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Extraction and identification of endopeptidases in convection dried papaya and pineapple residues: A methodological approach for application to higher scale

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ABSTRACT

The use of agro-industrial waste for application in the obtention of products with high added value has become a trend in recent years, especially in tropical countries whose main economic sector is agricultural exports. In the present study, an applicable method to food industry of extracting proteolytic enzymes from dryed papaya and pineapple residues by convection was developed. Different to other scientific reports the heat treatment at 40 °C of waste residues, to reach 20% moisture, allowed an increase in total soluble protein content and did not alter the proteolytic activity of the extracts when phosphate buffer pH 7.0 was used as solvent. In the residues evaluated as candidates for the extraction of endopeptidases, we observed that green dried papaya peel and dried pineapple core, had higher activity values (914.34 ± 25.47 U/mg and 2152.36 ± 75.99 U/mg, respectively). These results, combined with one-dimensional electrophoresis and protein identification methods by MALDI TOF-TOF, showed the presence of signal peptides characteristic of papain, bromelain and other endopeptidases previously reported in extracts of fresh papaya and pineapple residues. These findings show that the drying of the residues by convection does not alter neither the activity nor the structure of the proteolytic enzymes. Finally, it is confirmed that the use of 20% ammonium sulfate as a precipitating agent allows to reach an efficiency of 74% in different work scales the use of purification and identification protocols in a more adaptable way, making them the most promising waste in Colombia, due to its potential for the production of bromelain on a larger scale.

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1. Introduction

Plant species such as papaya and pineapple are important sources of endopeptidases (Salas et al., 2008; Soares et al., 2012), which are important inputs into the food industry. Papain (EC 3.4.22.2), chymopapain (EC 3.4.22.6), glycyl endopeptidase (EC 3.4.22.25) and caricain (EC 3.4.22.30) are among the proteases identified and characterized in papaya, mainly in latex (Azarkan et al., 2003) which have been widely used for meat tenderization, edema treatment and shrink proofing of wool (Braia et al., 2013). In pineapple, stem bromelain (EC 3.4.22.32) and fruit bromelain (EC 3.4.22.33) have been reported, and comosain (EC 3.4.22.31) in a lesser extent (Larocca et al., 2010), which have anti-inflammatory, antithrombotic and fibrinolytic effects (Baez et al., 2007). Bromelain extracted from fruit and other crop residues has also been used for meat tenderization, beverage clarification and in baking (Ketnawa et al., 2010; Ketnawa and Rawdkuen, 2011).

The production of papaya and pineapple around the world, is characterized by generating a volume of waste close to 25% for papaya (seeds and peels) and 50% for pineapple (Ketnawa et al., 2012; Ordoñez et al., 2016), not counting the harvest surpluses and the rejected fruits (green and ripe). In Colombia both species are cultivated, despite not being native fruits. "Honey gold" pineapple is grown in the Orinoquía, Pacific and Amazon regions, with a total production of 619.048 tons in 2015, with the main producers being Santander and Valle del Cauca (MADR, 2017a). The production of papaya in 2015 was 176.226 Ton (MADR, 2017b), representing 1% of world production (Dane, 2016). Although it is a low figure, the annual increase in cultivated hectares, as well as the increase in demand, has led to an increase in the generation

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of waste. In the case of pineapple, the waste generated by the fruits and juice processing industries are mainly peel, core, stem, crown and leaves. In recent years, interest in agro-industrial waste has increased, as these generate economic and environmental problems for the different production chains. In this sense, the integral management of these can be applied to the production of biofuels and active compounds with high added value, contributing not only to the development of sustainable businesses but also to those cataloged under the concept of bio refinery (Gopinath et al., 2016).

Among the high value compounds derived from agricultural residues there are enzymes with industrial application. The extraction processes have been focused on the development of new strategies that guarantee high recovery rates with maximum activity (Nadar et al., 2017). The residues of these two fruits have been widely used to obtain proteolytic enzymes and other products. Recently, the use of fresh pineapple peels and core in enzymatic and fermentative processes, for obtaining wine and cooking vinegar with antioxidant properties, has been reported (Roda et al., 2017, 2016). In addition to the use of pineapple stems for the extraction of starch with high content of amylose and amylopectin, and higher solubility than cassava or corn starch (Nakthong et al., 2017). Additionally, the extraction of bromelain from fresh peels, core, stem and crown has been evaluated (Ketnawa et al., 2012) and different methods have been applied to obtain purified extracts using biphasic systems (Ketnawa et al., 2010), precipitation methods (Seguí and Fito, 2018) and ultrafiltration methods (Nor et al., 2016).

Papaya latex has been used for the extraction of papain. For this purpose precipitation methods with polyethylene glycol and ammonium sulfate have been evaluated (Nitsawang et al., 2006) as well as polyvinyl sulfonate (Braia et al., 2013) and recovery processes of papain using alginate as macro-ligand (Rocha et al., 2016). Other parts of the plant (green fruits, leaves, stems and petioles) have been used to obtain crude extracts with proteolytic activity (Galindo-Estrella et al., 2009).

Most of the methods developed for enzyme extraction have focused on the development of aqueous biphasic systems for purification using neutral salts, polymers or ligands (Nadar et al., 2017), similar to those previously described with pineapple and papaya. However, in these cases, only the use of fresh residue for extraction is contemplated, because of the ease with which the enzymes tend to degrade or denature during pretreatment of the same.

In the issue of waste generation, one of the critical aspects is its storage and transport, so it is necessary to think about thermal treatments that guarantee the stability of the residues in the stages prior to their use. Therefore, the present study was focused on the development of a platform for extracting proteolytic enzymes from papaya and pineapple residues for which the effect of the solvent type, the type of residue and the effect of the percentage of moisture after heat treatment on the proteolytic activity of crude extracts, were evaluated. The residue with the highest activity for each plant species was then selected and the enzymes with proteolytic activity present in these protein extracts were identified by SDS PAGE separation and further analysis by mass spectrometry (MALDI-TOF-TOF). Finally, a purification and scaling model was developed for the extraction of proteolytic enzymes from pineapple residues, this was necessary due to their higher production and utilization at the local level, as well as being a less commercially available and higher added value product.

2. Materials and methods

2.1. Vegetal material

Pineapple waste (*Ananas comosus* L.) and papaya waste (*Carica papaya*) acquired at Plaza Central Mayorista located in Itagüí, Antioquia was used for the development of the experiments. For the study of the solvent type effect, Papaya trees from Sopetrán, Antioquia (6°30′15‘‘N, 75°44′40″W) located at 949 m, were used. The fruits and their parts were washed repeatedly with neutral soap and deionized water and then cut into 2×2 cm cubes and stored at −20 °C prior to processing.

