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Edible pectin film added with peptides from jackfruit leaves obtained by high-hydrostatic pressure and pepsin hydrolysis

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N-tert-Butyldimethylsilyl-*N*-methyltrifluoroacetamide (MTBSTFA >97% PubChem CID: 2724275)
Glycerol (PubChem CID: 753) sodium hydroxide (NaOH PubChem CID: 14798)
Hydrochloric acid (HCl PubChem CID: 313)
Acetonitrile (PubChem CID: 6342 HPLC grade ≥99.8%)
Low-methoxyl (LM) pectin (29–33% Food grade E-440)

ABSTRACT

Jackfruit (*Artocarpus heterophyllus* Lam.) is an evergreen tree that produces a high waste of leaves. This study evaluated the obtention of peptides from jackfruit leaves using pancreatin and pepsin, their antifungal activity, and their effect on pectin films. The protein content was 7.64 ± 0.12 g/100 g of jackfruit fresh leaves. Pancreatin produced a higher yield than pepsin in the obtention of peptides ($p \leq 0.05$). However, peptides obtained after 2 h by pepsin hydrolysis (Pep-P) had six essential amino acids and inhibited > 99% of mycelial growth and spore germination of *Colletotrichum gloeosporioides*. Pectin films with Pep-P showed a slight brown color, lower thickness, water vapor permeability, and moisture content, as well as higher thermal stability and better inhibition properties against *C. gloeosporioides* than pectin films without Pep-P ($p \leq 0.05$). Pectin films added with Pep-P from jackfruit leaf could be a green alternative to anthracnose control in tropical fruits.

1. Introduction

Fruits and vegetables are essential in the human diet due to the nutritional and health benefits associated with their consumption (Rai et al., 2016). The maintenance of the quality and nutritional values of fruits and vegetables is a challenge to the food industry and scientists. In addition to their physiological process after harvesting, these foods are highly susceptible to microbiological deterioration (Sapper & Chiralt, 2018). *Colletotrichum gloeosporioides* is a fungus that can produce losses of 100% of tropical fruits such as avocado, papaya, mango fruit among others depending on storage conditions during the postharvest stage (Iñiguez-Moreno, González-Gutiérrez, et al., 2020). Its control usually is through the application of synthetic waxes (polyethylene and petroleum origin) and chemical fungicides. However, the continuous application of these treatments could be harmful to human health and the environment. Therefore several methods and technologies based on biodegradable alternatives have been assessed (Sapper & Chiralt, 2018).

The use of edible films (EFs) based on polysaccharides such as pectin and sodium alginate is an effective eco-friendly alternative to extend the shelf-life of fresh food products (Iñiguez-Moreno et al., 2021; Iñiguez-Moreno, Ragazzo-Sánchez, et al., 2020; Shahrampour et al., 2020). EFs create semipermeable barriers to maintain the organoleptic properties

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and provide a good appearance to the coated food products. Moreover, one of the main interests is the inclusion of substances with antimicrobial activity such as natural extracts, essential oils, bacteriocins, metals, enzyme systems (Sapper & Chiralt, 2018), or biocontrol agents (Iñiguez-Moreno et al., 2021) within polymeric matrices. However, the study of the effect of peptides from agro-industrial wastes on the inhibition of fungal growth has been rarely assessed.

Jackfruit (*Artocarpus heterophyllus* Lam.) is an evergreen tree cultivated in the world's tropical regions, including China, India, Malaysia, Bangladesh, and Mexico. Agro-industrial wastes (seeds and leaves) from this tree are discarded during the processing of jackfruit. It has been estimated that every year 10377.76 tons/ha of jackfruit leaves are generated (Zhang et al., 2019), making it a sustainable source to obtain leaf flour with a high protein content (24.06%, dry basis) (Calderón-Chiu et al., 2021). Besides, increasing the use of leaf wastes will contribute to reducing greenhouse gas emissions, loss of biodiversity, and climate change (Oliveira Filho et al., 2019). Otherwise, to obtain proteins from plant sources, the first stage is extraction. In this sense, high hydrostatic pressure has been demonstrated to be an effective technique to obtain proteins from jackfruit leaves (Moreno-Nájera et al., 2020). However, antimicrobial activity is mainly related to peptides more than proteins. Several studies have been assessed the antioxidant effect of peptides from plant and animal sources in beef, pork, and turkey meat (Tkaczewska, 2020). Only few studies have been reported the use of enzymes such as pepsin and pancreatin for the obtention of peptides with technological and antioxidant properties from agro-industrial waste (Calderón-Chiu et al., 2021). Hence, the use of enzymes to obtain peptides from underutilized proteins is a promising alternative to produce active ingredients for the development of bioactive packaging. In line with this, peptides from cotton seeds have been obtained with a subtilinase and added into active films to inhibit *Staphylococcus aureus*, *C. gloeosporioides*, and *Rhizopus oligosporus* (Oliveira Filho et al., 2019). Hence, the development of natural films added with peptides from agro-industrial wastes will provide an alternative to inhibit the growth of *C. gloeosporioides*, which is one of the main phytopathogenic fungi in tropical fruits. Therefore, the aims of this study were to i) obtain peptides using pepsin and pancreatin at different hydrolysis times, ii) evaluate their antifungal activity against *C. gloeosporioides*, and iii) characterize a biocomposite film of pectin added with peptides from jackfruit leaves.

2. Materials and methods

2.1. Plant material

Jackfruit leaves without damage and impurities were collected from "Las Varas", Compostela, Nayarit, Mexico (21.3328 N; −104.588 W) in February 2019. The leaves were washed, disinfected by dipping in sodium hypochlorite (2%, v/v; for 2 min), and rinsed with distilled water. The leaves were grounded using a blender (NutriBullet® SERIE 900, Los Angeles, CA, USA) and sieved below 75 μm diameter.

2.2. Phytopathogen fungus

Colletotrichum gloeosporioides, previously isolated from *Carica papaya,* was cultivated on potato dextrose agar (PDA, Oxoid, Basingstoke, UK) for 7 days at 28 °C. The spore suspension was obtained according to the protocol proposed by González-Estrada et al. (2017), concentration was adjusted to 1×10^6 spores/mL by microscopic counting in a hemocytometer.

2.3. Chemical substances

Pepsin (EC 3.4.23.1), pancreatin (EC 232–468-9), Bradford reagent, serine standard (PubChem CID: 5951), amino acid standards (AA-S-18), L-Norleucine (Nor, ≥98%; PubChem CID: 21236), *N-tert*-Butyldimethylsilyl-*N*-methyltrifluoroacetamide (MTBSTFA, >97%; PubChem CID: 2724275), and glycerol (PubChem CID: 753) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Sodium hydroxide (NaOH; PubChem CID: 14798), hydrochloric acid (HCl; PubChem CID: 313), and acetonitrile (PubChem CID: 6342, HPLC grade, ≥99.8%) were purchased from Thermo Fisher Scientific Inc. (Waltham, MA, USA). Low-methoxyl (LM) pectin (29–33%, Food grade E-440) was purchased from Cargill Mexico (El Salto, Mexico).

