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Towards enzymatic release of polyphenolic components with antioxidant power from Guava Leaves

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ABSTRACT
Plants for thousands of years have been used to enhance health and for medicinal purposes. *Psidium guajava Linn* is one which has an enormous wealth of medicinal value. Enzyme-assisted release of polyphenolic antioxidants from guava leaves was examined. The enzymes used were cellulase, pectinase, acid protease, neutral protease and alkaline protease. Among these enzymes, the activity of four of them were inhibited and only acid protease catalyzed degradation of proteins and increased the recovery of phenolic compounds. Total phenols released were ranged from 4.8 to 75.2 mg GAE /g dry guava leaf and varied in response to enzyme type, time and temperature treatment and the particle size of guava leaves. The guava leaf extract significantly showed high antioxidant activity as determined by $DPPH^{\cdot}$ at 515 nm.

Keywords: guava leaf; polyphenols; cellulase; pectinase; protease; $DPPH^{\cdot}$; antioxidant activity

1. INTRODUCTION

Guava (botanical name: *Psidium guajava Linn*) is a plant belonging to the family *Myrtaceae*, which has been used for centuries as health tea, with its leaf having a character of strong superoxide-dismutase-like activity and having been used to treat many ailments including cough, diabetes, diarrhea, dermatitis, etc. [1, 2]. *P. guajava* used as an important food as well a medicinal plant in tropical and subtropical countries, therefore its nickname as the poor man's apple. The scientific evidences of the medicianl uses of *P. guajava* began in 1940's and reports, maintain a tradition of repeating the data each decade [2]. In earlier works, guava leaf has been reported to contain several compounds, e.g. sesquiterpene, triterpenoid, flavonoids (quercetin, guaijaverin, and leucocydin, amritoside), coumarins (ellagic acid), alkaloids and tannin. Polyphenols, in particular, quercetin and its glycosides are thought to be the active ingredients [3].

Phenolic compounds are secondary plant metabolites and are becoming increasingly recognized as important in maintaining human health. In most plant cells, phenolic compounds are contained in vacuoles [4]. Common extraction methods include using solvents, solid phase extraction and supercritical extraction. For guava leaves extraction, there are reports about using water, methanol, ethanol, acetone process [5]. At present, the extraction of phenolic compounds recovery by using enzyme assisted extraction has not investigated thus far.

Enzymes are widely used to assist extracting all kinds of bioactive chemicals from plants or animals. Cellulase, xylanase and pectinase are commonly used to cell wall degradation. Protease is used to hydrolyze proteins or some peptides in juice or meat industry. In polyphenolic compounds extraction there aren't many reports about using enzymes because of the paradox that phenolics can form complexes with proteins or related biopolymers. The fact that enzymes which are also proteins may be inactivated by phenols, it has been reported that polyphenols

found in beans and peas inhibit the activity of amylases *in vitro* [6].
The purpose of this study was to examine enzyme-assisted extraction of phenolic compounds from guava leaves. Various enzymes were studied and conditions optimized in extracting phenols such as enzyme type, particle size, freshness, time of hydrolysis, temperature, E/S ratio. The yields of selected samples were then determined for the antioxidant level by means by a 2,2-diphenyl-1-picrylhydryzyl (DPPH·) colorimetry with detection scheme at 515 nm.

2. MATERIALS AND METHODS

2.1. Guava leaves: Dry guava leaves were obtained from Burundi, Bujumbura city.

2.2. Chemicals and Enzymes: Cellulase from *Trichodema longibrachiatum*, acid protease from fungi, neutral protease from *Bacillus amyloliquefaciens*, bacteria alkaline protease from were generally presented by Genecor (Wuxi) Bio-Products Co., Ltd. Folin reagent was prepared by ourselves. DPPH· was purchased from Aldrich Chemical Co. (free radical, 95%). Pectinex 5 XL was purchased from NOVO Enzyme Co., Ltd. Gallic acid and other reagents used were of analytical grade.