2.2. Reagents

For the extraction of the enzymes, 0.1 M phosphate buffer, pH 7.0 was used and for the precipitation ammonium sulfate ($(NH_4)_2SO_4$ J.T. Baker) was used. For the determination of proteolytic activity, a solution of papain (1200 U/mg), Citric Acid (0.05 M), Trichloroacetic Acid, Merck (30%) and Hammerstein Sigma-Aldrich Casein was used as standard. Casein was prepared in a 0.05 M Na_2PO_4 solution and its pH value was adjusted to 6.0. The papain solution was prepared and the purified protein pellet was suspended in Sodium-EDTA-L-cysteine Buffer Phosphate (FSEC) (0.1 M) (EDTA disodium salt and L-cysteine Merck). For fractionation of enzymes, a pH 7.6 (SSB) lysis buffer was used, consisting of 20 mM Tris-HCl pH 7.6, 150 Mm NaCl, 0.5 M sodium deoxycholate, 8 M urea, Sigma-Aldrich and 0.2 M NaOH Merck, to increase solubility. The enzymes were solubilized in Polyvinyl Pyrrolidone (PVP) Calbiochem and Triton X-100, Amresco. Pellet washing was performed with acetone, and the protein extract was purified with Methanol and Chloroform Merck. The pellet resuspension buffer obtained in the purification was the buffer (SSB1), composed of 7 M Urea, 2 M Thiourea, 4% CHAPS and 20 mM Tris-Hcl, pH 8.8, Sigma-Aldrich reference.

2.3. Effect of solvent type in extraction of papaya waste on proteolytic activity

For this study, an asymmetric 7×4 factorial design was evaluated. The study variables on the proteolytic activity were 4 types of solvents: (Methanol-Water (10–90), Methanol-Water (30–70), Methanol-Water (50–50) and Phosphate Buffer pH 7.0) and 7 parts of the papaya tree: Mature Pulp, Green Pulp, Ripe Peel Root, Green Peel, Mature Seeds and Green Seeds. In the extraction process the solvent substrate ratio (1:1), the cold-mortar cell disruption method, the filtration with polyester fiber at 4 °C and the centrifugation at 4 °C and 4000 rpm for 20 min were maintained as constants. The experiment was performed in triplicate. A validation test of the results obtained with papaya was carried out with pineapple residues (crown and core).

2.4. Effects of moisture percentage in papaya and pineapple residues on proteolytic activity

In order to evaluate the effects of the moisture percentage in the residue and to determine the feasibility of applying a thermal treatment that facilitates its transport from the place of origin to the enzyme extraction plant, a 6×2 asymmetric factorial design was performed in which the variables were the moisture content of the residues in 2 levels (100%–20%) for 6 types of residue (Ripe papaya peel, Green papaya peel, Green papaya pulp, Mature pineapple crown, Core of ripe pineapple; Ripe pineapple peel). In order to do this, drying curves at 40 °C were executed to determine the drying time for each residue in which the moisture content was 20%. The drying temperature was set on the basis of not exceeding the maximum activity temperature of the cysteine proteases (20–75 °C) (Barry, 2002). For the extraction of enzymes the use of Buffer phosphate pH 7.0 (SSB) as solvent, the ratio of solvent substrate (1:1), filtration with polyester fiber at 4 °C, centrifugation at 4 °C and 4000 rpm for 20 min and storage at 4 °C were maintained as

constants prior to the determination of the proteolytic activity for a time not greater than 24 h.

2.5. Identification of proteolytic enzymes in selected extracts of papaya and pineapple

Obtaining concentrated and purified proteolytic extracts: based on the previous experimentation, the protocol was adjusted, including steps of concentration and partial purification of the crude extracts. To obtain the different extracts, 25 g of dried (%H = 20%) pineapple core and green papaya peels were mixed with 25 mL Buffer (SSB). Disruption was performed with mortar and liquid nitrogen (5 cycles of 15 s). Subsequently, the extract was filtered with polyester fiber at 4 °C and centrifuged at 4 °C, 4000 rpm for 90 min. The supernatant was recovered and 50 μL/mL of 10% PVP and 16 μL/mL of Triton X-100 were added. It was stirred until complete homogenization and the mixture was allowed to stand for 1 h at 4 °C. Then, 200 μL/mL of 100% TCA was added, the vortex mixture was stirred and allowed to stand for 1 h for the precipitation process. Centrifugation was performed at 4000 rpm for 30 min and the pellet was recovered, which was washed with 7 mL of ice-cold acetone. After another centrifugation, the acetone was removed and the pellet was allowed to dry for one minute. 4 mL of resuspension buffer (FSEC) was then added.

Subsequently, to a fraction of the extracts obtained previously (2 mL), a purification protocol was applied adding 800 μL of chloroform and 800 μL of methanol to each extract. The mixture was homogenized and centrifuged at 4000 rpm for 30 min. The interface formed was recovered and washed with 800 μL of methanol to remove excess chloroform. The resulting pellet was resuspended in 800 μL of SSB1 buffer and preserved at 4 °C until separated by electrophoresis. At this point, several gels were run and evaluated for the presence of a protease inhibitor (PMSF) and the addition of glycerol to improve solubility. Based on these results, those that showed better bands and greater activity were selected for identification by MALDI-TOF-TOF mass spectrometry.

1D or conventional SDS-PAGE electrophoresis: From the residues selected for their higher proteolytic activity (pineapple core and green papaya peel), fractionation of proteins extracted by 1D electrophoresis was carried out under the improved extraction method in the previous stages.

The protein fractions were evaluated by one-dimensional electrophoresis under denaturing conditions. For this, 10 μg of protein was mixed with a loading buffer (4% SDS w/v, 20% glycerol v/v, 10% β-mercaptoethanol w/v, 4% SDS w/v, 0.125 M Tris-HCl pH 6.8 and 0.05% bromophenol blue w/v), then the mixture was subjected to 95 °C for 5 min. Protein separation was performed using Mini-Protean electrophoresis cameras (BIO-RAD, Hercules, CA-USA or Scie-Plas 1D, UK) using 12% (w/v) SDS-PAGE polyacrylamide gels, and were prepared according to the method of Laemmli (Laemmli, 1970). The separation was performed at 120 V for one hour. Finally, the obtained gels were stained for further analysis.

The gels were stained with Coomassie blue (0.1% Coomassie G-250 blue in 50% methanol: 10% acetic acid). Then gel discoloration was performed in a 40% methanol solution, followed by washing in water by immersion and shaking the gel for 1 h (Candiano et al., 2004; Pecet, 2010; Smejkal, 2004). Silver nitrate staining was also performed, which is a highly sensitive method for the detection of proteins in polyacrylamide gels. Silver nitrate staining comprises a series of steps between multiple washes, such as fixation (methanol-acetic acid), sensitization, staining (silver nitrate) and development (formaldehyde 2% sodium carbonate) (Orrego, 2010).

Mass spectrometry (MALDI-TOF-TOF): After separation in polyacrylamide gels, bands of interest were cut out based on molecular weight and intensity, corresponding to proteases previously reported for pineapple and papaya. Subsequently, tryptic digestion of the proteins in gel was performed. The selected bands were dried and faded with acetonitrile: water (ACN: H_2O, 1:1), and then reduced with 10 mM DTT for 1 h at 56 °C, and alkylated with 50 mM Iodoacetamide for 1 h at 26 °C. Digestion was performed in situ using trypsin-grade sequencing (Promega, Madison, WI) (Shevchenko et al., 2007).

The samples were analyzed on a MALDI-TOF-TOF (BrukerDaltonics) Ultraflextreme mass spectrometer, operating in positive linear mode, in a mass range of 700–3500 m/z. The identification of the proteins was carried out using the software Flexanalysis 3.4 and MASCOT 2.0, using the National Center for Biotechnology Information (NCBInr) database and the taxonomy Viridiplantae.

