed an average thickness of 0.120 ± 0.005 mm and were characterized in terms of WVP, tensile strength, elongation percentage at break point, solubility and transparency properties (Table 1). Values of Table 1 shows significant differences ($p \leq 0.05$) between the water vapour permeability and mechanical properties of films through Tukey's mean comparison test. The tensile strength values were 9.8 and 11.4 MPa for films of CPI and CPI + MMT, respectively. These results are below those found by Kvien *et al*. (2007), who found a tensile strength of 12.5 MPa in films of starch-based nanocomposites. These same values are above the values obtained by Sothornvit *et al*., (2009), who studied the effect of nanoclay on the type of physical and antimicrobial properties of composite films of whey protein isolate and found lower values for tensile strength, which were 1.55 and 3.29 MPa when Cloisite 20A and Cloisite 30B, were used respectively. In the range of values obtained by Cortez-Vega *et al.* (2013), who studied mechanical and barrier properties in the nanocomposite biofilms obtained from Whitemouth croaker preoteim isolate.

The results for tensile strength in this study are above those found by Al-Hassan and Norziah (2012), who mixed starch with gelatin in different proportions to obtain films, using glycerol as plasticizer (1.28 to 1.70 MPa) and below these values when using sorbitol as a plasticizer (18.06 MPa). The values found for Elongation at break point are among the values found by Souza *et al*., (2011), who evaluated films formed from polysaccharides such as chitosan, incorporated with different lipid fractions finding values between 7.1 and 19%.

The solubility values are below the values found by Hendrix *et al*. (2012), who developed biopolymer films based on defatted mustard flour, treated with high pressure homogenization, ultrasonic irradiation, and finding values between (30.7 - 34.1%), (31.4 - 31.8%)and (30.3 - 34.4%) of solubility, respectively. The transparency values are above those found by Sothornvit *et al*. (2009), who studied the effect that the type of nanoclay on the physical properties of antimicrobial and composite films of whey protein isolate and found lower values for transparency using Cloisite 30B (6.30 mm^{-1}). Sothornvit *et al.* (2010), evaluated composites based on whey protein isolate / Cloisite 30B clay with different amounts (0, 5, 10 and 20 g/100 g CPI) and the transparency values found 14.38; 6.30; 4.89 and 4.22 mm^{-1} respectively, when clay concentrations values were used, the values were lower than those found in this study. These authors showed that with increasing concentration of clay there was also a reduction in transparency of the films.

The values found for WVP in this study are above the values found by Souza *et al.* (2011), who evaluated films formed from polysaccharides such as chitosan, incorporated with different lipid fractions finding values between 1.32 and 1.80 g mm/m^{-2} d^{-1} KPa^{-1} and in the range of values obtained by the same authors when they evaluated pure chitosan films (3.8 g mm/ m^{-2} d^{-1} KPa^{-1}). Yano *et al*. (1997), also demonstrated that the WVP of nanocomposite polymer/clay films decreased exponentially when the clay content was increased. This is because the layered structure of the nanoclay prevents the transmission of water vapor through the film matrix or promotes delayed diffusion of water vapor due to the difficulty of the track (Park *et al*., 2003; Sorrentino *et al*., 2006; Sothornvit *et al*., 2010).

Figure 1 (a) shows the micrograph of the film of pure protein isolate plasticized with glycerol, showing the internal structure of the matrix of the film without addition of MMT. (b) corresponding to the nanocomposite CPI film with MMT showing the film surface. The pure CPI film (Figure 1 (a)) shown to be, free of air bubbles, having a homogeneous

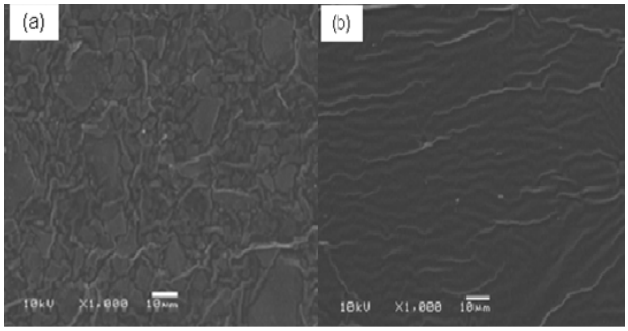

Figure 1. (a) Scanning electron micrograph of the pure croaker protein isolate film plasticized with glycerol, showing the film surface; (b) micrograph corresponding to the CPI nanocomposite film and MMT showing the surface of 10 mm film. 10KV x1000 10 μm.

surface with granular and porous structure, and surface irregularities. These imperfections possibly facilitated diffusion of water vapor, which explains the higher WVP (8.57 g/mm^{-2} d^{-1} KPa^{-1}) of the film. Figure 1 (b) shows that the nanocomposite films of CPI and MMT had a smooth and continuous surface, without granular and porous structure, minimum surface irregularities, without the presence of coarse rough filaments, internal cracks or bubbles which may be an indicative of better interaction between the polymer and MMT, positively influencing the good performance of the mechanical properties of the films.

Souza *et al.* (2011), evaluated films formed from chitosan, incorporated at different lipid fractions, finding that the micrographs of the film surface made of pure chitosan was free of air bubbles with a continuous smooth surface without granular and porous structure. Whereas the lipid composed films (oleic acid, refined rice oil and refined fish oil) showed some irregularities, however, without increasing the permeability to water vapor. Kampeerapappun *et al.* (2007), in the preparation of composite films of cassava starch/montmorillonite saw in the scanning electron microscopy that the size of the finest clay particles was obtained in composites with chitosan. The results indicated that the chitosan, due to their hydrophobicity and the ability to attach to the surface of clay had a role in the compatibility between starch matrix and montmorillonite.