2.3. Sample preparation, enzyme hydrolysis and extraction of phenols: Dry guava leaves were milled at low temperature. Particle size distribution of crushed leaves was analyzed using a four-sieve sieving tower with aperture sizes of 900, 450, 300 and 200µm, respectively. The effects of the particle size of samples, 2g dry leaves, were examined at 37°C for 7hr incubation in 20ml deionized water.
For enzymatic hydrolysis, samples (20000g) were incubated in deionized water (20ml) in a constant temperature shaker. The selected hydrolysis conditions were obtained when comparing with the total phenols determination. Enzymes were dosed on the basis of enzyme /substrate ratio (E/S), that is, the total volume of the enzyme preparation related to the weight of the dry crushed guava leaves. E/S ratios of 0 or 1 corresponding to enzyme concentrations of 0 or 1 ml/20000g dry leaves in 20ml deionized water. All treatments were conducted with a heat treatment (100°C, 5min) prior to extraction of phenols. The effects of different enzymes were tested with E/S of 0.43 ml/g dry leaves, 50°C for 5 hr. To study also the influence of the other parameters on the extraction, the enzyme hydrolysis was optimized further in a 3^3 and 2^2 factorial design. Extracts were obtained by filtering through Whatman paper for two times. All investigations were carried out in two parallel and identical systems.

2.4. Phenol quantification: Total phenols from guava leaves were determined by the Folin-Ciocalteu procedure [5], with total phenols expressed as mg gallic acid equivalents (GAE)/g dry guava leaf [5].

2.5. Protein determination: The total proteins were determined by using international method. Protein content was analyzed according to **AOAC** methods [7].

2.6. Analysis of enzyme activity: Protease activity was assayed by the modified method [8], using casein as the substrate [9]. Cellulase activity was assayed using the dinitrosalicylic acid (DNS) method to determine reducing sugars released from 50mg of Whatman filter paper. The activity of each enzyme was determined within 60 min every 5 min after being treated in the guava leaves solution.

2.7. Antioxidant activity: Antioxidant activities of enzyme-hydrolyzed guava leaves extracts were determined by direct using DPPH· at 515nm [10]. One ml methanol with 22µg gallic acid equivalents of guava leaves extracts and 22µg EGCG were placed in 1-cm cuvettes and 3ml 7.1×10^{-5}mol/L were added. Absorbance measurements commenced immediately. The decrease in absorbance at 515nm was determined

continuously with data acquisition at 2-s intervals with a spectrophotometer (Genesis 2 Thermospectronic, Analis). All determinations were performed in triplicate. The percent inhibition of the DPPH radical by the extract and EGCG was calculated according to the formula: %inhibition=$[(A_{c(0)}-A_{A(T)})/A_{C(0)}]\times100$, where $A_{c(0)}$ is the absorbance of the control at t=0 and $A_{A(t)}$ is the absorbance of the reaction solution at 20min.

3. RESULTS

3.1. Effect of crushed guava leaves on phenol yield

The yield level of total phenols was 35.5mg GAE/g dry guava leaves when whole guava leaf was extracted with water without enzyme treatment. When the leaves were crushed by milling prior to extraction, the level of total phenols increased by 18.87% (Table 1). Crushed guava leaves were therefore used as a substrate for enzyme hydrolysis in all subsequent experiments. The particle size distribution of the crushed leaves is shown in Table 2. The crushed powder was subjected to the following analysis.

Table 1.Yield of phenols as affected by milling of guava leaves prior to extraction with water

	Not crushed	Crushed[b]
Phenols GAE(mg/g)[a]	35.5±0.5	42.2±0.5

[a]GAE, gallic acid equivalents [b]. crushed for 30s by using a high speed-crushing machine

Table 2. Particle size distribution of crushed Guava leaves

Particle size(μm)	>900μm	450-900μm	300-450μm	200-300μm	<200μm
Distribution(%)	4.8±0.2	13.2±0.2	50.2±0.1	25.5±0.1	6.3±0.3

3.2. Influence of particle size of guava leaves on enzymatic release of phenols

The effects of the particle size were examined in the same conditions. Phenols were extracted with water (1:10, per gram dry leaf/10ml deionized water), at 37°C for 7hr. Results are shown in Table 3.

Table 3. Particle size effect

Size	Not crushed	450-900μm	300-450μm	200-300μm	<200μm
Phenols (GAEmg/g)	31.5±0.1	46.5±0.3	43.6±0.2	24.9±0.3	26.1±0.1

3.3. Effect of Enzyme Type on Yield of Phenols

The effects of cellulase, pectinase, protease at the same condition were tested. Results are shown in Table 4.

Table 4. Effects of enzyme type and yield of phenols[a]

2.8. Statistical analysis: The SAS (statistical Analysis System) software was used to aid the statistical design of factorial experiments and to fit and analyze the data by multiple linear regression. Differences in effects of particle size and types of enzymes were tested by one-way analysis of variance.