2.4. Jackfruit leaf protein concentrate extraction and quantification

Jackfruit leaf protein concentrate (LPC) was obtained using the isoelectric precipitation method assisted by high hydrostatic pressure (HHP) according to the protocol described by Calderón-Chiu et al. (2021) with some modifications. Briefly, 100 g of grounded green leaves were mixed with 720 mL of distilled water, pH was adjusted at 9.0 with 0.2 M NaOH. The mixture was homogenized at room temperature for 20 min, vacuum-sealed, and placed in the HHP equipment (Isostatic press Flow Autoclave System, Avure Autoclave Systems, Model LCIP402260NCEP1MLN, Eri, PA, USA) at 300 MPa for 20 min at 25 °C. Then, the mixture was centrifuged at 15,000 *g* for 20 min at 4 °C (Hermle Z 326 K, Wehingen, Germany) and the supernatant was recovered. The supernatant was fractionated using an ultrafiltration cell (XFUF04701, Millipore®; Burlington, MA, USA) with a 30 kDa membrane constituted of regenerated cellulose (Ultracel®, Jeisys Medical, Inc., Seoul, Korea). The pH of the filtrate was adjusted to pH 4.0 with 1 N HCl, allowed to stand for 12 h, then was centrifuged at 9000 g for 5 min at 4° C (Hermle Z 326 K, Wehingen, DEU). The supernatant was discarded and the precipitate was washed with acetone, ethanol, and diethyl ether. Finally, the precipitate was diafiltered through a 1 kDa membrane (Ultracel®, Jeisys Medical, Inc., Seoul, Korea) and lyophilized at −50 °C and 0.12 mbar in a freeze-drier (Labconco FreeZone 4.5, Kansas, MO, USA). Bradford method was used to quantify the protein content in LPC (Bradford, 1976). The results were expressed in g of protein/100 g of fresh leaf. The protein content was determined in triplicate in two samples of LPC.

2.5. Obtention of peptides from LPC

Peptides were obtained by hydrolysis with pepsin and pancreatin according to the method described by Calderón-Chiu et al. (2021). Briefly, LPC (1%, w/v) was suspended in distilled water; then, the suspension was incubated in a shaking water bath (Shaking Hot Tubs 290200, Boekel Scientific, Feasterville, PA, USA) at 37 °C, at 115 rpm/30 min. The pH of the solutions was adjusted to 2.0 (for pepsin) and 7.0 (for pancreatin) with 1 N HCl and NaOH, respectively. The enzymes were added at an enzyme:substrate ratio of 2:100 (w/w). Hydrolysis was carried out at time intervals of 2, 4, and 6 h. Inactivation of the enzyme was achieved by heating at 95 °C for 10 min and the pH mixture was adjusted to 7.0, then, the suspension was centrifuged at 6195 *g* for 10 min at 4 °C. The supernatant was fractionated with a 10 kDa membrane (Ultracel ®, Jeisys Medical, Inc., Seoul, Korea) and freeze-dried to obtain the peptides with pepsin (Pep-P) and pancreatin (Pan-P) at different hydrolysis times. Hydrolysis yield was calculated with Equation (1). The test was carried out in triplicate and repeated twice.

$$Yield(\%) = \frac{Weightofhydrolyzate(g)}{WeightofLPC(g)} \quad (1)$$

2.6. Antifungal activity of peptides

2.6.1. Inhibition of mycelial growth

Antifungal activity of peptides was assessed against *C. gloeosporioides*. Mycelial plugs (7 mm in diameter) from the active fungus growth cultivated on PDA (7 days at 28 °C) were aseptically placed on a new PDA plate added with peptides (340 or 680 μg/mL,

these concentrations were determined based on the results of preliminary tests, data not shown). Then, the inoculated plates were covered with parafilm to avoid dehydration. The growth diameter was registered during 7 days at 28 °C. PDA with and without fungus plug were used as positive and negative controls for fungal growth, respectively (Iñiguez-Moreno, Ragazzo-Sánchez, et al., 2020). The percentage of inhibition of mycelial growth was calculated according to Equation (2):

$$Inhibition(\%) = \left(\frac{dc - dt}{dc}\right)100 \quad (2)$$

where dc (cm) is the mean of the colony diameter for the control and dt (cm) is the mean of the colony diameter for the treatment. The test was carried out in triplicate and repeated twice.

2.6.2. Inhibition of spore germination

For this, 5 mL of potato dextrose broth (Oxoid, Basingstoke, UK) with 340 or 680 μg/mL of peptides were inoculated with 500 μL of spore suspension (1×10^6 spores/mL) of *C. gloeosporioides* to give a final concentration of 1×10^5 spores/mL. Tubes were incubated on a rotary shaker bath at 110 rpm and 25 °C. The germination rate was microscopically determined by measuring the percentage of germinated spores after 8 h of incubation in ~ 200 spores (Vázquez-González et al., 2020). A spore was considered germinated when its germ tube was longer than half the length of the spore (Iñiguez-Moreno, Ragazzo-Sánchez, et al., 2020). The test was carried out in triplicate and repeated twice. The peptide fraction with higher inhibition of *C. gloeosporioides* was chosen for the next tests.

2.7. Amino acid profile and GC–MS analysis

The identification and quantification of amino acids were carried out in Pep-P with sizes higher and lower than 10 kDa. The peptides were subjected to acid hydrolysis with 6 M HCl for 24 h at 110 °C (Lamp et al., 2018). The hydrolyzed samples were derivatized with MTBSTFA following the methodology proposed by Mykhailenko et al. (2020). Briefly, 100 μL of hydrolysate and 10 μL of Nor as internal standard (concentration 0.2 mg/mL) were transferred into a 2 mL PTFE-lined screw-capped vial and evaporated to dry residue under a gentle stream of nitrogen. The resulting precipitate was dissolved in 100 μL of acetonitrile and 100 μL of MTBSTFA. The solution was heated at 100 °C for 2.5 h in a glycerol bath. The mixture of L-amino acids standards was assessed under the same conditions. The chromatographic analysis was carried out in GC equipment 7890A (Agilent Technologies; Palo Alto, CA, USA) coupled to mass spectrometry (MS) 240 Ion Trap. 1 μL portion of the derivatized extract was injected in split mode (20:1) at 260 °C. The free amino acids were separated by using a capillary column Agilent J&W VF-5 ms (30 m length, 0.25 mm I.D., 0.25 μm film thickness) coated with 5% phenyl 95% dimethylpolysiloxane. Helium (99.9998 %) was used as carrier gas at a flow rate of 2 mL/min. The oven temperature was set at 150 °C and kept for 2 min, and then increased at 3 °C/min to 280 °C. The time analytical run was 45.33 min. GC–MS parameters were as follows: energy of ionization (70 eV), full scan mode (range 35–650 *m/z*), ion trap (150 °C), manifold (80 °C), and transfer line (130 °C). The amino acids in the samples were identified by the retention time and their linear retention index data (LRI) of the amino acid standards, NIST (version 2.3) software was used to confirm the identification by mass spectrum comparison. Quantification was performed by standard curves for each of the compounds and adjusting for the response of the internal standard. AMDIS (version 2.69) and Agilent MS Data Review (version 7.0.1) software were used. The amino acid profile was reported as g of amino acid/100 g of protein. The test was carried out using two samples and repeated twice.

