Enzyme-assisted Polyphenol Extraction from Mangosteen Peels and Evaluation of Its Antioxidant Activity

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Abstract: Enzyme-assisted extraction was investigated to enhance polyphenol yield from mangosteen peels. Different enzyme type (Viscozyme and cellulase), enzyme concentration (v/w) (0.5% and 1%) and reaction time (0-6 h) were tested. In addition, total polyphenol content and antioxidant activity were assayed to evaluate the extraction performance. The maximum total polyphenol content was achieved by 1% Viscozyme at 4 h: 1062.6 ± 205.6 μg GAE / mL. The maximum antioxidant activity was achieved by 0.5% Viscozyme at 0.5 h: 92.35% ± 0.11% DPPH quenched. Enzyme type has a significant influence on both total polyphenol content and antioxidant activity. Extraction assisted by Viscozyme performs better than cellulase in both total polyphenol content and antioxidant activity assay results. But, Viscozyme-assisted extraction does not show significant difference from 70% ethanol extraction and water extraction. The result indicates enzyme-assisted extraction cannot significantly enhance polyphenol extraction efficiency from mangosteen peels in this experimental set-up.

Keywords: Enzyme-assisted extraction, polyphenol, mangosteen peel, cellulase, total polyphenol content, antioxidant activity

Introduction

Mangosteen peel

Mangosteen (*Garcinia mangostana L.*) is one of the most admired tropical fruits and known widely for its beautiful purple blue pericarp and delicious flavor(Ketsa & Paull, 2011). Mangosteen consumed as dietary supplement may have the potential health beneficial properties(Li, Thomas, & Johnson, 2013). Polyphenols are considered as major bioactive compounds in mangosteen fruit(SUKATTA et al., 2013). Mangosteen peel is rich in polyphenols. It was reported that, xanthone, a polyphenolic compound has a high concentration in yellow gum and pericarp of mangosteen (**Figure 1**) (SUKATTA et al., 2013). However, mangosteen peel is normally discarded as agricultural waste. So, mangosteen peel could become a potential polyphenol source.

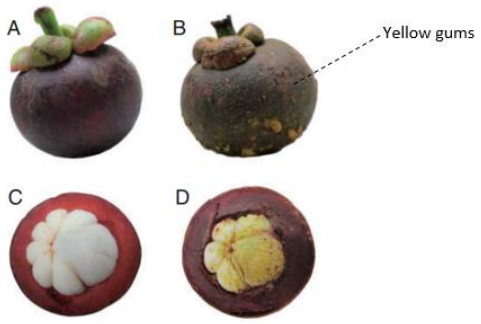

Figure 1 Mangosteen, yellow gum and pericarp (A. Fruit without yellow gums; B. Fruit with yellow gums; C. Inside of fruit without yellow gums; D. Inside of fruit with yellow gums. Picture is adapted from Sukatta U. et al.(SUKATTA et al., 2013))

Beneficial effects of polyphenol

Polyphenols have a lot of beneficial effects as food additives. A significant amount of polyphenol intake is claimed can protect chronic diseases by its antioxidant activities(Pietta, Minoggio, & Bramati, 2003).

In addition, polyphenols have a lot of potential functionalities in food industry besides health benefits: (1) they effectively inhibit lipid oxidation because they can scavenge free radical and chelate metals(Decker, Elias, & McClements, 2010). (2) they can interact with proteins and could protect protein oxidation(Lund, Heinonen, Baron, & Estévez, 2011). (3) they can inhibit Maillard reaction(Jansson et al., 2017).

Polyphenols structures and solubility

Polyphenol is a large group of chemical compounds. Common plant polyphenols can be classified into 5 groups based on their structures: (1) Hydrobenzoic acids derivatives (2) Hydroxycinnamic acid derivatives (3) 4-oxo-flavonoids: flavanols, flavones, isoflavones, flavanones, chalcones and dihydrochalcones (4) Flavanols (FIavan-3-ols) (5) Anthocyanidins, anthocyanins, proanthocyanins, tannins(Pietta et al., 2003).

Most polyphenols are insoluble in water, but soluble in organic solvents because benzene ring in polyphenol structure is non-polar. However, some of the polyphenols, especially in their glyosidic forms are polar and soluble in the water, e.g. glycosylated isoflavones, glycosylated anthocyanidins(Pietta et al., 2003).