2.6. Effects of the concentration of ammonium sulfate as a precipitating agent of proteolytic enzymes in pineapple extracts

Based on the results obtained with the different types of residue, the pineapples were selected for evaluation of the later stages that allowed the approach of the product to a stage on a larger scale. In this instance, ammonium sulfate (AS) was evaluated as a precipitating agent, since it is the only agent applicable to the food industry. For this purpose, a factorial design 3^2 was performed, where the effect of the concentration of AS in three concentrations (10–20–30%) and the storage time at 4 °C in three levels (0, 14 and 28 days) were evaluated. The response variable was the total soluble protein content and proteolytic activity in the pellet and the supernatant obtained after precipitation. For this test, dry pineapple at 20% moisture, the ratio of substrate: solvent in a ratio of 1:1, disruption with blender, filtration with polyester fiber at 4 °C and centrifugation at 4 °C at 4000 rpm for 20 min, was used. The precipitation process was performed on the crude extract by adding the corresponding ammonium sulfate for 30 min at 750 rpm in an ice bath. The supernatant was preserved and the pellet obtained was washed and then resuspended with FSEC buffer and stored at 4 °C. The experiment was performed in triplicate.

2.7. Development of a protocol for extracting proteolytic enzymes from pineapple residues: An approach to higher scale

In order to evaluate the effects of the working scale and to determine the feasibility of scaling the extraction process, a unifactorial design was performed, in which the extraction scale as a response variable was studied (50–500–5000 mL). For this test, dry pineapple from the fluidized bed drier with 20% moisture, SSB buffer as solvent, 1:1 substrate: solvent ratio (solubility criterion as extraction limit), mortar disruption at 4 °C, filtered with polyester fiber at 4 °C, centrifugation at 4 °C at 4000 rpm for 90 min, was used The extraction phase was performed in 100 and 1000 mL with 50% working volume. A 10 L tank bioreactor, provided with a marine propeller were used for the extraction at 5000 mL. The precipitation process was performed on the crude extract by adding 20% ammonium sulfate at 750 rpm for 30 min in an ice bath. The supernatant was discarded and the pellet obtained was washed and then resuspended with FSEC buffer and stored at 4 °C. The experiment was performed in triplicate.

2.8. Analytical techniques

2.8.1. Determination of moisture curves for fruit residues

To determine the initial moisture content in the residues and to determine the moisture kinetics in the convection oven, approximately 250 g of each of the following residues were weighed: pineapple (crown, shell and core) and papaya (green shell, pulp green and ripe peel). The residues were fractionated into 2 × 2 cm sections and dried for 400 min in a redline forced convection oven Model RF115, removing the samples every 20 min to deter-

mine their total moisture content (drying of each sample until constant weight for moisture calculation). Drying was performed at 40 °C, temperature adequate to maintain proteolytic activity.

The calculation of the humidity was performed with the following formula:

$$\%H = \left(\frac{W_i - W_f}{W_i}\right) \times 100$$

where:

% H: Percentage of moisture lost (Kg water remaining/Kg solid moisture)

W_i: Wet sample weight (kg)

W_f: Final dry sample weight, constant weight (kg).

2.8.2. Determination of total soluble protein content

Quantification of the total soluble protein was performed using the Bradford colorimetric method previously standardized using Coomassie Brilliant Blue G-250, which in the presence of proteins forms a blue complex that can be monitored spectrophotometrically at 595 nm, using as standard a serum albumin solution (BSA) (Bradford, 1976).

2.8.3. Determination of proteolytic activity

The proteolytic activity was determined by the standardized AOAC method 971.16 and expressed in units per mg of soluble total protein. One unit of proteolytic activity corresponds according to the reference method to the amount of enzyme required to convert 1 μmol casein per minute. According to the reference standard used, 1 mg contains an activity of 30,000 U, and is capable of releasing 40 μmol of tyrosine/mL.

The formula used to determine the specific activity is as follows:

$$A = \frac{A_{Ref} \times C_M \times V_{rxn}}{V_{ext} \times C_W} \times FD$$

A: Specific activity in U/mg

A_{Ref}: Reference activity (30,000 U)

C_M: Final concentration of the sample obtained in the papain curve (mg/mL)

V_{rxn}: Final reaction volume (mL)

V_{ext}: Volume of enzyme extract (mL)

C_W: Analyzed Sample Concentration (mg/mL)

F.D: Dilution factor.

2.9. Statistical analysis

Statistical analysis of the data was performed with the GraphPad Prism 5.0 package. Statistical differences between treatments were determined using ANOVA and the Bonferroni test.

3. Results and discussion

3.1. Effects of solvent type in the extraction of papaya residues on proteolytic activity

Fig. 1 shows the results of the solvent type and the type of residue on the proteolytic activity of crude extracts from papaya residues. The use of phosphate buffer pH 7.0 is widely recommended as a solvent extraction or resuspension of enzymes in general and proteolytic enzymes obtained from pineapple and papaya, given their ability to maintain the pH of the enzyme in the range of maximum activity (pH: 6–7) (Chaurasiya and Umesh Hebbar, 2013; Galindo-Estrella et al., 2009; Quinde and Sánchez, 2012; Soares et al., 2012). Solvents such as methanol are used in combination with chloroform for the purification of enzymes based on a liquid-liquid extraction system whose interface formed between the organic phase and the aqueous phase turns out to be the purified enzyme pellet, which was initially assumed to be effective for extraction.

However the solvent which obtained the highest proteolytic activity was the phosphate buffer pH 7.0, when the extraction was performed from green papaya peel and mature seeds, recording activity values of 1037.5 ± 88.4 and 972.0 ± 18.1 U/mg, respectively. With the exception of green papaya seeds, where the lowest activity was recorded, this buffer also showed high activity levels, with no significant differences compared to the methanol-water solvent in the 10:90 ratio. Although it is not completely clear that the phosphate buffer pH 7.0 is better solvent for extraction of proteolytic enzymes than the methanol-water mixture, for the evaluated residues, is known that phosphates are involved in the extraction of more soluble protein and dynamic fractions (Bastida et al., 2018).

For that reason a subsequent assay with pineapple residues (crown and core) and green papaya peel was performed to show the effectiveness of the phosphate buffer pH 7.0 (SSB) as an extraction solvent, using pure water as a control solvent (Fig. 2). Regarding the total soluble protein content, it was observed that the highest concentrations of total protein were obtained using Buffer Phosphate pH 7.0, instead of water, for the three residues (pineapple crown: 406.2 ± 25.1 μg/mL; pineapple core: 204.8 ± 1.5 μg/mL and mature papaya peel: 464.5 ± 2.0 μg/mL), due to the fact that the buffer solution possesses a greater dissolution power of the enzymes than water, as a result of the presence of phosphate salts.

