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Low-cost Recovery of Bromelain Solids from Industrial Pineapple Peel, Pulp, and Core Wastes Using Ethanolic Cashew Leaf Polyphenol

Le Grande O. Dolino[1,2], **Doris B. Montecastro**[1], and **Antonio M. Basilio**[1]*

[1]Ateneo de Davao University, Jacinto Street, Davao 8000 Philippines
[2]Philippine Science High School Southern Mindanao Campus
Tugbok District, Davao 8000 Philippines

Bromelain, a valuable protease present in all parts of the pineapple fruit, was previously shown to be separated from pineapple juice and protected by complexation with polyphenols. This study aimed to establish a simple, low-cost procedure for the collection of solids with bromelain using industrial pineapple waste and polyphenols extracted from cashew leaf. An alternative method was introduced that allows for solids that have higher protease activities from pineapple core and pulp to be obtained from pineapple extract compared to typical extraction and purification methods. Using this modified method, solids were obtained from pineapple waste with the following protease activities: 5343 CDU/g (core), 5147 CDU/g (peel), and 5732 CDU/g (pulp). Given the simplicity and low cost of the procedure, this method can be of benefit to smaller pineapple producers who could profit from obtaining an otherwise expensive material using their production's waste.

Keywords: beneficial reuse, enzyme from food wastes, ethanolic extraction, protease

INTRODUCTION

The Philippines is one of the largest producers and exporters of pineapple fruit in the world. For the quarter of July–August 2019, the country produced up to 712000 metric tons of pineapple. For Mindanao, Northern Mindanao held an approximate 60% share of production, while SOCCSKSARGEN held approximately 28% (PSA 2019). Along with the production of the fruit comes the production of waste, be it a prior sale or after consumption. It is estimated that rough handling of fruits alone potentially contributes up to 55% of product waste (Nunes *et al.* 2009).

These wastes can be a potential source of the enzyme bromelain. It is a crude protease mixture majorly found in the stem and flesh (Bala *et al.* 2012). It is used for various applications such as in cosmetics, food, and dietary supplements (Uhlig 1998; Walsh 2002; Ketnawa and Rawduken 2011). Bromelain has also been previously isolated from pineapple waste, particularly from the core and peel (Ketnawa *et al.* 2012; Lakshminarasimaiah *et al*. 2014). Exploration of methods to retrieve bromelain from pineapple, particularly pineapple waste, then proves to be an interesting venture for the Philippines due to its large pineapple production.

Previous studies have shown that bromelain can be complexed with and protected by polyphenols. Liang *et al.* (1999) showed that bromelain could be complexed with tea polyphenols, allowing it to be separated from pineapple juice and improving bromelain's thermal stability. Up to 80% of pineapple juice's bromelain activity was recovered as a solid after the addition of tea

*Corresponding Author: ambasiliosj@addu.edu.ph

polyphenols. Since tea is generally expensive, the present study explored cashew leaf as an alternative polyphenol source. The leaf was previously shown to have high amounts of phenolics (Chotphruethipong *et al*. 2017) and is commonly available, thus making it an attractive option since it can still improve the thermal stability of bromelain, as previously studied by Poh and Abdul Majid (2011).

DAVECO Agrarian Reform Beneficiaries Cooperative (DAVECO-ARB) is a pineapple growing and processing cooperative in Calinan, Davao City, Philippines. Aside from producing fruit, it provides pineapple products such as juice, jam, and vinegar. The present study was motivated by the search for a simple and low-cost method for obtaining bromelain from pineapple wastes. This is to benefit small pineapple fruit and product producers such as DAVECO-ARB in generating additional value through marketing the obtained bromelain for potential textile, food, or health purposes. The usage of polyphenols was pursued as it allows for the generation of bromelain solids that can be easily obtained, handled, and stored, making it a potential product to be sold. This paper reports a simple method for obtaining bromelain-containing solids that forego complicated extraction and purification techniques and largely avoids the usage of expensive chemicals and equipment such as freeze dryers, mechanical shakers, and chromatography apparatuses.

