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Proceeding Paper

Production of Protein Hydrolysates from Spent Coffee Ground via Microwave, Enzymatic, Subcritical Water Extractions, and Their Combination †

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Abstract: Spent coffee grounds (SCGs) are currently considered abandoned landfill waste, despite retaining valuable organic compounds, especially with a high protein content of 16.64 ± 0.13 g/g dried SCGs and a high oil content of 15.48 ± 0.17 g/g dried SCGs. As a result, SCGs could serve as a potential source of valuable ingredients. However, utilizing a single technical strategy of alternative green extractions was insufficient for extracting the target compounds and hydrolyzing proteins. This work aimed to optimize the operating parameters of enzyme-assisted (EAE), microwave-assisted (MAE), and subcritical water (SWE) extractions using a response surface methodology. The results showed that EAE, at a papain to substrate ratio (E/S) of 0.5 and a duration of 15 min, generated the %DH of 93.39% and provided the water soluble protein concentration (WSPC) in the range of 400 to 800 μg/mL. Besides, MAE provided the highest %DH of 9.72% at 600 watts and 10 min, while SWE produced the maximum %DH of 13.41% at 160 °C at 17.5 min. However, the WSPC of MAE and SWE extracts were comparable at ~250 μg/mL. A combination of MAE−SWE enhanced %DH of hydrolysates than combined SWE−MAE, SWE, and MAE did. However, the effects of differential hydrolysis on bioactivity are not directly correlated with %DH. In this study, the highest antioxidant activity was found at E/S of 0.5 and 15 min for EAE, 350 W and 10 min for MAE, and 160 °C and 30 min for SWE. This work demonstrated that the valorization of SCGs not only reduces the amount of waste but also yields functional cosmeceutical and nutraceutical ingredients.

Keywords: protein hydrolysate; enzyme-assisted extraction; microwave-assisted extraction; subcritical water extraction; spent coffee grounds

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1. Introduction

Spent coffee grounds (SCGs) were a waste generated by the coffee industry, despite still retaining various bioactive compounds associated with potential health advantages. Consequently, SCGs could act as a viable source of high-value ingredients for various applications.

Typically, enzymatic hydrolysis is performed via proteases with high specificity, operating under mild conditions. In this study, papain was used as a plant-derived proteolytic enzyme sourced from papaya (*Carica papaya* L.) which is used to facilitate the breakdown of peptides into lower molecular weight [1]. Nonetheless, challenges such as the elevated cost of enzymes, allergenic properties, requiring additional downstream processes, and the necessity for various chemicals to maintain the extraction condition, remain a concern point.

Extraction techniques such as subcritical water hydrolysis (SWH), and microwave-assisted hydrolysis (MAH) represent non-conventional proteolysis techniques aligned with sustainable principles. In practice, SWH is employed at (110–300 °C), pressure (20–100 bar), and short reaction time (5–60 min) in a liquid state to release bioactive peptides [2]. For protein hydrolysis, occurred biocatalyst with high ionization of the reaction namely, H+ combined with the N-terminal of the protein chain resulted in peptide bond cleavage, then at that position, OH^- to the carbon cation of the C-terminal. While MAH using electromagnetic waves not only increased extraction yields in a shorter time due to superheating water molecules but also improved the %DH, antioxidant capacity. Presently, MAH is considered as a simple, rapid, and highly efficient extraction method.

This work aimed to optimize the operating parameters of enzyme-assisted (EAE), microwave-assisted (MAE), and subcritical water (SWE) extractions using a response surface methodology. The combination of those techniques was investigated as well.

2. Materials and Methods

2.1. Preparation of Spent Coffee Grounds (SCGs) SAMPLE

SCGs (Arabica coffee variety) were donated by Starbucks, Bangkok, Thailand used as starting material. Initially, it was dried by natural convection for 24 h at room temperature (35 ± 5 °C), and then dried by vacuum hot air oven at 60 °C for 6 h. The proximate analysis was determined according to the official AOAC method (2000). SCGs consisted of 2.58 ± 0.12% (*w*/*w*) of moisture, 16.64 ± 0.13% (*w*/*w*) of crude protein, 15.48 ± 0.17% (*w*/*w*) of lipids, 28.14 ± 1.00% (*w*/*w*) of water insoluble fiber, 31.18 ± 0.82% (*w*/*w*) of carbohydrate, and 1.98 ± 0.02% (*w*/*w*) of ash.