Polyphenol extraction methods

1. Non-enzymatic extraction methods

The routine extraction technique of polyphenol is solid-liquid extraction (SLE)(Ameer, Shahbaz, & Kwon, 2017). Organic solvent is used to extract polyphenols from the matrices.

'Green' extraction methods can also be used to extract polyphenols from plant matrices and their by-products(Ameer et al., 2017). 'Green' in this context means less organic solvent is used. These green extraction methods include supercritical fluid extraction, ultrasound-

assisted extraction, microwave-assisted extraction, pressurized liquid extraction, and pressurized hot water extraction(Ameer et al., 2017).

2. Enzyme-assisted polyphenol extraction and its principles

Enzyme-assisted extraction (EAE) has been explored to extract antioxidants from plant matrices. The benefits of EAE include: (1) High yield and efficiency. Some bioactive compounds in the plant matrices are secondary metabolites, e.g. polyphenol(Pietta et al., 2003). They are dispersed in cell cytoplasm, and some compounds are retained in the polysaccharidelignin network by hydrogen bonding or hydrophobic interactions, which are not accessible by solvent in a routine extraction process(Chen, Xing, Huang, & Xu, 2011). Enzymatic treatment has been considered as an effective way to release bounded compounds to increase overall yield. (2) EAE works in mild environment and is environmentally compatible and easy operated (Selvamuthukumaran & Shi, 2017).

Hydrolytic enzymes, including cellulase and pectinase, are commonly utilized to hydrolyze and degrade cell wall constituents and improve the release of intracellular contents(Selvamuthukumaran & Shi, 2017).

Enzymatic polyphenol extraction is still not fully applied in industry for a large scale compared with other extraction methods(Selvamuthukumaran & Shi, 2017). Part of the reason could be: complexity of plant matrices makes it difficult to have a standard method. Some existed researches include polyphenol extraction from peanut sheel(Zhang et al., 2013), apple(Zheng, Hwang, & Chung, 2009), Lonicerae Flos (Kong, Yu, Bi, Huang, & Huang, 2016) (a Chinese herbal which is rich in polyphenols).

Objectives

This research aimed at investigating effects of different factors (different enzyme type, extraction time and enzyme amount) on polyphenol yield and their antioxidant activities. A schema of research is shown in the table. Detailed experimental procedures are described in 'Materials and Methods' section.

Table 1 Schema of experimental design and set-up

Factors							**Responses**	
Type of extraction	Cellulase		Viscozyme® L		Ethanol	Water	Polyphenol yield	Antioxidant activity
Enzyme concentration%* (v/w)	0.5	1	0.5	1	N/A	N/A		
pH	3.7		5.5		7	7		
Temperature	66°C		50°C		20°C	20°C		
Extraction solvent	Acetate buffer		Acetate buffer		70% ethanol	Water		
Extraction time	0.5 h, 1 h, 2 h, 3 h, 4 h, 5 h, 6 h							

***percentage of enzyme to substrate, volume to mass ratio**

Materials and Methods

Chemicals

Acetic acid (Bie&Berntsen A-S, 3240103), sodium carbonate (EMSURE, 1.06268.1000), gallic acid (Sigma-Aldrich, CAS: D21,140-0), 2,2-Diphenyl-1-picrylhydrazyl (DPPH), 0.1 M HCl (VWR, BDH Chemicals, 20255.290) and NaOH (EMSURE, 1.06469), ethanol 96% vol (UN 1170, ethanol solution, Plum A/S 5610 Assens, Denmark), Folin-ciocalteu's phenol reagent solution (Sigma-Aldrich, F9252)

Apparatus

Spectrometer (Spectramax i3x, Molecular Devices), centrifuge (type: 154.RF, Ole Dich, Denmark), hot water bath (type: WNB22, memmert Germany), pipette (GILSON), weight scale, Vortex mixer, ultrasonic bath.

Mangosteen peel preparation

Mangosteen peels was obtained from Thailand. They were freeze dried and stored in a plastic bag at ambient temperature. Dried peel sample was grounded by a mortar and then stored in a sealed bottle in 4°C until use.

Enzyme

Cellulase and Viscozyme® L were tested in the experiment. Cellulase (E.C. 3.2.1.4) is produced by Trichoderma reesei, CAS NO. 9012-54-8. Viscozyme® L is a commercial product from Novozyme. It is a complex of multi carbohydrate degrading enzymes, including arabanase, cellulase, β-glucanase, hemicellulase, and xylanase. Both were liquid and stored in the 4°C fridge. In polyphenol extraction experiment, they reacted in their separate optimal pH and temperature. Optimal pH and temperature data was obtained from literatures(Zhang et al., 2013; Zheng et al., 2009). The optimal pH for cellulase and Viscozyme® L is 5.5 and 3.7 respectively. The optimal temperature is 66°C and 50°C respectively for cellulase and Viscozyme® L.