2.8. Preparation of films

Films with and without peptides were prepared using LM pectin (1%, w/v), glycerol (50%, w/w, on the dry basis to the weight of pectin), and calcium chloride (20 mg/g of pectin), peptides (680 μg/mL). The mixture was stirred at 350 rpm in sterile distilled water for 12 h at 35 °C. The film-forming solution was poured into Petri dishes with a Teflon bottom and allowed to dry for 12 h at 25 ± 2 °C in a biosecurity cabinet (Novatech, Model CFLV-120, Kingwood, USA). Before each test, films were conditioned by storage in a desiccator (25 °C, 50% relative humidity, RH) for 72 h.

2.9. Films characterization

2.9.1. Thickness and water vapor permeability (WVP)

Film thickness was measured using a micrometer (Digimatic Micrometer Mitutoyo 252–164-62, Kawasaki, Japan) at six random positions of the films. The precision of the thickness measurements was ± 0.5 μm. Otherwise, the WVP of the films was gravimetrically determined according to AFNOR NF *H*00-030 standard (AFNOR 1974) modified by González-Estrada et al. (2017). The tests were carried out using six samples. Both tests were repeated twice.

2.9.2. Moisture content (MC) and solubility

MC was gravimetrically determined (Oliveira Filho et al., 2019). For the solubility test, films of 4 cm^2 were placed in a dilution flask with 50 mL distilled water at 25 °C/2h in continuous stirring at 100 rpm. The solution was filtered through filter paper (Whatman #1), and the undissolved matter was desiccated at 105 °C/24 h. The weight of solubilized dry matter was calculated by subtracting the weight of the insolubilized dry matter from the initial weight of the dry matter and expressed as a percentage of the total weight (Iñiguez-Moreno et al., 2021). The tests were carried out using six samples and repeated twice.

2.9.3. Film color and browning index

Color values of the films were measured with a CR-CHROMA METER CR-400 (Konica Minolta Sensing Inc., Tokyo, Japan). Six random locations of each sample were measured. The test was carried out using six samples and repeated twice. Color was expressed as L^* (lightness/brightness), a^* (redness/greenness), and b^* (yellowness/blueness). A white standard color plate ($L^* = 95.44$, $a^* = -0.44$, and $b^* = 2.42$) was used as background for the color measurements of the color film. The total color change (ΔE) was determined with Equation (3):

$$\Delta E = \sqrt{(L^* - L_c^*)^2 + (a^* - a_c^*)^2 + (b^* - b_c^*)^2} \quad (3)$$

where L^*, a^*, and b^* are the color parameters of pectin films with peptides; L_c, a_c, and b_c are the color parameters of pectin films without peptides. The obtained values were then used to calculate the browning index (BI) according to Equation (4) and (5) (Kchaou et al., 2019):

$$BI(\%) = \frac{(z - 0.31)}{0.172}100 \quad (4)$$

with

$$z = \frac{a^* + 1.75(L^*)}{5.645(L^*) + a^* - 3.012(b^*)} \quad (5)$$

2.9.4. Thermal properties of the films

Thermogravimetric analysis (TGA) was conducted with ~ 5 mg of sample, heating from 25 to 500 °C, at a heating rate of 5 °C/min under a nitrogen flow (40 mL/min) using TGA 550 equipment (TA Instruments, New Castle, USA) (Iñiguez-Moreno et al., 2021). The derivative thermogravimetric (DTG) curve was obtained directly from the TGA curve with the software TRIOS 5.0.0.44616 (TA Instruments Universal Analysis, New Castle, USA). Thermal tests were carried out with pectin films

with and without peptides and with all their components. All samples were analyzed in duplicate.

2.10. Activity of pectin films against C. gloeosporioides

The effect of the pectin films without and with peptides (680 μg/mL) on the mycelial growth and spore germination of *C. gloeosporioides* was assessed. For the spore inhibition, 50 μL of spores suspension (1×10^5 spores/mL) were spread onto PDA disks (1.4 cm in diameter) and then let dry for 10 min in a biosafety cabinet. After that, the disks were covered with films containing or not Pep-P. Subsequently, the Petri dishes were incubated at 28 °C for 8.5 h. After incubation time, the films were aseptically removed to determine the germination rate of *C. gloeosporioides* by microscopy. The percentage of germinated spores was measured as in Section 2.6.2. Otherwise, the technique described in Section 2.6.1. was used for the assessment of the effect on mycelial growth. As above, mycelial plugs were placed onto a new PDA medium. The inoculated medium was covered with films containing or not peptides. The growth diameter was registered during 7 days at 28 °C. Inoculated but uncoated PDA Petri dishes were used as positive controls for fungal growth (Iñiguez-Moreno, Ragazzo-Sánchez, et al., 2020). The percentage of inhibition of mycelial growth was calculated according to Equation (2). The test was carried out in triplicate and repeated twice.

2.11. Statistical analysis

Data were processed by one-way analysis of variance (ANOVA). Before the ANOVA, values expressed as percentages were arcsine-square-root transformed before their analyses, aimed to have better approximations to the normal distribution and improve the homoscedastic of the data (Lin & Xu, 2020; O'Neill & Mathews, 2002). The statistical data analysis was performed using the software Statgraphics Centurion XVI.I (Statpoint Technologies, Inc., Warrenton, USA), the post-hoc least significant difference (LSD) Fisher test ($p \leq 0.05$) was used for means comparison.