Enzyme type	blank	Cellulase	Pectinase	Neutral protease	Acid protease	Alkaline Protease(novo)
Phenols GAE(mg/g)	29.2±0.2	19.5±0.3	18.0±0.2	30.6±0.1	35.1±0.2	8.4±0.2

[a]E/S=0.43 ml/g , temperature=50°C,time=5hr

3.4. Enzyme activity determination after adding to guava leaf extraction

Within 60 minutes, the activities of the above five enzymes were tested. Cellulase was totally inactivated within 20min (Results are shown in the Fig. 1.), alkaline protease within 5 min, pectic enzyme within 10 min. Neutral enzyme was partially inactivated. Only acid protease was not observed and it may be inactivated (results not shown).

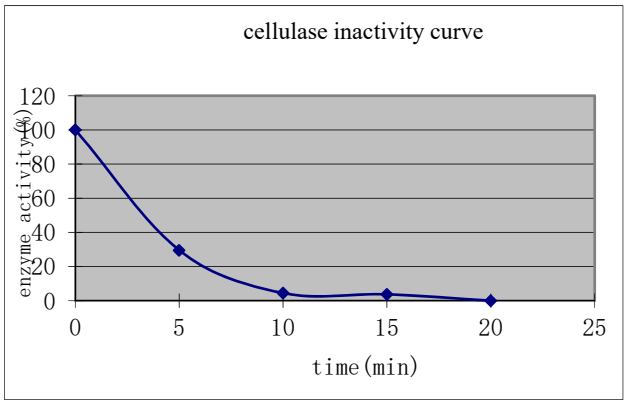

Fig.1. Cellulase inactivity curve

3.5. Influence of some parameters on yields of phenols by using acid protease

The effects of acid protease and time of enzyme treatment on the degradation and the release of phenolic compounds from crushed guava leaves were tested in one way analysis and a 3-level plus a two-level full factorial experimental design (3^3 and 2^2 design). As expected, the yield of total phenols varied in response to different treatments

and ranged from 7.1 ± 0.1 to 57.9 ± 0.2 mg GAE/g dry guava leaf in the extracts (Table 5,6).

Table 5. Acid protease treatment at different pH

PH[a]	PH3.5 blank	PH3.5	PH4.0 blank	PH4.0	PH5.0 blank	PH5.0	PH6.0 blank	PH6.0
Phenols(GAE mg/g dry leaf)	38.7±0.2	48.1±0.1	34.1±0.3	42.6±0.2	7.1±0.1	36.4±0.1	24.1±0.2	10.2±0.1

[a] citrate buffer, acid protease/citrate buffer = 1:1.15, temperature = 50℃，time = 5hr

Table 6. Acid protease treatment parameters

Exp. nb.	Acid protease (E/S)[a]	Temperature (℃)	Time (h)	Phenols (GAE mg/g dry leaf)
1	0	40	5	46.2±0.1
2	0.5	40	2	34.9±0.1
3	1	40	3	25.1±0.3
4	0	45	3	33.5±0.2
5	0.5	45	5	35.8±0.2
6	1	45	2	28.9±0.1
7	0	50	2	42.3±0.1
8	0.5	50	3	39.5±0.2
9	1	50	5	57.9±0.2
10	0.5	50	5	34.4±0.2
11	0.5	55	5	36.2±0.1
12	0.5	50	6	52.5±0.2

E/S, acid protease volume/ g dry guava leaf, enzyme was diluted by deionized water

Table 7. Multiple linear regression describing the yields of phenols by acid protease assisted extraction in response to enzyme hydrolysis parameters[a]

Term	Parameter estimate	Standard error	Parameter=0	Prob>|T|
INTERCEP	-7.800000	4.00998753	-1.945	0.1912
E/S	1.440000	1.11139552	1.296	0.3245
Temperature	0.216000	0.08119113	2.660	0.1170
Time	0.120000	0.37523326	0.320	0.7794

[a] as estimated from data in Table 6

3.6. Antioxidant activity determined by using DPPH·

The antioxidant activities of water and enzyme extracts treatments from guava leaves were compared with EGCG *in vitro* at equival molar phenol concentrations of 22µg GAE (table 8).

Table 8. Antiradical activity of guava leaf extract and EGCG with the DPPH· assay

Compound	% Inhibition	Reduced DPPH molar (10^{-5})
Water extract	82.5	22.50
Enzyme assisted extract	85.67	22.82
EGCG	86.4	23.57

4. DISCUSSION

It is known that phenolic compounds accumulate in the vacuoles or in the cell walls. In the vacuoles there are other ingredients including proteins. In this study, acid protease was used to assist extracting phenols from the guava leaves whose recovery was 57.9g GAE/g dry guava leaf. What's more, the antioxidant activity released from enzymatic way was higher than deionized water extract.