2.2. Protein Hydrolysate Production

EAE was performed as described in literature [3] with modification. Briefly, samples at a solid/solvent ratio of 1:20 (*w*/*v*) were hydrolyzed by papain at enzyme to substrate (E/S) ratio of 0.1–0.5 in phosphate buffer (PBS) for 15–180 min with constant rotational shaking of 150 rpm at 50 °C. The hydrolysis reactions were terminated by heating at 85 °C for 20 min with occasional agitations. For microwave hydrolysis, it was carried out by Mars 6 synthesis (CEM Co., Matthews, NC, USA) at 100–600 watts for 2–10 min. For subcritical assisted hydrolysis, it was performed by using a laboratory-scale extractor [4]. Briefly, the sample was loaded into the extraction vessel (Parr Instruments Company, Illinois, USA). The investigated temperatures were in the ranges of 110 ± 5–160 ± 5 °C for 5–30 min at an initial pressure of 20 bar. In all experiments, the solution was removed insoluble portion by filtration and centrifugation at 10,000× *g* for 30 min at 4 °C, and the supernatant was collected and stored at −20 °C until further preliminary investigation.

2.3. Assays

2.3.1. Water Soluble Protein Content (WSPC) and Degree of Hydrolysis (%DH)

The soluble protein concentrations were determined by using Bradford's assay [5]. The absorbance was read at 595 nm using a standard curve of bovine serum albumin (BSA). The %DH was determined using the o-phthalaldehyde (OPA) method in which the change of solution color was detected at 340 nm.

2.3.2. Total Phenolic Content, Antioxidant Capacity, and Total Polysaccharide Content

The total phenolic content (TPC) was assessed using the modified Folin-Ciocalteu assay [6]. It was measured in 96-well flat bottom plate using a spectrophotometer (Thermo Fisher Scientific, Multiskan GO) at 725 nm and expressed as mg gallic acid equivalent (mg GAE) per dry weight sample. For antioxidant activities, DPPH radical scavenging ability was determined following the approach described in literature [7]. The change of solution color was measured at 517 nm and expressed as mg of L-ascorbic acid equivalent (mg AAE/g SCG) and a half maximal inhibitory concentration (IC_{50}). Total polysaccharide (TPS) was determined using phenol sulfuric acid method described elsewhere [8] with D-glucose as reference standard, the absorbance was measured at 490 nm, and reported as g GLU/100 g SCGs.

2.4. Experimental Design and Data Analysis

The optimal conditions were established through a response surface methodology employing a face-centered composite design, which involved two factors at three levels each. Design Expert® software (Stat-Ease, Inc., Minneapolis, MN, USA) was utilized to create an experimental design consisting of 11 treatments, as detailed **i**n Table 1.

Table 1. Experimental units and response variables of protein hydrolysate.

Methods	Factors	
	X_1	X_2
Microwave-assisted extraction	100–600 watt (power)	2–10 min (time)
Subcritical-assisted extraction	110–160 °C (temperature)	5–30 min (time)
Enzymatic-assisted extraction	0.1–0.5 (E/S)	15–180 min (time)

3. Results and Discussion

3.1. Preliminary Study of Protein Extractioing Conditions

The parameters under investigation included a papain to substrate ratio ranging from 0.1–0.5 and 15–180 min for EAE. Power levels varied from 100–600 watts and 2–10 min for MAE. The temperature range was in the range of 110–160 °C and 5–30 min for SWE.

As it is shown in Figure 1(a1), the rate of hydrolysis at 0.5 E/S was complete at 93.39 ± 0.29% within 15 min and remained constant around 84.65–93.39% over 180 min. Moreover, %DH increased with E/S ratios from 0.1 to 0.5. The highest antioxidant activity was also found at E/S of 0.5 and 15 min (See Figure 1(b1)). Generally, SCGs were processed through a roasting process and espresso machine with roughly water pressure of 15 bars leading to partial hydrolysis into smaller polypeptides. Moreover, MAE and SWE hydrolyze protein by thermal energy and microwave energy, respectively. These methods assist by associating of hydronium ion. MAE showed that %DH linearly increased with extraction time (See Figure 1(a2)). The highest %DH was observed at 600 W and 10 min. However, at microwave power of 600 W, the boiling of solution was observed at 10 min and the DPPH activities were in downward trend (See Figure 1(b2)). Although MAE exhibited relatively high hydrolysis at 600 watts for 10 min, it demonstrated a diminishing trend in DPPH activity. Consequently, the optimal condition was 350 watts for 10 min. For SWE, the extraction at 160 °C for 17.5–30 min showed the highest %DH (See Figure 1(a3)). The highest DPPH activities were detected at temperature of 160 °C as well (See Figure 1(b3)). Notably, while %DH plays a significant role in peptide bond cleavage into shorter peptides, chain length, and its associated DPPH activities emerged as crucial factor.