3. Results and discussion

3.1. Protein content in jackfruit leaf and yield of its peptides

The protein content in fresh leaves of jackfruit was 7.64 ± 0.12 g/100 g of leaves, this result is similar to reported to fresh leaves of *Olea europaea* (8.65 ± 0.40%) (Contreras et al., 2020). The slight discrepancy between these studies is related to the chemical composition of leaves varies depending on plant species, age, environmental conditions, and cultivation practices (Tamayo Tenorio et al., 2018). Therefore, jackfruit leaf can be considered as a good source of nitrogen for animal feeds and peptides obtention.

Pepsin and pancreatin were used to obtain peptides from the LPC of jackfruit (Fig. 1A, B). The yield of the enzymes was different ($p \leq 0.05$). However, both enzymes showed similar behavior, with a slight increment in the yield from 2 to 6 h ($p > 0.05$; Table 1). The high yield obtained with pancreatin in comparison to pepsin ($p \leq 0.05$; Table 1) was attributed to the enzymatic complex in the pancreatin with numerous activities including amylase, lipase, RNase, DNase, phosphatase, and various protease activities. These protease activities include: i) trypsin activity that cleaves protein at cationic amino acids such as arginine and lysine, ii) chymotryptic activity that cleaves at the aromatic amino acid and branched-chain sites (tyrosine, phenylalanine, tryptophan, leucine), iii) carboxypeptidase A which cleaves at all amino acid sites (except asparagine, glutamine, arginine, lysine), and iv) elastase which breaks down at alanine site (Andriamihaja et al., 2013). Otherwise, pepsin is a protease that acts on peptide bonds of the amino group of L-tyrosine or L-phenylalanine or in bonds of the carbonyl group of aromatic amino acids (Fruton et al., 1961). Otherwise, the low increment in the yield of both enzymes as increases the time is due to during the first minutes the

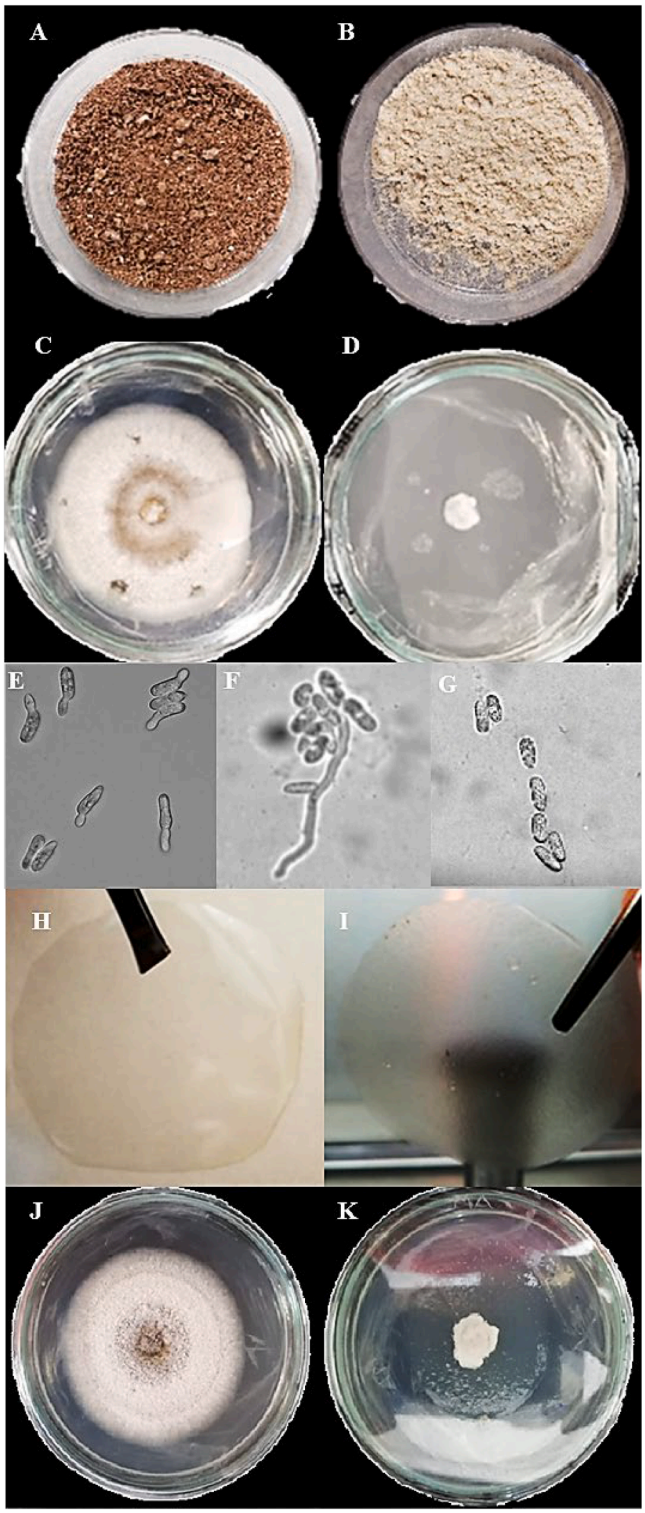

Fig. 1. Peptides from jackfruit leaves and their antifungal activity. Peptides with pepsin (A) and pancreatin (B). Growth of *Colletotrichum gloeosporioides* in potato dextrose agar (PDA) without (C) and with peptides (680 μg/mL) from leaf protein concentrate (LPC) of jackfruit after 2 h of exposition to pepsin (D). Spores germination of *C. gloeosporioides* in potato dextrose broth without (E) and with peptides at 340 (F) and 680 (G) μg/mL, respectively, after 8 h of incubation at 28 °C. Growth of *C. gloeosporioides* in PDA covered with pectin film without (H) and with peptides (680 μg/mL) from LPC of jackfruit after 2 h of exposition to pepsin (I). Tests in PDA were incubated for 7 days at 28 °C.

Table 1
Yield of peptides obtention from LPC of jackfruit and their effect on the *Colletotrichum gloeosporioides*.