Regarding the proteolytic activity, it was observed that there is no significant difference between the activity obtained for the pineapple core extract when using SSB buffer or pure water, obtaining values of 2233.50 ± 78.86 and 2287.84 ± 449.60 U/mg, respectively. The same results were observed with pineapple crown extracts, for which an activity of 1634.91 ± 58.54 and 1634.34 ± 150.85 U/mg were obtained with phosphate buffer and water, respectively. Other reports where pure water was used as solvent showed activities of 36.111 ± 1.62 and 172.96 ± 1.29 U for both pineapple core and crown wastes, respectively (Ketnawa et al., 2012) however, to obtain enzymes from mature papaya shells, it was observed that the activity is higher when the SSB buffer (396.10 ± 11.36 U/mg) is used. Additionally it was confirmed that under this method, it is possible to extract proteolytic enzymes in the same range of activity previously obtained.

The classical methods of extracting plant active compounds by soxhlet mainly involve the use of solvents. However, due to the new tendencies to use environmentally friendly methodologies and to reduce the use of chemical compounds, extraction methods assisted by supercritical fluids, ultrasound, electric pulses, microwaves and enzymatic digestion have been developing (Azmir et al., 2013). Some of these innovative developments in extraction technologies are applicable to the extraction of enzymes. Even though the use of ultrasound, for enzymatic inactivation or elimination of microbial load in foods even at low frequencies have been reported (Zhang et al., 2017), some works have showed an increase of 18.17% of enzyme activity with the use of ultrasound at 15 W, 24 KHz for 10 min (Wang et al., 2012).

Even though pineapple residues have been used for the extraction of proteolytic enzymes for several years, currently they continue evaluating alternative methods of extraction from heart waste and pineapple peels remaining from saccharification processes in which they have been reported activities between 340 and 805 GDU (Gelatin Digestion Unit) for lyophilized and previously purified raw extracts (Seguí and Fito, 2018).

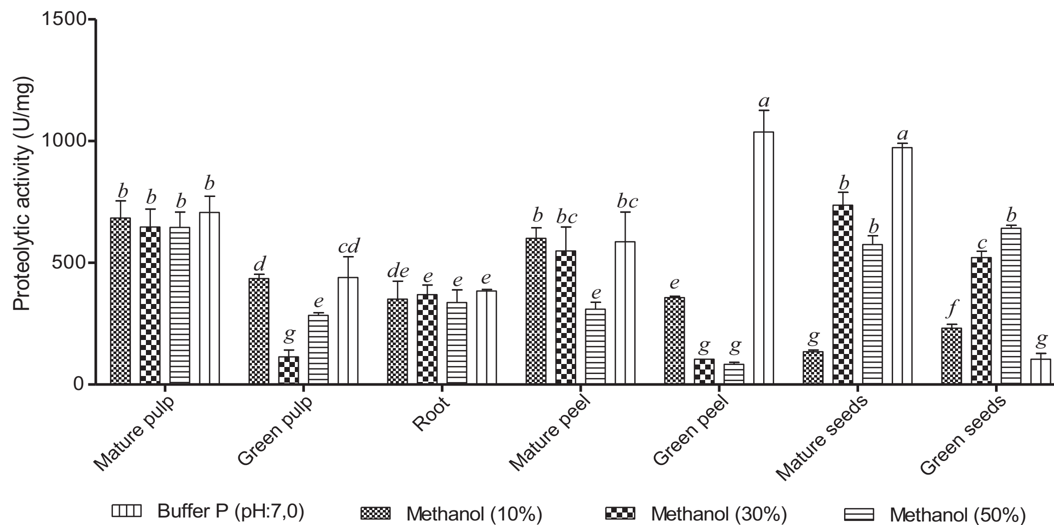

Fig. 1. Effect of the type of solvent and the type of residue on the proteolytic activity of papaya extracts. Different letters on the bars show significant difference between treatments (n = 3).

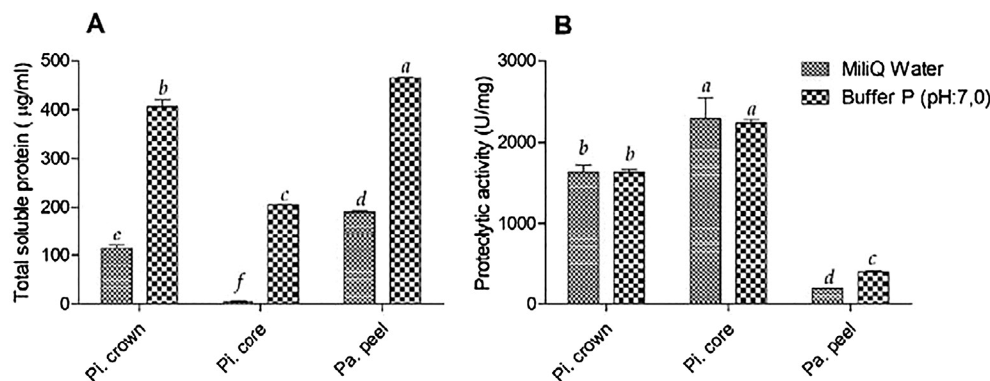

Fig. 2. Extraction of proteolytic enzymes with Buffer Phosphate pH: 7.0 and MiliQ-Water in residues of pineapple (Pi) and papaya (Pa). (A) Total soluble protein content. (B) Proteolytic activity. Different letters on the bars show significant difference between treatments (n = 3).

3.2. Effects of moisture percentage of papaya and pineapple residues on proteolytic activity

Fig. 3 shows the moisture content for each of the residues during drying in a convection oven at 40 °C. During the first 120 min the highest moisture loss corresponds to 47.0 ± 7.1% (mature papaya peel), 50.7 ± 1.2% (green papaya peel), 48.1 ± 1.0% (green papaya pulp), 41.0 ± 1.7% (pineapple crown), 43.3 ± 4.2% (pineapple core) and 43.3 ± 4.2% (pineapple peel). From this time the loss of moisture decreases. This is because the removal of unbound water in a solid is greater before the air stream reaches the saturation process.

The drying curves for each residue were adjusted to an exponential decay model, which was adjusted to the following formula:

$$\%H = (\%H_0 - Plateau)exp^{-kt} - Plateau$$

where:
%H: Percentage of moisture (kg water/kg solid wet)
$\%H_0$: Initial moisture content (kg water/kg wet solid)
k: Decay constant
t: Time (min).

The kinetic parameters for the residues evaluated during drying are presented below (Table 1). Drying times were determined at 40 °C, required for each residue to reach a 20% moisture percentage: 113 min for mature papaya peel, 99 min for green papaya peel, 109 min for pulp of green papaya, 89 min for pineapple crown, 100 min for pineapple core and 101 min for pineapple peels.

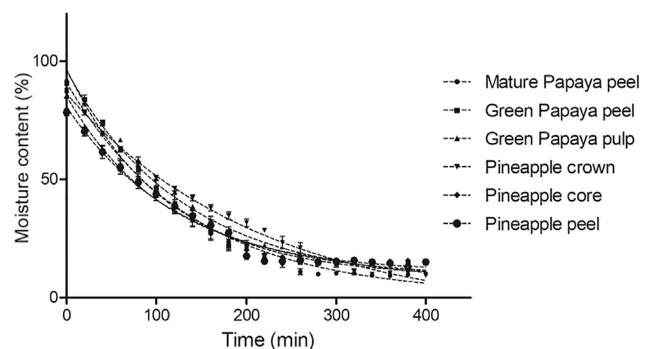

Fig. 3. Moisture curve for different residues. Batch drying for 400 min. Forced convection oven. Particle size 2 × 2 cm.