The plant cell walls are composed of cellulose, hemicelluloses, pectin and lignin arranged in a complex network [11]. Cellulase and pectinase were tested in extracting polyphenolic compounds. Unfortunately, from Table 4 and Fig. 1, cellulase and pectinase were inactivated very quickly suggesting that they can't help the hydrolysis of the cell walls. Although there are reports about using pectinolytic enzyme or cellulase, yet the present data showed that the extraction of phenolic compounds was negatively correlated to adding the two enzymes. Furthermore, the phenols recovery was much less than the blank. Previous results showed that some polyphenols like flavonoids from wine and grape seeds have been found to interact with grape juice proteins [12, 1]. Catechin and grape seed

dimeric phenols could react with wine proteins. Most of the polyphenolic compounds have different properties, especially the ability to precipitate proteins. The characteristic affinity of tannic compounds with proteins is due to hydrogen bonding. These bonds are formed between the tannin's phenol group and the carbonyl group of the proteins peptide. It has been demonstrated for both condensed and hydrolysable tannins with metabolic enzymes and extracellular enzymes. The inhibition of the two enzymes could result in that there must be new complexes between phenolic compounds and extracellular enzymes [13]. However, acid protease was positively related to the recovery of phenol yields, while neutral protease almost had no effect and alkaline protease was negatively correlated with extraction of phenolic compounds (Table 4). It's well known that enzyme show three levels of specificity: for the reactants, for the susceptible bond involved in the reaction, and the type of product produced. Some groups of enzymes show a fine specificity. It's not easy to understand how the protease can help to extract phenols from leaves. The reason may lie in that the protein content in guava leaf was rather high (7.71%). These proteins may combine around the phenolic compounds and hinder the diffusion of other ingredients from the leaf. Proteins were hydrolyzed by acid protease and then helped to liberate other compounds including phenolics from the vacuoles. Neutral protease only showed a little effect and seemed not to react with phenolic compounds. The alkaline protease was inhibited very quickly and must react with phenolic compounds for the phenols recovery when it is much less than the blank [14].

The optimal acid protease pH was pH 3.5 and the phenols recovery was the high according to table 5, but it was thought that deionized water was more economical and better for phenolic compounds recovery. At pH 5.0, the acid protease activity was the most powerful. However, we selected deionized water as solution, because pH of the water solution is between pH3-4.8, during which the acid protease has high activity. Furthermore, if buffers used, deionized process must be added in the final step. Therefore we use deionized water as the solution. From the data of Table 6 and 7, acid protease was helpful in phenols recovery. It seemed that the most important parameter was E/S for the estimate parameter was the biggest. Too long time and high temperature may not be advantageous because phenols were easily oxidized and protease activity may be reduced. Optimal conditions are highly suggested to perform the extraction process.

Another significant parameter affecting phenol yields was the reduction in particle size diameter of the guava leaf according to table 1 and table 3. The objective in extracting phenolic compounds is to liberate these compounds from vacuolar structures where they are found, either through rupturing plant tissue or through a process of diffusion [15]. The first case is accomplished by carrying out particle reduction. Thus, significant correlations were found between reduction in particle size and yield of total phenols. Compared with the extraction results using deionized water, medium size was proper to be used. 450-900μm size was better to be penetrated by solvents. The distribution showed that the main particle was 300-450μm and the extraction recovery was almost the same with 450-900μm, so we used this size to later extraction.

The antioxidative activity of the guava leaf extract by using water and enzyme was compared with EGCG which was believed to be one of the main antioxidants (table 8 and fig. 2). In Fig. 2 the reactions with rapid kinetic behaviour is shown. The water and enzyme assisted extract reacted rapidly with the DPPH·(reaching a steady state in less than 20 min). The steady state for EGCG was approximately 3 min. On the one hand, guava leaf extract showed high antioxidative activity from the DPPH· % inhibition. On the other hand, acid protease assisted guava leaf extract showed a little higher activity, which may result from the extract of different chemicals from the water extract.

5. CONCLUSION

The present work demonstrated that guava leaf phenolic components could be assisted by one acid protease and the guava leaves extracts showed high antioxidative activity. Furthermore, phenols recovery is greatly affected by the parameters including enzyme type, particle size, pH, time, E/S and temperature. Our results have opened further research areas in the fulfillment of the extraction of antioxidant substances from various plants. Work in progress is aimed at optimizing conditions of use of the acid protease assisting the extraction of phenolic compounds from guava leaves and studies of varieties of guava leaves that could release more polyphenols components from enzymatic extraction are suggested.

6. ACKNOWLEGMENTS

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