MATERIALS AND METHODS

Preparation of Ethanolic Cashew Leaf Polyphenol

Cashew leaves were collected, air-dried for 48 h, and ground into a fine powder using an industrial mill. The powder was then sieved using a #20 mesh (0.84 mm diameter). The powder was extracted with reagent-grade 95% ethanol for 24 h in a 1:20 (g/mL) ratio with no agitation. Afterward, 200 mL of this extract was then evaporated to dryness in a rotary evaporator. To ensure solubility of the polyphenol in the pineapple extract, ease of handling, and reproducibility, the dried cashew leaf extract was redissolved in 20 mL 95% ethanol. This ethanolic cashew leaf polyphenol (CLP) was subsequently filtered through Whatman No. 1, sealed, and stored at 4 °C until use.

Preparation of Crude Pineapple Extract

Ripe MD-2 variety pineapple samples were sourced from DAVECO-ARB in Calinan, Davao, Philippines. DAVECO-ARB processed pineapple (delivered from the field to the laboratory without the crown) by manually peeling using a knife, then expressing the chunks to collect the juice. The resultant pulp (post-juicing pineapple flesh), as well as the pineapple peel and pineapple core, were considered as wastes and were thus explored in this study.

This study's methods were adapted from Ketnawa *et al.* (2012) and Al-Sa'ady *et al.* (2016) with some modifications. Crude pineapple extracts were prepared by mixing 300 g of pineapple core, peel, or pulp with 300 mL of 0.100 M pH 7.0 phosphate buffer in a blender for 3 min. This mixture was then filtered through triple layer cheesecloth and stored at 4 °C until use. The phosphate buffer was controlled at pH 7.0 as initial reports for protease activity of bromelain from other pineapple variants show a significant decrease at extreme acidic and alkaline conditions (Ketnawa *et al.* 2012), with activity peaking at or near pH 7.0 (Koh *et al.* 2006; Al-Sa'ady *et al.* 2016).

Typically, pineapple extracts are centrifuged at 10000g and decanted to remove cellular residues and other fine suspended particles. Aside from this method (which will hereafter be referred to as a typical method), this study also prepared extracts without centrifugation and decantation to determine the significance of these steps in preserving protease activity. This latter method was referred to as a modified method and was explored to see if the disposal of suspended solids significantly affected the protease activity recovered from the pineapple waste. Specifically, usage of a modified method may allow small cooperatives to perform similar pineapple waste preparations without having to purchase a centrifuge.

Determination of Optimal Polyphenol Concentrations

To determine the appropriate amount of CLP that can be added to the pineapple extracts, 4 mL of the extract was mixed with enough CLP to give final concentrations at 1.00% CLP increments (up to 8.00%). Four replicates were prepared per increase. The mixtures were stored at 4 °C for 24 h and subsequently centrifuged at 10000g for 5 min. The supernatants were discarded and the masses of the precipitate were measured. The optimum amount of CLP is determined to be the minimum amount of CLP necessary between methods such that any increment above this amount would lead to a statistically similar precipitate weight.

Complexation of Bromelain with CLP

In calibrated test tubes, 4 mL of specific pineapple extract was mixed with the optimal amount of CLP using a vortex mixer. The samples were prepared in triplicate for each type of pineapple waste and each type of preparation method. The tubes were sealed and stored at 4 °C for 24 h. As this study is only concerned with the solids as a final product, the samples were then centrifuged at 10000g for 5 min and the supernatants were discarded.

To perform measurements on the solids, the remaining solids were dissolved and diluted to 6 mL with 0.100 M pH 7.0 phosphate buffer. In the case of undissolved solids, samples were centrifuged at 10000g for 5 min and only the liquid was retrieved; liquids were not diluted to 6 mL. The resultant liquids will be referred to as crude bromelain-cashew leaf polyphenol complex (BCLP). It must be emphasized that BCLP is not pure bromelain as it contains polyphenols and cellular residues, particularly for the modified method.