FT-IR spectroscopy was used to examine the interactions between CPI and MMT. The infrared spectra of pure CPI and CPI + MMT film are presented in Figure 2. In this study the spectra was used for qualitative analysis and comparison of the structure of the biomaterials in the range of 4500-500 cm^{-1}, being focused on the mid-infrared. The spectra of the films (pure CPI and CPI with MMT) were compared. The exact position of the absorption band reveals important details of the structure, as well as

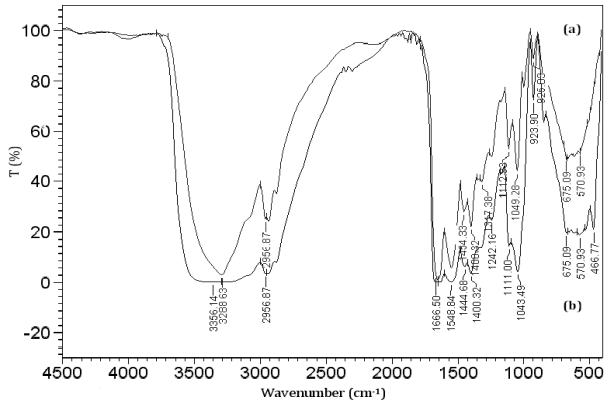

Figure 2. Comparison of the spectra of CPI films in the wavelength range 4500-500 cm^{-1}, where (a) pure CPI film, and (b) CPI and MMT nanocomposite film

changes in the contours of the bands. The two most important areas for the preliminary examination of the spectra are the regions 4000-1300 cm^{-1} (2.5 to 7.7 μm) and 909-650 cm^{-1} (11.0 to 15.4 mm). This last range of shorter wavelength (high energy) is called the region of functional groups. The absorptions corresponding to important functional groups such as OH, NH and C=O occur in this region. The intermediate region of the spectrum, 1300-909 cm^{-1} (7.7 to 11.0 μm) is known as the "fingerprint". The spectrum includes many bands, is complex and the vibration modes are generally coupled. This spectral region is very important for determining the structure, provided that it is compared to other regions (Silverstein *et al*., 1987). Figure 2, shows spectra that range from the shortest wavelength (high energy) until the fingerprint. One can see in 3356.14 and 3288.63 cm^{-1} the overlapping in axial stretching of OH and NH present in the protein (protein isolate). The peaks in the region of 2956 cm^{-1} are relative to axial stretching CH. The acute and less intense band centered at 1666.50 cm^{-1} shows the carbonyl stretch, coming from the interaction of clay with the isolated protein. The peaks at 1317.38 and 1242.16 cm^{-1} are relative to the symmetrical angular deformation of C-H. The peak area of 570.93 cm^{-1} may be related to the angular deformation of N-H amino acids present in the isolated protein.

Figure 3 shows the type of crystal structure of the protein isolate films croaker. It can be observed from Figure 3 that for pure CPI films, a polymer type structure without the presence of peaks predominates. For nanocomposite films of CPI and MMT a broad band in the region between 2θ = 26.9° and 2θ = 50.4° is exhibited where the broad bands observed in this region reflect that the films showed a very low percentage of the degree of crystallinity. The XRD pattern of the composite film also shows

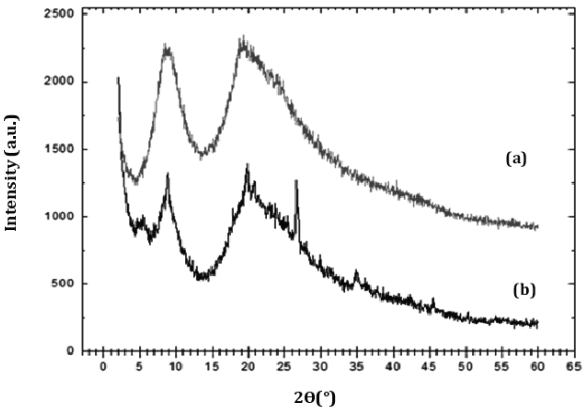

Figure 3. Diffractogram of the protein isolate croaker film, where (a) pure CPI film; (b) CPI and MMT nanocomposite film

a peak 2θ = 8.05° which corresponds to the spacing between the interplanar montmorillonite silicate layers. Generally, the variation of the interplanar spacing in CPI and MMT nanocomposite films was between 10.38 Å = D001 and D001 = 1.67 Å, which suggests the formation of a nanocomposite with low crystallinity. At values above 5% of MMT in the matrix, the interplanar montmorillonites spacing tends to stabilize or decrease (Dean *et al.*, 2007; Bordes *et al*., 2009).

Chivrac *et al.* (2010), reported that in the starch-based nanocomposite plasticized with glycerol and Na-MMT (Cloisite Na^+), when glycerol is present in concentrations above 10% (w/w), the formation of structures interspersed is favored as demonstrated by the XRD.

Conclusions

The incorporation of montmorillonite decreased the solubility, transparency and water vapor permeability (WVP) as compared with pure CPI film). However, the films with montmorillonite showed increase in mechanical properties, tensile strength and elongation percentage at break point, than the pure CPI film. The films produced from CPI with MMT showed better properties of WVP, tensile strength, scanning electron microscopy (SEM), structural analysis in Fourier Transform Infrared (FT-IR) spectroscopy and X-ray diffraction (XRD).

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