Reaction solvent and buffer preparation

0.1 M Acetate buffer was used to keep the optimal pH during extraction. For cellulase, the buffer was adjusted to pH 5.5; for Viscozyme L, pH was adjusted to 3.7. 70% (v/v) ethanol was used to compare effects of different extraction methods, pH adjusted to 3.7. Blank group contains only distilled water, with pH 7.

Enzyme precipitation test

Viscozyme® L was mixed with 70% (v/v) ethanol to test whether enzymes would precipitate.

Enzymatic extraction and sampling

1 gram sample and 50 mL buffer (or extraction solvent) were mixed in a sealed bottle, then pre-heated in the water bath at optimal temperature for 10 min. Proper amount of enzymes, 50 μL for 0.5% (v/w) groups and 100 μL for 1% (v/w) groups were added into the bottle. Sealed bottles were shaken manually every 10 min to make sure the sufficient contact between enzymes, solutions and substrates.

Duplicate samples were collected at 0.5 h, 1 h, 2 h, 3 h, 4 h, 5 h and 6 h. Samples were instantly stored in -18°C freezer to inactivate the enzymatic activities.

At the meantime, 70% ethanol group and blank group were incubated at 20°C and shaken, sampled by the same way.

Sample pre-treatment

Samples were taken out to thaw for 30 min. Then, they were centrifuged by 10,000 rpm for 20 min. Supernatants were taken. Then, all samples were diluted by 96% ethanol to 10-fold. The determination of optimal dilution factor will be discussed in later.

Total polyphenol content (TPC) assay

Total polyphenols contents were determined by Folin–Ciocalteu (F-C) method(Singleton, Rossi Jr., & Rossi J A Jr., 1965) with minor modifications. F-C solution was pre-made and stored at 4°C. Then, samples and reagents were loaded into 96-well plate[1]. First, 20 μL 96% ethanol were added to Blank wells. Second, 20 μL diluted extracts were added to Sample wells. Third, 240 μL distilled water and 20 μL F-C solution were added to Blank and Sample wells. Then, the plate was kept in dark for 3 min. In the end, 30 μL 10% Na_2CO_3 was added to Blank and Sample wells. Further, the plate was incubated at room temperature for 1.0 h. Then, absorbance was measured at 765 nm by UV spectrophotometer. The results were expressed as gallic acid equivalents (μg gallic acid/mL), which was converted by the standard curve of gallic acid.

The mechanism underlying F-C method is Mo(VI) react with polyphenol forming blue color Mo(V) complex, which has the λ_{max} around 765 nm.

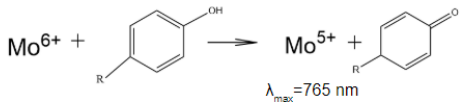

Figure 2 Reaction mechanism of F-C method

Antioxidant activity assay

Antioxidant activities were determined by DPPH radical scavenging capacity assay. DPPH solution is a dark color free radical reagent. The discoloration of DPPH can indicate the antioxidant activities.

0.2 mmol/L DPPH in 96% ethanol was prepared freshly and stored in dark. Then samples, solvents and DPPH solvents were loaded into 96-well plate. First, 200 μL 96% ethanol was added to Blank wells. Second, 100 μL 96% ethanol was added to Control wells. Third, 100 μL diluted samples were loaded to Sample wells. Then, 100 μL of 0.2mM DPPH solution was added to Blank, Control and Sample wells. The plate was gently shaken for 5 s and kept in

[1] The methods were modified from ' Dr Prieto's DPPH Microplate Protocol'. This protocol is an unpublished document and can be accessed at ResearchGate.

dark for 30 min. In the end, the absorbance was measured at 515 nm. The result was expressed as %DPPH quenched, calculated by the following equation:

$$DPPH\ quenched(\%) = 100 * \left[\frac{A_i}{A_c - A_s}\right]$$

A_i : absorbance of sample;
A_c : absorbance of control;
A_s : absorbance of blank.