Determination of Enzyme Activity

The methods used here were adapted from Merck. Bromelain activity in this study is expressed in CDU/mL, where one CDU (casein digestion unit) is defined as 1 μg of L-tyrosine liberated per min per mL of a sample when casein is hydrolyzed at 37 °C and pH 7.0 for 10 min. From the prepared enzyme solutions, 1 mL of each was pipetted out in triplicate and used to react with 5 mL of 0.65% casein solution in a test tube for 10 min to liberate L-tyrosine. To stop the release of L-tyrosine, 5 mL of 110 mM trichloroacetic acid (TCA) was added to denature the casein after 10 min. This was done immediately and at an angle such that the TCA would reach the bottom part of the test tube. The solution was placed in a 37 °C water bath for 30 min. The denatured casein was filtered out using 0.45 μm polyethersulfone syringe filters. Afterward, 1 mL of filtrate was made to react with 1 mL Folin-Ciocalteau reagent (FCG), which would impart a blue coloration to the solution depending on the L-tyrosine concentration. Dilution of the original sample may have been necessary. Immediately after, 5 mL of 500 mM sodium carbonate was added to regulate any pH drop caused by the FCG. The samples were read using a ultraviolet-visible (UV-Vis) spectrophotometer at 660 nm against an L-tyrosine calibration curve.

Since the FCG can be utilized to estimate polyphenol concentration (Singleton *et al*. 1999), the prepared CLP was measured in triplicate against a standard gallic acid calibration (GAC) curve. In a 50-mL volumetric flask, 0.5 mL CLP was added with 2.5 mL FCG and about 30 mL water. After 1 min, 7.5 mL of 20% sodium carbonate was added and diluted to 50 mL. Five replicates were read after 2 hr against a GAC curve at 760 nm using a UV-Vis spectrophotometer.

Determination of Protein Content

Protein content was investigated using the bicinchoninic acid (BCA) assay by Sigma-Aldrich. In the standard 2.1 mL assay protocol (Sigma-Aldrich), 20 parts of BCA working reagent were mixed with 1 part of either bovine serum albumin (BSA), blank, or CLP. The BCA working reagent was prepared by mixing 50 parts BCA stock and 1 part 4% copper (II) sulfate pentahydrate. The mixtures were incubated at 60 ºC for 15 min to develop a purple color and were cooled to room temperature. Dilution of the original sample may have been necessary. Sample concentrations were subsequently determined using a spectrophotometer against a BSA calibration curve at 562 nm.

Statistical Analysis

All statistical tests were performed at an alpha value of 0.05. Results for enzyme activity and protein content were fit into analysis of variance models. Differences between means were determined using Tukey's honest significant difference test for triplicate runs. All statistical tests were performed using R.

RESULTS AND DISCUSSION

The prepared CLP was determined to have 14.7 ± 0.1 mg GAE per mL extract. This is markedly lower than the literature-reported value of 62.87 mg/g (Poh and Abdul Majid 2011), but this was expected as the present paper diluted the extract in ethanol to ensure solubility and ease of handling.

Results for the determination of optimal CLP concentration are visualized in Figure 1 and summarized in Table 1. The addition of CLP to the various extracts exhibited a generally positive trend on the amount of solid recovered until the trend plateaus. Since the bromelain in the sample is fixed, the addition of more CLP will not increase the obtained mass as there comes a CLP amount where all the bromelain will be converted to solid. This plateau was consistent with earlier reported behavior of the recovery of bromelain activity (Poh and Fadzilah 2011). The lowest percentage of CLP that yielded solid weights not significantly different from higher CLP values was taken as the optimum amount and applied to both typical and modified method preparations for consistency in further experimentation. For comparability of samples across the typical and modified methods, the higher optimum value from the typical and modified methods is applied for both methods of a particular pineapple waste type. All types of pineapple waste across both methods demonstrate the trend of the recovered mass flattening with increasing CLP.