Enzyme	Hydrolysis time (h)	Yield (%)	Peptides concentration (μg/mL)	Inhibition (%) Mycelial growth	Spore germination
Pepsin	2	12.50 ± 0.98^{d}	340	65.06 ± 0.08^{d}	96.00 ± 2.56^{b}
			680	99.20 ± 0.05^{a}	100.00 ± 0.00^{a}
	4	13.00 ± 0.56^{cd}	340	59.13 ± 0.15^{e}	83.21 ± 1.27^{d}
			680	82.46 ± 0.09^{b}	98.16 ± 1.09^{b}
	6	13.50 ± 0.32^{c}	340	54.39 ± 0.17^{f}	76.94 ± 2.31^{e}
			680	79.82 ± 0.05^{c}	91.89 ± 3.39^{c}
Pancreatin	2	46.00 ± 0.67^{b}	340	−25.85 ± 2.38^{g}	0.00 ± 0.00^{f}
			680	−35.73 ± 2.65^{h}	0.00 ± 0.00^{f}
	4	47.00 ± 0.23^{a}	340	−27.64 ± 1.56^{g}	0.00 ± 0.00^{f}
			680	−37.72 ± 1.30^{h}	0.00 ± 0.00^{f}
	6	47.50 ± 0.15^{a}	340	−35.53 ± 1.47^{h}	0.00 ± 0.00^{f}
			680	−41.91 ± 2.11^{i}	0.00 ± 0.00^{f}

Values are expressed as means ± standard deviation (n = 9). Values in the same column followed by different superscript lower-case letter are significantly different according to Fisher's LSD test at $p \leq 0.05$.

enzymes hydrolysate the polypeptide fractions attached to the protein surface. Then, the protein fraction compacted in the core is slowly hydrolyzed, producing low increments as the hydrolysis time increases (Wubshet et al., 2019). Calderón-Chiu et al. (2021) reported that the higher increment in yield of protein hydrolysates from dried jackfruit leaves was achieved after 0.5 h. Then slight increments were observed until reach constant values after 3 and 4 h. Therefore, the high yield reached with pancreatin is due to its wide activity sites in comparison to pepsin.

3.2. Antifungal activity of LPC peptides

The effect on the fungal growth of the peptides with a molecular weight lower than 10 kDa was assessed against *C. gloeosporioides*. Peptides, obtained from pancreatin activity, stimulated the growth of *C. gloeosporioides* until 40% concerning the control. The growth increased as increased the concentration of peptides and hydrolysis time ($p \leq 0.05$, Table 1). The broad number of sites to pancreatin activity produce high amounts of free amino acids, di- and tripeptides, and small peptides (>7 amino acids). Increasing their numbers as the hydrolysis time increases (Morais et al., 2013). Therefore, pancreatin produced peptides that could be used by the fungus as a nitrogen source, stimulating its growth. This explains the high growth percentage of *C. gloeosporioides* with Pan-P obtained after 6 h of hydrolysis in comparison to the control (Table 1).

Otherwise, Pep-P obtained after 2 h showed the higher inhibition percentage of mycelial growth (99%, Fig. 1D; Table 1) and spore germination (100%, Fig. 1G; Table 1). The antimicrobial activity of peptides is highly related to their molecular weight. Usually, peptides with antimicrobial properties are sequences of 12–50 amino acids with a net positive charge (+2 to + 11) due to the presence of cationic amino acids (Tkaczewska, 2020). Moreover, around 50% of the amino acids in peptides include leucine, isoleucine, valine, phenylalanine, and tryptophan are hydrophobic, enabling them to interact and penetrate cell membranes (Liu et al., 2015). These peptides inhibit fungal growth through morphological alterations of the mycelium and by inhibition of protein synthesis, α-amylase, and trypsin activity (Srivastava et al., 2021). Based on the obtained results of antifungal activity, the Pep-P obtained after 2 h of hydrolysis were selected for amino acid identification and for its addition into pectin films aimed to improve their functional properties.

3.3. Amino acids analysis

The GC–MS chromatograms allowed to establish the presence of 12 and 11 of 20 amino acids; including five and six essential amino acids in Pep-P with molecular weight > 10 kDa and < 10 kDa, respectively. The amino acids were identified by comparing their retention times with the standard amino acids in specific MS chromatograms, quantitative analysis was performed using Nor as an internal standard (Table 3). Due to the polar nature of amino acids, derivatization is required before GC analysis. Higher sensitivity can be reached during the analysis of amino acids for GC–MS by using derivatization agents, such as MTBSTFA that produces derivatives more stable and contain less moisture (Mykhailenko et al., 2020). According to a comparative analysis of the composition of amino acids in Pep-P, the fraction > 10 kDa showed a higher content of amino acids than Pep-P < 10 kDa. In the Pep-P > 10 kDa fraction can exist peptides with higher size and therefore more variation in the presence of the amino acid. Besides, if the peptides do not have sites for the enzyme activity, these amino acids will not be present in peptides of low molecular weight obtained by this way. Moreover, in Pep-P > 10 kDa, proline and hydroxyproline were identified but not in Pep-P < 10 kDa, because these amino acids can be part of peptides of higher molecular weights or were present in low concentrations. The high variability of amino acids identified in this research can be related to the sample. It was previously demonstrated that amino acid distribution decreases at leaves > corms > flowers > stigmas (Mykhailenko et al., 2020). The obtained results are similar to the reported *Crocus sativus* leaves extract and Juno dry leaves extract in which 13 amino acids were identified (Mykhailenko et al., 2020). The predominant amino acids in jackfruit leaves were amino acids with hydrophobic groups such as glycine, alanine, valine, leucine, isoleucine, proline, and phenylalanine (Table 2). This agrees with the results reported to *Crocus sativus* leaves (alanine 2.71 mg/g, valine 3.02 mg/g, leucine 2.40 mg/g, isoleucine 2.32 mg/g, and proline 4.68 mg/g) (Mykhailenko et al., 2020). These amino acids can inhibit free radicals by proton donation (Pompella et al., 2003). In line with this, it was reported that pepsin hydrolysates obtained from dry jackfruit leaves exhibited a high *in vitro* antioxidant activity through 1,1-diphenyl-2-picrylhydrazyl ($DPPH^{+\bullet}$, ~60%) and 2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) ($ABTS^{+\bullet}$, ~82%) at 0.1 and 1 mg/mL, respectively (Calderón-Chiu et al., 2021). This activity can be achieved in several ways: by reducing hydroperoxides, scavenging free radicals, reactive oxygen species inactivation, prooxidative transition metal chelation, and modifying the physical properties of the products (Sohaib et al., 2017). In addition, both samples assessed in this research have a high content of aliphatic amino acids (glycine, alanine, valine, leucine, and isoleucine), which contribute to improving the properties of a natural EF. Because these compounds could reduce the moisture content developing a tight network due to their non-polar and hydrophobic behavior (Tkaczewska, 2020). Therefore, the addition of Pep-P from jackfruit leaves could provide an antioxidant effect to pectin EFs. Moreover, it is important to take into account that the incorporation of peptides with essential amino acids into edible coatings could provide additional dietary value to the coated food (Tkaczewska, 2020).

3.4. Films characterization

The incorporation of active agents can modify the physicochemical and technological properties of films. Therefore, the properties of pectin films added with Pep-P from jackfruit leaves were compared with the

Table 2
Amino acid composition of peptides obtained with pepsin from jackfruit leaves.