Standard Curve and determination of optimal dilution factor

For total polyphenol content, standard gallic acid solution was diluted by 96% ethanol to gradient concentration (15.63, 31.25, 62.5, 125, 250, 500 µg/mL and 1 mg/mL). Then, they were assayed by the same F-C assay procedure as samples.

For antioxidant activity, standard trolox solution was diluted by 96% ethanol to gradient concentration (1.82, 3.64, 7.725, 14.55, 29.1, 58.2 and 116.4 µM). Then, their antioxidant activities were estimated by the same DPPH radical scavenging capacity assay procedure as samples.

To determine optimal dilution factor, samples were diluted by 96% ethanol to 5, 10, 20, 40 and 80-fold. Then, they were assayed by TPC and antioxidant activities.

Statistical analysis

Multi factor ANOVA was carried out by JMP 13; two-way ANOVA was carried out by Microsoft Excel 2017.

Results and Discussion

Enzyme precipitation test

The ethanol solution gradually turned grey when Viscozyme® L was added into 70% (v/v) ethanol solution. It indicated that 70% ethanol can precipitate the enzyme. Because ethanol can significantly lower the dielectric constant of the aqueous solution(Zellner et al., 2005). Hence, the enzyme changed the structures and lost catalytic activities. So, 70% ethanol is not compatible with Viscozyme® L.

However, ethanol can significantly increase performance of enzyme-assisted extraction by influencing the solubility of substrate and bioactive compounds(Chen et al., 2011). As described before, enzyme-assisted extraction increases mass transfer of bioactive compounds from intracellular fluids into extraction solution by degrading membranes or cell walls. Once substrate has a better solubility in ethanol, enzyme has more contacting areas with substrate. In addition, if bioactive compounds have a higher solubility in ethanol, the difference of solubility becomes driving force motivating mass transfer and diffusion. A research paper investigated the effect of ethanol concentration on enzyme activity. It showed that, when ethanol concentration was increased, transglycosylation activities get a significant improvement(Chen et al., 2011). But, when ethanol concentration exceeds a limit, the extraction rate decreases due to the change of enzyme structures(Chen et al., 2011).

Ethanol as extraction solution cannot buffer pH change. It is also a problem can influence extraction efficiency. On contrary, acetate buffer (NaAc/HAc) is a good buffer solution for enzyme because Ac^- can work as proton acceptor and HAc can work as proton donor. Therefore, pH can be buffered and does not fluctuate during the reaction.

Calibration curve

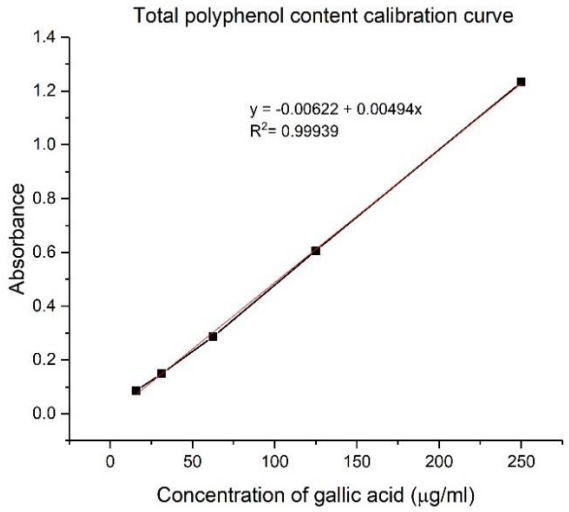

Figure 3 Total polyphenol content calibration curve

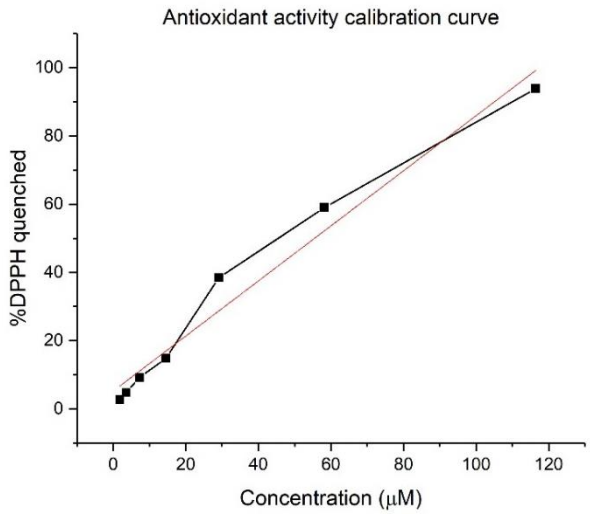

Figure 4 Antioxidant activity calibration curve

Calibration curves (Figure 3, Figure 4) get good fittings: total polyphenol content with R^2=0.9994 and antioxidant activity with R^2=0.9708.