Among the residues most often used to obtain extracts rich in cysteine proteases (endopeptidases), peels, crown, stem and core of pineapple have been reported (Amid et al., 2011; Quinde and Sánchez, 2012); as well as papaya peels and latex, mainly green papaya (Jagtiani et al., 1988). Green papaya latex, although it is

the substrate richest in proteases presents production yields of only between 20 and 100 kg of dry papain per hectare, is expensive to obtain and already involves practicing sagittal cuts on the green fruits and their subsequent collection in containers prior to their use. It should then be dried in the sun or oven at 55–60° (Silva et al., 1997). This process can be replaced by the use of both green and mature papayas peel which are obtained directly from the food processing industries (juices, preserves, pulps, etc.). By-products of pineapple cultivation and processing such as stem, crown, peels and core, which represent 55% of the dry weight and are considered as residues, are promising for use in the process of obtaining proteases with the same purpose (Quinde and Sánchez, 2012).

When the residues are fresh (100% moisture), it was observed that the highest protein content is extracted from the crown of the pineapple (1694.90 ± 100.52 μg/mL). When the residues are subjected to a heat treatment giving a moisture content of 20%, the highest total protein concentration is extracted from mature papaya peels, followed by green papaya peels, with values of 1494.73 ± 27.43 and 1005.00 ± 19.07 μg/mL, respectively (Fig. 4A).

The effect of drying at 40 °C increases the total soluble protein content extracted by up to 322% for the residues of mature papaya peel, which is probably due to the difficulty and gelling phenomenon that occurs during the extraction of protein from fresh papaya residues, attributed primarily to its high polysaccharide content (Jagtiani et al., 1988). This is corroborated by the increase in the concentration of protein extracted from the peels and the green papaya pulp, which present the same problem. For pineapple residues with a high sugar content, although no gelation is present, an increase also occurs when the heat treatment is applied. Only for the pineapple crown residues, where the main component is cellulose, a reduction is observed.

With respect to the proteolytic activity, it was observed that this is greater with the heat treatment for all residues (Papaya: 881.70 ± 23.69 U/mg mature husks, 914.34 ± 25.47 U/mg green peels; 545.93 ± 48.88 U/mg green pulp - Pineapple: 1464.67 ± 45.45 U/mg crown, 2152.36 ± 75.99 U/mg core, 2618.36 ± 679.41 U/mg shell) (Fig. 4B). The most active extracts are obtained from core and pineapple peel. However, there is also a high activity for green papaya residues. The results obtained prove that any of the residues evaluated could potentially be used as a source of enzymes with proteolytic activity. In relation to pineapple residues, it was observed that the proteolytic activity is greater in dry peels and the core, turning them into residue with high applicability for obtaining bromelain, of low supply in the Colombian market.

Most of the reports on extraction of proteolytic enzymes from residues (peel, core, crown and stem) (Ketnawa et al., 2012) or pineapple core (Quinde and Sánchez, 2012), are made from fresh tissue, since in general the enzymes are sensitive to the drying processes. The results show that the highest total protein content was obtained in the crown (220.5 ± 3.65 mg/100 g) and peel (132.4 ± 1.40 and 70.7 ± 0.46 mg/100 g) from two different regions of Thailand, Nang Lae and Phu Lae, respectively (Ketnawa et al., 2012). These results agree with those previously reported in this study. In the case of the pineapple crude extract from pineapple from Ecuador, a soluble total protein concentration of 5.21 mg/mL was obtained (Quinde and Sánchez, 2012). There are other reports in which the core of pineapple is evaluated to extract other types of enzymes such as Polyphenol Oxidase and Phenyl Ammonium Liasa (Zhou et al., 2003).

In none of the reports a thermal treatment to the residue has been made, prior to the extraction of enzymes. In this study it was demonstrated that, not only is it possible to increase the total protein concentration in the extract, but also its activity, which makes the transportation of the waste from its place of origin without carrying with it the problems of the extra cost in the transportation and degradation of the residues until the arrival at the plant for extraction of enzymes very viable.

3.3. Protein separation on SDS-PAGE and identification of proteins by mass spectrometry (MALDI-TOF-TOF)

For the identification of proteases, the extracts of dried pineapple core (CPi) and green dried papaya peel (CPa) were selected because they were the ones with the highest total protein content and with the highest activity, in addition to their easy handling characteristics. In Figs. 5 and 6, the separation of the proteins present in the CPa and CPi extracts, respectively, with and without protease inhibitor (PMSF), can be observed. We can also see that in all lanes (1–6), in both gels, the same pattern of bands is found, predominating a band with greater intensity between 23 and 25 kDa for the samples obtained from papaya, which coincides with the molecular weights of the proteases present in papaya (Fig. 5).

In Fig. 6, we see similar results with the bands found in pineapple core, where bands of greater intensity between 30 and 35 KDa are observed. This coincides with the weight of bromelain and is reported by other authors, who have found it from 28 to 35 kDa using fresh pineapple extracts (Arumugam and Ponnusami, 2013). Other authors have identified bands at 23, 27, 30 and 33 kDa, identified as chymopapain (27 kDa), caricaina (24 kDa), CC-IV (28 kDa) and CC23, Ccib and CMS1MS2 (23 kDa) (Gutiérrez et al., 2017). These results confirmed that thermal pretreatment did not affect the nature of the proteolytic enzymes.

In both gels we observed that the presence of PMSF as a protease inhibitor does not significantly affect the banding pattern or the intensity of the bands of interest. PMSF is a compound used as a serine and cysteine protease inhibitor, which binds specifically to the serine and cysteine residues of the active site. Its use is widely recommended for protein extraction protocols to avoid protein degradation, especially in the processes of disruption and storage. However, after analyzing the results, it can be determined that the use of this inhibitor for protein extraction in papaya and pineapple is not necessary, since in both cases the bands are intense and well demarcated.

3.4. Identification of proteins present in extracts obtained from pineapple and green papaya residues, by mass spectrometry (MALDI-TOF)

An analysis of the proteins obtained in the protein extracts of pineapple core and green papaya shell was performed by mass spectrometry, for which, after obtaining the characteristic bands

Table 1
Kinetic analysis for drying curves.