In the experiments, the pulp generally had minuscule amounts of solids after centrifugation, which may explain why results for both the typical and the modified methods are comparable. Cellular debris can add to the solid weight, which explains why modified method weights are mostly higher than the corresponding typical method

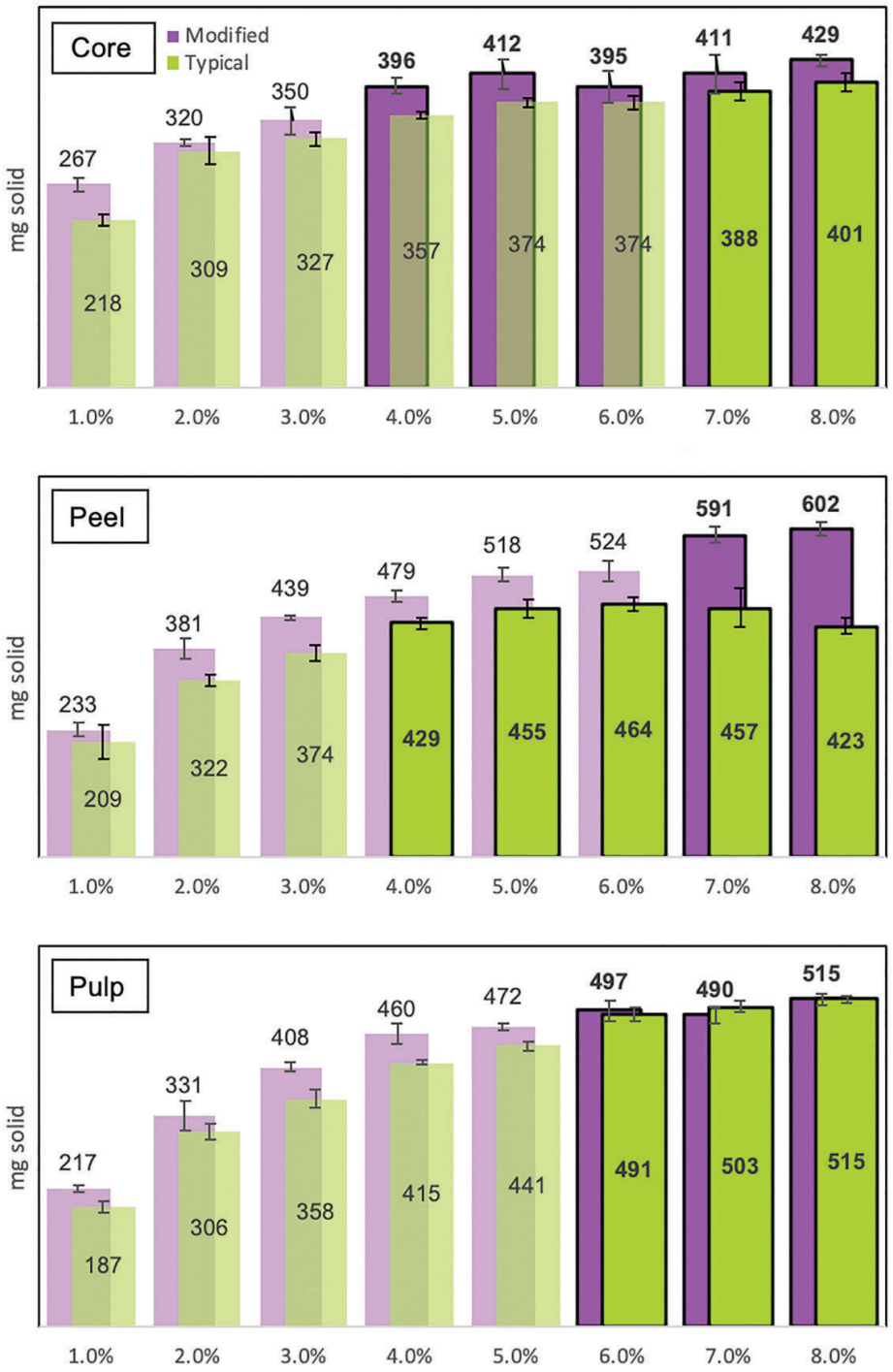

Figure 1. Recovered amount of solids in milligrams with respect to percentage of CLP across two methods. All values in bold in the same method are not significantly different from each other.

