Waste Management 78 (2018) 58–68

Contents lists available at ScienceDirect

Waste Management

journal homepage: www.elsevier.com/locate/wasman

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

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ARTICLE INFO

Article history:
Received 24 January 2018
Revised 11 April 2018
Accepted 11 May 2018

Keywords:
Agro industrial waste
Proteolytic activity
Bromelain
Papain
Convection drying

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.

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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https://doi.org/10.1016/j.wasman.2018.05.020
0956-053X/

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