Paremeter	Mature papaya peel	Green papaya peel	Green papaya pulp	Pineapple crown	Pineapple core	Pineapple peel
Initial moisture	90.83 ± 1.63	96.62 ± 1.50	95.85 ± 1.28	86.21 ± 0.93	85.28 ± 1.41	80.68 ± 1.61
Steady state	7.163 ± 1.768	8.551 ± 1.141	6.313 ± 1.300	7.409 ± 1.070	10.650 ± 1.070	8.320 ± 1.530
K	0.0072 ± 0.0004	0.0090 ± 0.0004	0.0075 ± 0.0003	0.0089 ± 0.0005	0.0090 ± 0.0005	0.0078 ± 0.0005
Half life[1]	95.72	76.74	91.75	89.4	78.31	88.70
R^2	0.9779	0.9825	0.9864	0.9907	0.9783	0.9686

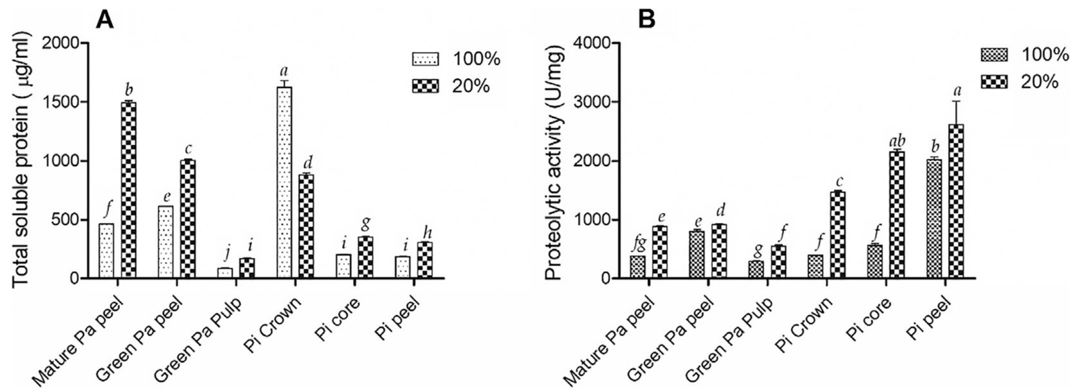

Fig. 4. Effect of moisture percentage during extraction of proteolytic enzymes on pineapple (Pi) and papaya (Pa) residues. (A) Total soluble protein content. (B) Proteolytic activity. Different letters on the bars show significant difference between treatments (n = 3).

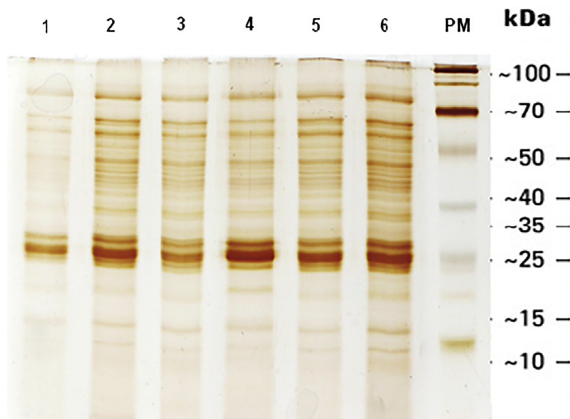

Fig. 5. Silver nitrate stained polyacrylamide gel, loaded with the different extracts of green papaya peel. (1) (2) (3) Without PMSF. (4) (5) (6) With PMSF.

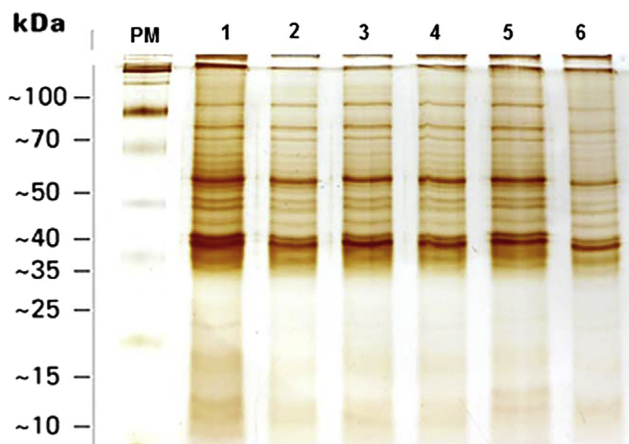

Fig. 6. Silver nitrate stained polyacrylamide gel, loaded with the different pineapple core extracts. (1) (2) (3) without PMSF. (4) (5) (6) with PMSF.

in the 1D electrophoresis, they were selected and then digested with trypsin. The obtained peptides were analyzed and compared with the available databases, as mentioned in the methodology (Jiang et al., 2004; Yin et al., 2008). This technology is robust and permits the resolution of complex samples and their identification. The samples of pineapple core and green papaya peel were subjected to this analysis to corroborate that the enzymatic activity was being determined by the presence of proteases of cysteine in papaya and pineapple, since its finding in the SDS PAGE gel is only presumptive.

The extracted peptides were ionized and the resulting ionic species were separated according to the ratio of their mass to their electrical charge (m/z) (Jiang et al., 2004; Yin et al., 2008). Exact values of the set of molecular masses of the peptides are the unique representation of a particular protein, this list of molecular masses is directly related to its primary structure (Del Chierico et al., 2012). The mass spectrum shows the ratio (m/z) of the peptides analyzed and is then converted into a fingerprint of the protein (mass fingerprint). Following that, the molecular masses measured by the mass spectrometry, are subjected to 2 proteomic analysis programs, in this case the software Flexanalysis 3.4 and MASCOT 2.0. Using the National Center for Biotechnology Information (NCBInr) database and the Viridiplantae taxonomy as the identification criteria, we carried out a comparison between the different proteins of the databases, which were digested *in silico*, generating a list of theoretical masses (Knowles et al., 2003). The obtained bands of papaya shell (CPa) and pineapple core (CPi), in triplicate, were selected for mass spectrometry and are described in Table 2.

After obtaining a list of molecular masses measured via mass spectrometry, they were subjected to the analysis program for comparison with different protein databases (Table 3). After analyzing the samples, it was found that the peptides signal obtained corresponded to proteases previously described for the genera of Ananas and Carica.

It is important to mention that the peptides AVPQSIDWR and NSWGSSWGEGGYVR, found in the three samples of core pineapple, had already been reported in extracts of this fruit and were considered biomarkers (Secor et al., 2012). In addition to the above, these peptides were identified in other extracts such as pineapple cysteine proteases in 2D gels subsequently analyzed by mass spectrometry (Dutta and Bhattacharyya, 2013; Larocca et al., 2010). Bromelain protein is currently being recombinantly obtained from *Ananas comosus* in *E. coli*, yeast, Baculovirus and mammalian cells,

Table 2
Description of the bands selected for analysis by mass spectrometry.

ID sample	Description and selection criteria
CPi1	Band in the approximate molecular weight range of bromelain (28–33 kDa)
CPi2	
CPi3	
CPa1	Band in the approximate molecular weight range of papain (23 kDa)
CPa2	
CPa3	

Table 3
Analysis of the different signal peptides with the Ananas and Carica genre. According to the mass spectrometers shown above.