values. Across the three pineapple sources, the peel had the most amounts of settled solids after centrifugation.

The experimental results for protease activity are presented in Table 2, where results are reported in terms of CDU per mL and CDU per g solid. The CDU per g was estimated using the results from Table 1. Statistical analysis showed that the modified method gives significantly higher enzyme activities per unit for both

Table 1. Optimal amount of CLP for solid precipitation*

Type	Optimum CLP (%)	Highest solid recovered from 4 mL extract (mg)	
		Typical method	Modified method
Core	7.0	401 ± 12	429 ± 7
Peel	7.0	464 ± 12	602 ± 14
Pulp	6.0	515 ± 7	515 ± 9

*Results are presented as mean ± SD.

core and pulp by as much as 80–100 units. However, these results were not seen with the peel. This means that the peel will benefit from centrifugation in recovering higher protease activities since the recovered solids do not have protease activities or prevent bromelain from exhibiting protease activity. Still, the protease activity of pineapple peel obtained using the modified method is comparable to that from pineapple pulp using the typical method and is significantly higher than the results using pineapple core and the typical method.

The pineapple peel shows the highest enzyme activity for the typical method against the pineapple core and pulp, which agrees with previous reports (Ketnawa *et al*. 2012; Lakshminarasimaiah *et al*. 2014). The pineapple pulp, which was originally sourced from pineapple chunks (expected to have high bromelain content), still exhibited high activity despite being a residue of the juicing process. The pineapple pulp displayed significantly higher activity than the core and shows promise as a source for bromelain extraction, especially since it is waste after juicing. The pineapple core performed the weakest among the sample types in terms of protease activity.

The enzymatic results of this study are compared to findings from studies with similar methodologies in Table 3. As far as the authors know, no other study reported on pineapple pulp as a residue of an initial industrial juicing, and so the data on the pulp has been excluded from the table for comparisons. The studies characterized waste sources from various pineapple varieties, but only the present study and Lakshminarasimaiah *et al*. (2014) analyzed solids. Ketnawa *et al.* (2012) and Nor *et al.* (2015) have previously established the presence of bromelain in extracts through molecular weight studies; hence, the present study did not repeat such. Reported values for enzyme activity for pineapple core extracts range from 76–259 CDU/mL, which are lower than the present study's reported value. The reported value for peel in the present study is either within or slightly below (for the typical and modified methods, respectively) the range of already reported values, which is 430–770 CDU/mL. Since there is no major difference between this study's findings with that of other methods that either report liquid extracts or use expensive technologies such as freeze-drying, the modified method shows

Table 2. Protease activity of various BCLP preparations*

Type	Protease activity (CDU/mL)		Estimated protease activity of recovered solids (CDU/g)	
	Typical method	Modified method	Typical method	Modified method
Core**	276 ± 13^{c}	382 ± 8^{b}	4130	5343
Peel**	661 ± 54^{a}	398 ± 35^{b}	6588	5147
Pulp**	395 ± 17^{b}	492 ± 35^{a}	4602	5732

*Results are presented as mean ± SD from triplicate runs.
^{a,b,c}Different letters in the same column signify significant difference ($p < 0.05$).
**Results across the rows are all significantly different ($p < 0.05$).