Amino acid	TBDMS-derivatized amino acid	RT (min)	LRI	Pepsin peptides(g/100 g of protein) >10 kDa	<10 kDa
Alanine	L-Alanine, N-(*tert*-butyldimethylsilyl)-, *tert*-butyldimethylsilyl ester	4.486	1524	10.77 ± 0.56^{BCa}	8.25 ± 0.23^{Eb}
Glycine	Glycine, N-(*tert*-butyldimethylsilyl)-, *tert*-butyldimethylsilyl ester	4.892	1548	17.42 ± 0.65^{Aa}	1.66 ± 0.06^{Ib}
Valine	L-Valine, N-(*tert*-butyldimethylsilyl)-, *tert*-butyldimethylsilyl ester	6.451	1642	9.56 ± 0.10^{Db}	10.85 ± 0.0^{Ca}
Leucine	L-Leucine, N-(*tert*-butyldimethylsilyl)-, *tert*-butyldimethylsilyl ester	7.254	1677	11.36 ± 0.00^{Bb}	12.37 ± 0.13^{Ba}
Isoleucine	L-Isoleucine, N-(*tert*-butyldimethylsilyl)-, *tert*-butyldimethylsilyl ester	7.915	1712	7.01 ± 0.25^{Fb}	9.95 ± 0.20 ^{Da}
Proline	L-Proline, 1-(*tert*-butyldimethylsilyl)-, *tert*-butyldimethylsilyl ester	8.822	1764	10.21 ± 0.23^{CDa}	ND
Methionine	L-Methionine, N-(*tert*-butyldimethylsilyl)-, *tert*-butyldimethylsilyl ester	13.883	1961	ND	1.22 ± 0.01^{Ia}
Serine	L-Serine, N,O-bis(*tert*-butyldimethylsilyl)-, *tert*-butyldimethylsilyl ester	14.376	1975	5.97 ± 0.07 ^{Gb}	13.13 ± 0.21^{Aa}
Threonine	L-Threonine, N,O-bis (*tert*-butyldimethylsilyl)-, *tert*-butyldimethylsilyl ester	15.121	2041	7.00 ± 0.52^{Fb}	12.28 ± 0.75^{Ba}
Phenylalanine	L-Phenylalanine, N-(*tert*-butyldimethylsilyl)-, *tert*-butyldimethylsilyl ester	17.162	2091	2.85 ± 0.34^{Ib}	4.58 ± 0.06 ^{Ga}
Aspartic acid	L-Aspartic acid, N-(*tert*-butyldimethylsilyl)-, bis(*tert*-butyldimethylsilyl) ester	19.116	2134	8.21 ± 0.77^{Ea}	6.08 ± 0.41^{Fb}
Hydroxyproline	L-Proline, 4-[(*tert*-butyldimethylsilyl) oxy]-1-(*tert*-butyldimethylsilyl)-, *tert*-butyldimethylsilyl ester	19.93	2196	0.17 ± 0.05^{Ja}	ND
Glutamic acid	L-Glutamic acid, N-(*tert*-butyldimethylsilyl)-, bis(*tert*-butyldimethylsilyl) ester	22.645	2251	4.14 ± 0.01^{Ha}	3.23 ± 0.12^{Hb}
Total				94.68 ± 0.27^{a}	83.59 ± 0.21^{b}

TBDMS: *t*-Butyldimethylsiloxy, RT: Retention time, LRT: Linear retention index. ND: No detected. Values of pepsin peptides are expressed as means ± standard deviation (n = 6). Values in the same column, followed by different superscript capital-case letters, are significantly different according to Fisher's LSD test at $p \leq 0.05$. Values in the same row, followed by different superscript lower-case letters, are significantly different according to Fisher's LSD test at $p \leq 0.05$.

Table 3
Physicochemical properties of pectin films.

Property	Pectin film	Pectin film with Pep-P
pH	3.81 ± 0.01^{a}	3.23 ± 0.01^{b}
L^*	94.01 ± 0.55^{a}	91.31 ± 0.39^{b}
a^*	−0.62 ± 0.44^{a}	−0.49 ± 0.06^{a}
b^*	5.27 ± 0.93^{b}	13.25 ± 0.59^{a}
ΔE	–	3.55 ± 0.30^{b}
Browning index	0.05 ± 0.00	0.15 ± 0.01^{a}
Thickness (μm)	68.60 ± 7.99^{a}	49.10 ± 2.51^{a}
WVP (g/(m·s·Pa) × 10^{-9})	8.02 ± 0.00^{b}	9.43 ± 0.67^{a}
Solubility (%)	90.85 ± 0.12^{a}	86.63 ± 0.18^{b}
Moisture content (g water/g dry matter content)	13.80 ± 1.92^{a}	9.75 ± 1.21^{b}

Values are expressed as means ± standard deviation (n = 15). Pep-P: peptides from jackfruit leaf obtained after 2 h of hydrolysis with pepsin, pH of film-forming solution, WVP: water vapor permeability, L^*: lightness/brightness, a^*: redness/greenness, b^*: yellowness/blueness, and ΔE: total color change. Values in the same row, followed by different superscript lower-case letters, are significantly different according to Fisher's LSD test at $p \leq 0.05$. Tests were carried out with conditioned films at 25 °C, 50% RH for 72 h.

properties of pectin films without Pep-P, and with the results reported in the literature. The pH of the film-forming solution remained constant at 3.81 and 3.23 to the solution without and with peptides, respectively ($p \leq 0.05$). This is in agreement with the characteristic pH (2.0 to 6.0) of LM pectin films formed in the presence of divalent cations such as Ca^{2+} (Gawkowska et al., 2018). Otherwise, thickness decreased in pectin films added with Pep-P in comparison to films without peptides ($p \leq 0.05$, Table 3). In contrast with our results, it was demonstrated that in sodium alginate films, the thickness increased (10, 50, and 110 μm) as increases the concentration of peptides (0.15, 0.30, and 0.60%) from cotton seeds, due to the increment in the total solids (Oliveira Filho et al., 2019). This could be related to the presence of hydrophobic amino acids in Pep-P. In pectin films, Pep-P could act as crosslinking agents, reducing the swelling of the pectin network peptides reducing the thickness, developing EFs with a tight network (Barbut & Harper, 2019). About this, pectin films with Pep-P showed a lower MC in comparison to pectin films ($p \leq 0.05$, Table 3). Similar MC values were reported to sodium alginate films without (12.22 ± 1.37%) and with peptides (10.75 ± 1.36%) from cotton seeds. However, in these films, the addition of peptides did not significantly decrease the MC (Oliveira Filho et al., 2019). The similitude between the MC of both studies is due to the gelation of LM pectin sodium alginate in the presence of Ca^{2+} ions, described by the "egg-box" model. At low pH (pH = 3), in this model, the gelling formation is due to pectin binding with Ca^{2+}, developing hydrophobic interactions, covalent and H-bonds, to the side chains of the polysaccharide (mainly to galactose and arabinose units), developing a

tight network and reducing the MC and thickness. In addition to this, the proportion of pectin to peptides had a significant impact on the MC of the composite films. As decreases, the ratio of pectin to peptides decreases the MC (Gawkowska et al., 2018).