ID sample	m/z	Peptide	Conf	Sco	%co	Identification by similarity with:	Code
CPi1	1541.7	NSWGSSWGEGGYVR	99	15	9.9	FB1035 (Fragment) *A. comosus*	tr|O23801
	1000.5	AVPQSIDWR	97.3	7	7.1		
CPi2	1541.7	NSWGSSWGEGGYVR	99	18		FB1035 (Fragment) *A. comosus*	tr|O23801
	1557.7	NSWGSSWGEGGYVR	99	8			
	1000.5	AVPQSIDWR	99	10			
	1071.6	AVPQSIDWR	99	9			
CPi3	1541.7	NSWGSSWGEGGYVR	99	12	7.1	FB1035 (Fragment) *A. comosus*	tr|O23801
CPa 1	1323.6	ASGNSPGVCGVYR	96.9	7	23	Papaya proteinase 4 *Carica papaya*	sp|P05994
	2036	GYQSTSLQYVAQNGIHLR	98.3	7			
	2775.5	VQSNNEGSLLNAIAHQPVSVVVESAGR	95	6			
	1226.6	NTYPYEGVQR	99	10	2.9	Papain *Carica papaya*	sp|P00784
	1064.5	YPQSIDWR	84.4	5	2	Chymopapain isoform II *Carica papaya*	tr|Q9SMI1
CPa 2	1064.5	YPQSIDWR	99	8	2.3	Chymopapain isoform II *Carica papaya*	tr|Q9SMI1
	1323.6	ASGNSPGVCGVYR	97.3	6	15.2	Papaya proteinase 4 *Carica papaya*	sp|P05994
	1226.6	NTYPYEGVQR	90.9	6	2.9	Papain Carica papaya	sp|P00784
CPa 3	1001.5	LPESVDWR	99	9	23	Papaya proteinase 4 *Carica papaya*	sp|P05994
	1593.7	NSWGPGWGENGYIR	99	10			
	1323.6	ASGNSPGVCGVYR	99	10			
	2775.5	VQSNNEGSLLNAIAHQPVSVVVESAGR	99	16			
	2036	GYQSTSLQYVAQNGIHLR	99	12			
	1077.5	IPEYVDWR	98.9	8	9.3	Papain *Carica papaya*	sp|P00784
	1226.6	NTYPYEGVQR	99	9			
	1064.5	YPQSIDWR	99	10	2.3	Chymopapain isoform II *Carica papaya*	tr|Q9SMI1

which contain the peptide AVPQSIDWR as an N-terminal sequence (mybiosource.com and NCBI recombinant).

Samples of papaya protein extracts also contained proteases previously described and reported. The different peptides found; ASGNSPGVCGVYR, LPESVDWR and VQSNNEGSLLNAIAHQPVSVV-VESAGR, were described and identified as glycerendopeptidases (Llerena, 2011) and have been reported since 1989 in papain peptide sequences (Ritonja et al., 1989). The sequence IPEYVDWR has been reported as the N-terminal residue of papain and the peptide NTYPYEGVQR has been found from the digestion of electrophoretic bands of papain (Llerena, 2011).

The above allows corroborating the presence of cysteine proteases in the enzymatic extracts of papaya and pineapple residues, specifically papain and bromelain, respectively. All extracts from which these proteins were identified were active and present in the bands at equal molecular weights in the polyacrylamide gels. The bands identification correspond to the most active samples and those that were present in a greater intensity.

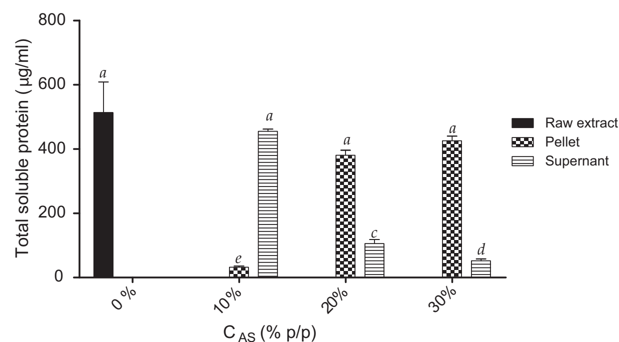

Fig. 7. Soluble total protein content of extracts of pineapple residues after the ammonium sulfate (SA) precipitation process in the solid (pellet) and liquid (supernatant) fraction. Different letters on the bars show significant difference between treatments (n = 3).

3.5. Effects of the concentration of ammonium sulfate as a precipitating agent of proteolytic enzymes in pineapple extracts

Ammonium sulfate (AS) is one of the most commonly used salts for the precipitation of proteins due to its high solubility (Duong-Ly and Gabelli, 2014). It has the advantage of having a low price and high availability in the market, and is freely allowed for use in the food industry. In order to verify its efficiency for the precipitation of the proteolytic enzymes present in the obtained extracts, the Total Soluble Protein (TSP) in the crude extract, pellets and supernatants after precipitation with AS at 10, 20 and 30% was quantified (Fig. 7).

TSP found in the crude extract was used to evaluate the efficiency of the precipitation process with each of the AS concentrations evaluated. The crude extract reference value was 514.0 ± 164.3 µg/mL. When precipitation was performed with 10% AS, only 6.2% of the protein (31.9 ± 6.9 µg/mL) was precipitated, leaving 89% of the protein in the same supernatant. The remaining 4.8% may have been degraded or a loss associated with an experimental error. When precipitation was performed with 20% AS, the efficiency rate was 74% (380.6 ± 26.6 µg/mL), leaving only 20% without precipitation. When the percentage of AS added was 30%, 82% of the protein (425.6 ± 25.1 µg/mL) precipitated, however when comparing the PTS values obtained in the pellet, no significant difference was observed (380.585 ± 26.631 µg/mL for 20% AS and 425.637 ± 25.052 µg/mL for 30% AS).

Protein precipitation occurs by the decrease in repulsive forces and the predominance of attractive forces at the isoelectric point, causing the protein aggregates to decrease their solubility (Arshad et al., 2014). In this research, the precipitation process was performed at 4 °C, which is favorable for reducing solubility since it has been reported that high temperatures can denature proteins (Walsh, 2002).

The proteolytic activity of the enzymes present in the pellet and the supernatant was monitored for 28 days in order to evaluate their stability over time and to propose a method of extraction and purification that guarantees a greater activity during the life of shelf proteins extracted in the residues.

Fig. 8 shows the activity data of the different fractions obtained (supernatant and pellet) after precipitation with AS at the different concentrations evaluated and at different times. For the applied concentration of AS of 10%, we observed that the highest activity was found in the supernatants resulting from the process on days 0, 14 and 28 with values of 2763.97 ± 365.50; 2725.73 ± 292.25 and 2099.94 ± 49.58 U/mL, respectively (Fig. 8A), confirming that most of the protein was not precipitated. The results of the activity found in the supernatants of extracts precipitated with AS at 20 and 30% presented values of 749.75 ± 142.60 and 529.28 ± 149.86 U/mL, respectively.

On the other hand, the activity found in the pellet, where a higher concentration of proteins was found (Fig. 7), was higher for the precipitates with 20 and 30% AS, with values of 3379.17 ± 431.45 and 3410.54 ± 372.33 U/mg, respectively (Fig. 8B).

When the activity was evaluated for the same extracts at 14 days after cold storage (4 °C), there was no significant decrease in activity with values of 3273.67 ± 36.61 and 3018.46 ± 488.73 U/mg, for precipitation with AS at 20 and 30%. However, for day 28, a decrease of 36% was observed for the pellet obtained with AS at 20% and of 50% with AS at 30%. For precipitated extracts with 10% AS, there was no decrease in activity, showing stability over time.

There have been several works that use precipitation of proteolytic enzymes in pineapple with ammonium sulfate. Such as the precipitation of a crude pineapple homogenate with 55% AS of ammonium sulfate (Pei et al., 2005). In addition to the partial purification of bromelain using a saturation percentage of AS between 30 and 80% (Seung-Cheol et al., 1996). Similarly, the bromelain precipitation of the fruit has been researched, improving the purity 2.81 times, with a 40–60% saturation of AS (Devakate et al., 2009).