Table 3. Protease activity across similar pineapple bromelain studies

Variety	Extraction medium	Protease activity (CDU/mL)		Source
		Core	Peel	
MD-2	pH 7.0 phosphate buffer and CLP complexation	276	661	Typical method; present study
		382	398	Modified method; present study
Nang Lae	Distilled water (no further pH adjustment)	206	554	Ketnawa *et al.* (2012)*
Phu Lae		259	770	
Smooth Cayenne	Cold milliQ water (no further pH adjustment)	76	430	Nor *et al*. (2015)*
Unspecified	pH 6.0 phosphate buffer	218	451	Lakshminarasimaiah *et al*. (2014)**

*Protease activities were calculated manually from the study's reported total activity and total extract volume. The study did not isolate the enzyme as a solid.
**As this study presented results for varying pH, only the largest values are presented.

Table 4. Protein results of various BCLP preparations.

Type	Protein content (µg/mL)		Specific activity (CDU/mg protein)	
	Typical method	Modified method	Typical method	Modified method
Core**	1800 ± 169[b]	3167 ± 119[a]	153	121
Peel**	3955 ± 167[a]	2724 ± 186[b]	146	167
Pulp**	1591 ± 56[b]	2231 ± 133[c]	248	221

*Results are presented as mean ± SD from triplicate runs.
[a,b,c] Different letters in the same column signify significant difference ($p < 0.05$).
**Results across the rows are all significantly different ($p < 0.05$).

feasibility in retrieving bromelain activity. The peel solids will have significantly higher protease activity, however, with prior centrifugation.

Similar trends for the enzyme activity were seen in the results for protein content, which are summarized in Table 4. The pineapple peel exhibited the highest amounts of protein against the other sample types but greatly decreased if the modified method was used, unlike the core and the pulp. Unexpectedly, the modified method result for pineapple core was much higher than the rest even if it did not exhibit significantly higher protease activity, as seen in Table 2. This suggests that the proteins retrieved from pineapple core, which were not discarded in the modified method, are not necessarily all proteases. Ketnawa *et al.* (2012) have reported that other non-protease proteins exist in pineapple extract such as peroxidase, which may explain the obtained values.

Since the modified method results for core and pulp were higher, this suggests that the solids discarded in the typical method contained bromelain. The opposite effect is clear for the peels. This suggests that the cellular residues in the peel interacted with the proteins that prevented the latter from being available. This claim is consistent across the protease activity and protein content results. Hence, to have a reference for purity, the CDU per mg protein is also reported in Table 4. Highly purified freeze-dried bromelain powder prepared through preparative high-performance liquid chromatography was reported to be 530 CDU/mg (Nadzirah *et al*. 2012) and that obtained using ion-exchange chromatography was reported to be 482 CDU/mL (Devakate *et al.* 2009). Without further purification, the present paper offers protease-containing solids that are 2–4 times less active than purified bromelain. However, Devakate *et al*. (2009) reported that the specific activity of commercial bromelain obtained from Thailand was 171 CDU/mg. Since present values are nearby this value and the method used did not require additional machinery, the present paper would like to suggest that the modified method be considered by small pineapple growers in obtaining bromelain solids for sale from the core, pulp, and even peel.

CONCLUSION

Ethanolic CLP was used to recover solids with protease, previously established as containing bromelain, from pineapple wastes. The pineapple peel showed the highest recoverable protease activity yet showed better results after the disposal of suspended solids in the extract. However, the modified method – where centrifugation was not performed – provided significantly higher protease activities from the core and pulp. It was also shown that the method using CLP complexation to generate solids produces results with comparable values to other studies that did not recover bromelain as a solid. Modified method results for peel, despite being lower than those for the typical method, are still comparable to typical method results of the core and pulp. This simple method recovers protease activity while avoiding expensive chemicals and machines such as centrifuges, freeze dryers, shakers, and other extraction and purification machines. The method presented in this study could be of benefit to small- and medium-scale pineapple businesses as they could generate profit from their main waste material without having to invest in machinery to isolate solids with bromelain activity.

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STATEMENT ON CONFLICT OF INTEREST

The researchers declare no competing interests.

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