WVP is one of the most important properties in determining a film's suitability for food packaging. A film should avoid, or at least reduce, the transfer of moisture between the environment and the food (Dashipour et al., 2015). The incorporation of Pep-P significantly decreased the WVP value ($p \leq 0.05$; Table 3). The interaction between pectin and Pep-P could limit the availability of hydrogen groups to form H-bonds with water, decreasing the affinity for water resulting in a decrement of permeability (Ngo et al., 2018). This effect was previously reported for chitosan films added with corn peptides, where the peptide addition decreased the hydrophilic properties of chitosan and, as a consequence, the water vapor transmission of the composite film diminished. Besides, corn peptides probably had a high level of non-polar amino acids, providing high water barrier performance to the films (Li et al., 2019, 2020). In line with this, the solubility of a film is an important property to determine its suitability for application as food packaging. The solubility of pectin films decreased with the addition of Pep-P of jackfruit leaves ($p \leq 0.05$), due to the presence of insoluble fractions of Pep-P, or the possible formation of covalent bonds in the film (Li et al., 2019). The addition of peptides could contribute to developing a rigid structure in which the glycerol was embedded into the matrix tightly. As a consequence, glycerol could not be leached out easily, reducing the solubility of the film (Qamruzzaman et al., 2021). Nevertheless, sodium alginate films added with peptides from cottonseed had higher solubility (>98%), related to the hydrophilic properties of sodium alginate and cleavage of the peptide bonds between amino acids, resulting in peptides with smaller molecular sizes and ionizable amino and carboxyl groups (Oliveira Filho et al., 2019; Qamruzzaman et al., 2021). The high water solubility of films could be considered a factor for their rapid biodegradability (Oliveira Filho et al., 2019).

Film color is an important property to be assessed due to the effect on the appearance of coated foods (Abdelhedi et al., 2018). The pectin films were transparent, while films added with Pep-P showed faint brown color (Fig. 1H, I). CIE Lab values to pectin films with and without Pep-P are shown in Table 3. The L^* value significantly changes with the Pep-P addition. In this sense, the b^* value also increased in comparison to pectin films ($p \leq 0.05$). The changes in the color are due to the brownish character of the jackfruit peptides previously reported by Calderon-Chiu et al. (2021). Then, peptides addition into pectin produces dark-colored films, originating a ΔE-value slightly higher than 3, this means that can be scarcely detected by the human eye (the human eye can perceive ΔE-values higher than 3) (Martínez-Cervera et al., 2011). The b BI gives information about the progressive development of colored compounds, and the relative crosslinking increment between Ca^{2+}, pectin, and Pep-P to pectin films (Kchaou et al., 2019).

The weight decrement during the heating process was determined from TGA (Fig. 2). Calcium chloride and glycerol were decomposed in one step (Fig. 2A). Otherwise, peptides and pectin showed a classical thermal decomposition in three steps. The first stage (25–120 °C) corresponds to the loss of moisture and water absorbed by the Pep-P and pectin (Fig. 1B), these results are in agreement with previous reports

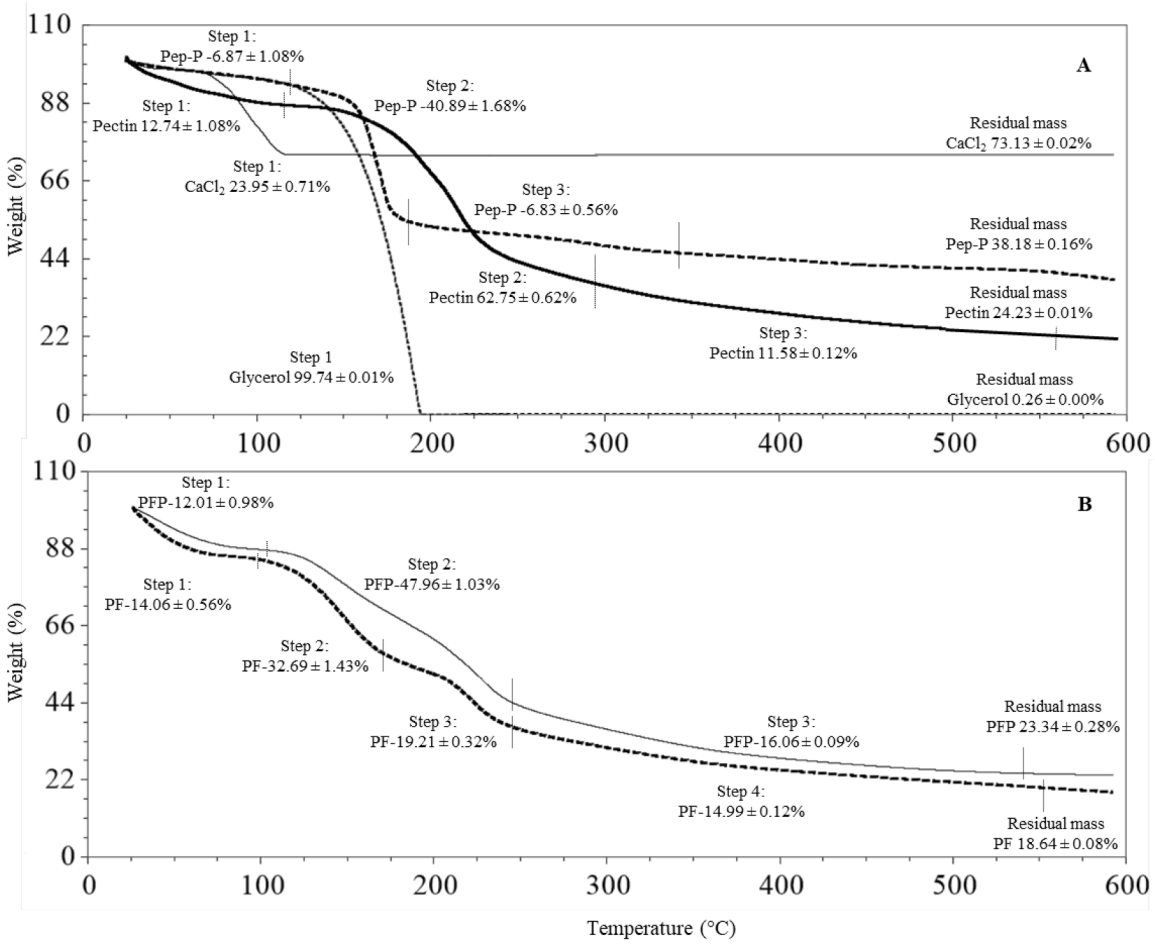

Fig. 2. Thermal analyses of pectin films with and without peptides from jackfruit leaves. (A) Thermogravimetry (TG) curves of components of films (glycerol —; calcium chloride —; pectin —; peptides from Jackfruit leaf protein concentrate obtained with pepsin (Pep-P, —). (B) TG curves pectin films with (PFP, —) and without (PF, —) peptides from jackfruit leaves.