The mechanism of precipitation by salting out is based on the addition of salt, which removes water from the hydrated protein, leaving its hydrophobic regions free to combine intermolecularly. Those proteins that have more hydrophobic regions on their surface form aggregates and precipitate faster than those with fewer hydrophobic regions. Due to this property there are proteins that can remain in solution at high concentrations of salt (Glatz, 1990), which is why the process reached only 82% of the efficiency with the concentration of SA at 30%.

The obtained results agree with reports where the level of saturation up to 50% with AS results in a better recovery and purification of bromelain (Chaurasiya and Umesh, 2013). In this test it was determined that there was no need to increase the AS concentration above 30% of the extract to enhance proteolytic activity. This salt concentration corresponds to a saturation percentage of about 55%.

It is well known that the NH_4^+ and SO_4^{2-} ions are at the end of the Hofmeister series and the stability of the structure of the proteins with this salt has already been demonstrated. This type of precipitation can be used with proteins of unknown sequence and proteins that do not possess molecular markers to be precipitated. Because the sample has a high concentration of ammonium sulfate, hydrophobic interaction chromatography may be used to continue the protein separation process. On the other hand, it is possible that the precipitated protein is accompanied by contaminants and for that reason it is necessary to perform ion exchange chromatography either by exclusion of size or chromatography in gel filtration if it is desired to purify the protein. Since samples end with a large amount of salt, dialysis is a very useful method for the removal of ammonium sulfate (Duong-Ly and Gabelli, 2014).

3.6. Development of a protocol for extracting proteolytic enzymes from pineapple residues: An approach to higher scale

Scaling is the development and implementation of an industrial-scale process based on experimental data obtained at the laboratory scale. In this sense, it is defined as the process by which numerical allocation criteria and rules are developed that determine the significant units of measure to take from a given size to another major or minor size, an operation or equipment (Rodríguez-Monroy and Orozco, 2014). Scaling focuses on different principles, which includes the principle of similarity that in turn is divided into geometrical similarity (proportional dimensions), mechanical (Static: proportional deformations, Kinematics: proportional times and Dynamics: proportional forces), thermal (proportional temperatures) and chemical (proportional concentrations). On the other hand, the solubility as a limit for the extraction could be used as a scale parameter. It is based as the relationship between the solvent volume and the material to be extracted being the diffusivity as a limit for the extraction in which the relation is evaluated between the solvent flow and the residence time in the extractor tank.

In the present study, a methodological approach was carried out to evaluate the extraction at different laboratory scales (50–500–5000 mL) using the solubility criterion as a scaling parameter and the extraction limit to obtain useful experimental data for observing the variation in activity with the change of scale. In this case the ratio used was 1.0 (g solvent (Buffer Phosphate pH 7.0)/g dry residue).

The results obtained show that the activity of pineapple enzymatic extracts for the different scales evaluated did not present significant differences, remaining at 2999.059 ± 155.163, 3168.151 ± 36.606 and 2797.539 ± 5.307 U/gDW, for the scales of 50, 500 and 5000 mL, respectively (Fig. 9).

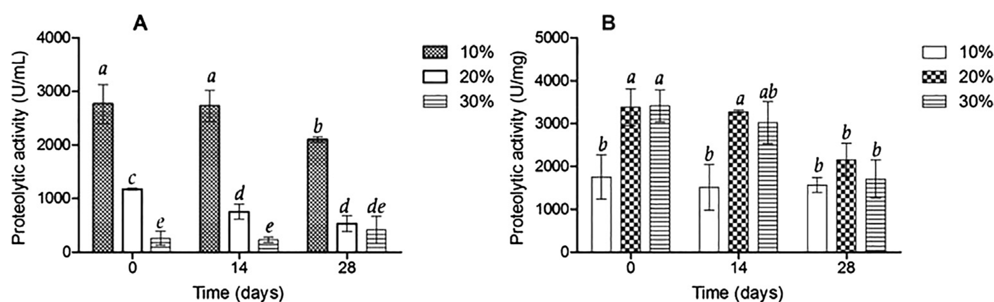

Fig. 8. Proteolytic activity over time of the enzymatic extracts of pineapple residues precipitated with ammonium sulfate A. Supernatant. B. Pellet. Different letters on the bars show significant difference between treatments (n = 3).

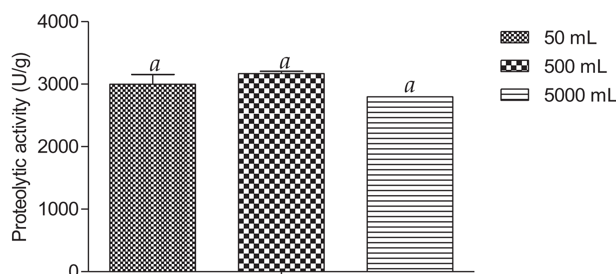

Fig. 9. Effect of the work scale on the proteolytic activity of the extracts obtained.

In this study, more than a scaling test, although the effectiveness of maintaining the solubility criterion as a limit for the extraction was constant, shows the technical validation of the extraction protocol in different laboratory scales, developed in this project in which the best operating conditions were found (Solvent type: 0.1 M phosphate buffer pH 7.0;; Residual moisture percentage: 20%; Percentage of $(NH_4)_2SO_4$: 20%) suitable for obtaining concentrated extracts of proteolytic enzymes that allowed the generation of a value-added model of pineapple by-products for the generation of enzymatic preparations with proteolytic activity.

This study allowed us to obtain a validated extraction protocol, where convection drying of the pineapple residue plays an important role, not only to increase the yield of the process of extraction of proteolytic enzymes, but also to facilitate the processing in places far from the zone of cultivation or the place of generation of this waste. A reduction in the moisture content of the tissues is quite used for obtaining active compounds or inorganic material, which can be achieved by drying or pressing methods (Boakye et al., 2017; Klavins et al., 2018); in this sense, its application of enzymatic extraction becomes an excellent contribution both to enzymatic technology and to the recovery of waste.

4. Conclusions

By means of the proteolytic enzyme extraction protocol developed (Extraction solvent: 0.1 M phosphate buffer, pH: 7.0, and residue moisture percentage: 20%), it was confirmed that the heat treatment does not affect the activity of the enzymes or the total protein content in the extracts. In addition, the green papaya peel and the honey pineapple peel were identified as the best residues for obtaining extracts of papain and bromelain.

Regarding the identification of signal peptides in green dry papaya and dry pineapple core residues, it was found that the peptides obtained AVPQSIDWR and NSWGSSWGEGGYVR correspond to previously described proteases for the genre of Ananas and Carica. Samples of papaya protein extracts are also a source of proteases as previously described and reported. The different peptides found; ASGNSPGVCGVYR, LPESVDWR and VQSNNEGSLLNAIAHQPVSVVVESAGR, were described and identified as glycylendopeptidases.

A scalable and applicable method to obtain proteolytic enzymes from pineapple was developed for the food industry. It was found that the use of 20% ammonium sulfate as a precipitating agent allowed recovery of the proteins of 74% and when evaluating the criterion of diffusivity as a limit for the extraction (relation between exhaust solvent flow and residue flow) it was found that to fix it at a value of 1.0, there was no significant difference in proteolytic activity at scales of 50, 500 and 5000 mL.

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Conflict of interest

The authors express they do not have any conflicts of interest to disclose.

Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.wasman.2018.05.020.

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