Total polyphenol content assay

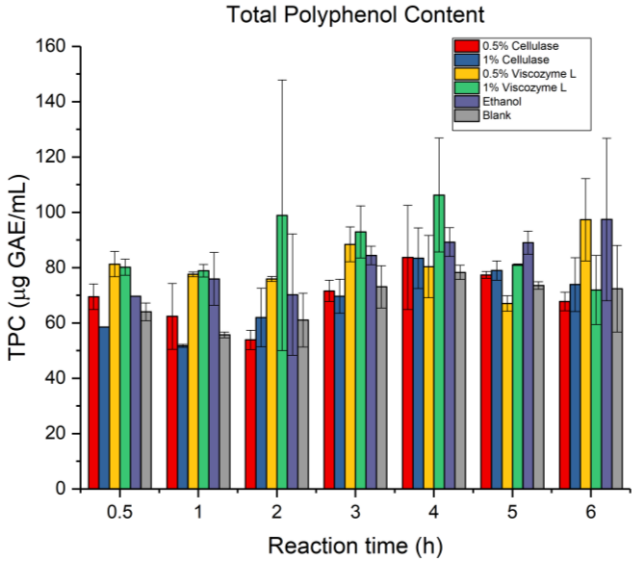

Figure 5 Total polyphenol content assay. Different colour indicate different treatments. Average and standard deviation were calculated based on duplicates.

TPC did not show a clear pattern in Figure 5. Some TPC results show high deviations e.g. 1% Viscozyme L at 2 h has TPC 98.92 ± 48.93. This could be unexperienced lab

Generally, 1% Viscozyme L shows a higher polyphenol extraction rate than other groups between reaction time 2-4 h. Statistical analysis shows that only enzyme type has a significant influence on TPC ($p<0.0001$). Enzyme-assisted extraction does not have a significant difference with water and 70% ethanol, which means enzyme-assisted extraction did not yield more polyphenols from mangosteen peels.

Antioxidant activities assay

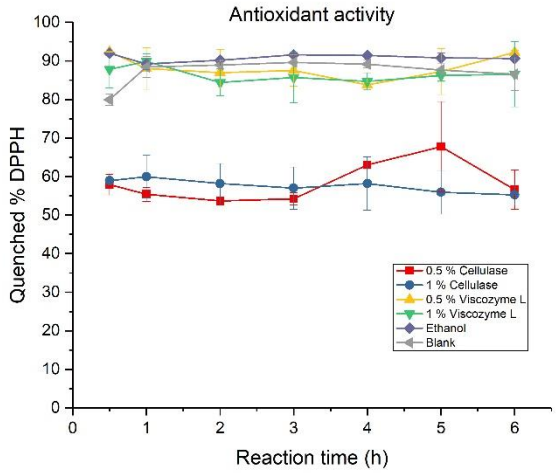

Figure 6 Antioxidant activity assay. Different lines indicate different treatments. Average and standard deviation were calculated based on duplicates.

It is significant that 0.5% and 1% cellulase groups quenched less DPPH than other groups, which indicates the cellulase-assisted extraction yielded lower antioxidant activities ($P<0.05$). The reason could be: (1) cellulase did not work in optimal pH and temperature (2) the pH and temperature combination inhibited the antioxidant activity

Statistical results show, enzyme type has very significant effects ($P<0.05$) on polyphenol antioxidant activity. Enzyme amount ($P=0.3108$), extraction time ($P=0.9584$) do not significantly influence polyphenol antioxidant activity.

Conclusions

Enzyme-assisted polyphenol extraction from mangosteen peel by Viscozyme® L and cellulase have significant differences ($p<0.05$) in total polyphenol content and antioxidant activities. Extraction assisted by Viscozyme® L both by 0.5% and 1% has higher total polyphenol content and antioxidant activities. However, enzyme-assisted extraction did not significantly affect extraction efficiency, compared with water and ethanol. The maximum polyphenol content was achieved by 1% Viscozyme® at 4 h: 1062.6 ± 205.6 μg GAE / mL. The

maximum antioxidant activity was achieved by 0.5% Viscozyme® at 0.5 h: 92.35% ± 0.11% DPPH quenched.

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