(Mendes et al., 2019). The higher weight loss in this step was observed in the pectin due to its hygroscopic properties (Skwarek et al., 2017). The second step located between 120 and 300 °C or 120 and 215 °C to peptides and pectin, respectively (Fig. 2B), indicates the decomposition of the polysaccharide chain (Skwarek et al., 2017). Otherwise, the weight loss of peptides was associated with progressive deamination, decarboxylation, and depolymerization by the breaking of polypeptide bonds (Mendes et al., 2019).

The TGA of pectin films and pectin films with Pep-P showed a decomposition in four and three steps, respectively. The first weight loss from 25 to 95 °C was associated with water evaporation (Giri & Mandal, 2018; Iñiguez-Moreno et al., 2021). The difference with MC (Table 3) is attributable to the better sensibility of the TGA technique. In pectin films, the second weight loss located between 98 and 194 °C was associated with loss of physical and chemical bound water (Giri & Mandal, 2018). Moreover in this stage, also occurs pectin depolymerization (Mendes et al., 2019). The polymer decomposition occurs between 194 and 270 °C (third weight loss). Otherwise, in pectin films and films with Pep-P the loss of linked/absorbed water, polymer, and Pep-P decomposition occur in one step between 105 and 270 °C. The loss of thermal stability of Pep-P; could be attributed to the decomposition of supramolecular β-sheet arrangement (Giri & Mandal, 2018). Besides, the decomposition occurs in one step due to the development of a complex between all film components. In line with this, serine and threonine are polar amino acids since they carry a ¯OH group. This polar group can contribute to H-bond formation with another polar group by donating or accepting protons. In addition, they participate in van der Waals interactions, which are essential for the stabilization of protein structures (Tkaczewska, 2020). Therefore, they can form bonds with pectin, calcium chloride, and glycerol. Finally, the last weight loss in both samples was associated with the decomposition of carbonaceous material (Iñiguez-Moreno et al., 2021; Mendes et al., 2019). Both films showed similar decomposition profile, although some differences could be noted. It was observed a reduction in the percentage of water evaporation, polymer decomposition occurred in one step and the onset temperature of its decomposition showed a slight increment ($p \leq 0.05$) in comparison to films without Pep-P. These findings suggest that Pep-P incorporation affected interactions with the polymer matrix of pectin films through the H-bonding and van der Waals interactions. This behavior was previously reported to pectin film reinforced with spent coffee grounds by the formation of hydrogen and covalent bonds with reactive groups of pectin (Mendes et al., 2019).

3.5. Effect of pectin films added with Pep-P on the growth of *C. gloeosporioides*

The use of peptides of low molecular weight has several advantages, such as antimicrobial activity. Antimicrobial peptides are a promising alternative to give natural protection to natural foods and crops against microbial damage. Pectin films with Pep-P inhibited in 82.3 ± 0.8 and 91.6 ± 1.2% the mycelial growth (Fig. 1K) and spore germination of *C. gloeosporioides*, respectively. These inhibitions percentages are higher than those obtained for *Aspergillus niger*, *Penicillium italicum*, *Penicillium digitatum*, *Geotrichum candidum*, *Mucor* sp., and *Rhizopus* sp. using EFs based on pea starch and guar gum added with blueberry or macadamia extract (1000 μg/mL) and similar to the same films added with epigallocatechin-3-gallate (catechin from green tea, 1000 μg/mL) (Saberi et al., 2017). Otherwise, a low inhibition percentage was reported to *C. gloeosporioides* (27.02 ± 2.83%) and *Rhizopus oligosporus* (25.91 ± 2.02%) with sodium alginate films added with cotton seeds hydrolysates (0.60%, w/v) after 72 h of incubation (Oliveira Filho et al., 2019). The antifungal properties of peptides depend on their size and composition. Peptides with antimicrobial properties usually are composed of<50 amino acids, nearly half of which are hydrophobic (Tkaczewska, 2020) as occurs in this research. The antimicrobial mechanism of action of peptides is based on their electrostatic interaction with the cell membrane of microorganisms, resulting in its disruption and the loss of cytosolic compounds. Besides, the antimicrobial activity of bioactive peptides could be related to the iron-chelating activity (Rai et al., 2016). These changes cause a disequilibrium in cellular contents which deregulates all metabolic processes of the fungus. In addition to the antifungal mechanism of peptides, the films represent a physical barrier to oxygen reducing its availability to fungal metabolism (Iñiguez-Moreno, Ragazzo-Sánchez, et al., 2020).

Conclusions

Enzymatic hydrolysis of jackfruit LPC with pepsin and pancreatin at different times allowed obtaining peptides with different hydrolysis degrees. The yield in enzymatic hydrolysis had a significant effect on the antifungal activity of peptides against mycelial growth and spore germination of *C. gloeosporioides*, at higher yield lower antifungal activity. The higher antifungal inhibition was reached with the Pep-P obtained after 2 h hydrolysis, these peptides had 11 amino acids, of which six are essential. Biocomposite films of pectin with Pep-P into pectin films showed lower thickness, water vapor permeability, and moisture content, and higher thermal stability, as well as higher inhibition of *C. gloeosporioides* in comparison to pectin films without Pep-P. Pectin films added with Pep-P from jackfruit leaves are the basis for the development of novel biocomposite edible coatings for anthracnose management in tropical fruits during the postharvest stage. That in addition, they provide some essential amino acids for the human diet. However, further studies should be carried out on the assessment of its effect on phytopathogenic fungi *in vivo* conditions and on the organoleptic properties of the fruits.

CRediT authorship contribution statement

Irving Antonio Brion-Espinoza: Conceptualization, Methodology, Investigation. **Maricarmen Iñiguez-Moreno:** Formal analysis, Data curation, Writing – original draft, Visualization. **Juan Arturo Ragazzo-Sánchez:** Conceptualization, Formal analysis, Writing – review & editing. **Julio César Barros-Castillo:** Methodology, Investigation, Formal analysis. **Carolina Calderón-Chiu:** Methodology, Investigation, Formal analysis. **Montserrat Calderón-Santoyo:** Conceptualization, Resources, Writing – review & editing, Project administration, Supervision, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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