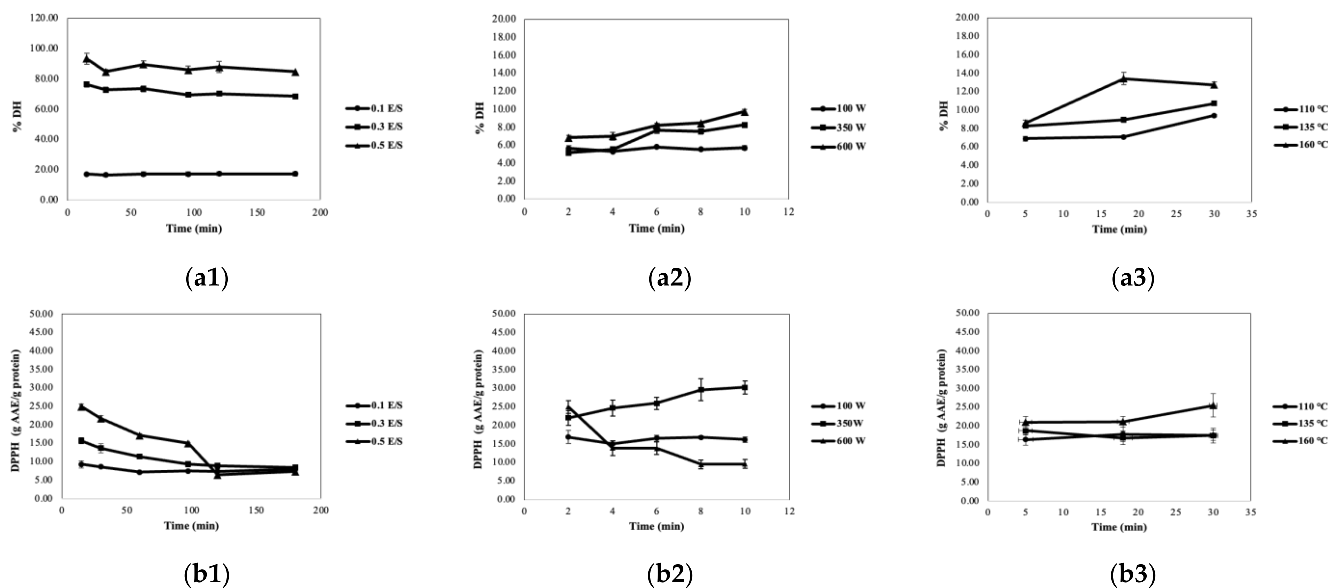

Figure 1. Influences of papain to substrate ratio (E/S) on %DH (**a1**) and DPPH activity (**b1**), microwave power level on %DH (**a2**) and DPPH activity (**b2**), and extraction temperature on %DH (**a3**) and DPPH activity (**b3**).

3.2. Process Optimization by RSM

In the context of RSM analysis, the optimization results are depicted in Figure 2.

(**a1**) (**b1**) (**c1**) (**d1**)

(**a2**) (**b2**) (**c2**) (**d2**)

(**a3**) (**b3**) (**c3**) (**d3**)

Figure 2. Response surface plots of (**a**) %DH, (**b**) protein content, (**c**) total phenolic content (TPC), and (**d**) DPPH for EAE (**a1**–**d1**), MAE (**a3**–**d3**), and SWE (**a3**–**d3**).

3.3. Optimal Conditions and Combination Technique

For the EAE technique, the highest %DH was observed at E/S ratio of 0.5 for duration of 15–60 min. Although %DH shows a significant efficiency in peptide bond cleavage, DPPH activities emerged still as a crucial factor. It was found that EAE exhibited lower DPPH radical scavenging activities compared to MAE and SWE techniques. Moreover, TPS reached 30.02–33.66 g GLU/100 g SCGs due to terminating enzyme activity at 85 °C for 20 min, which are suitable condition for polysaccharide extraction. Among the studied treatments, SWE emerged as the most prominent and effective method in terms of antioxidant activity followed by MAE, displaying an appropriate %DH, elevated protein content, and minimal TPS. Based on RSM optimization, conditions of MAE and SWE were selected to study the effects of the combined techniques. The hydrolysis efficiency achieved through the MAE-SWE combination exceeded that of SWE-MAE, as well as individual SWE and MAE. However, it is noteworthy that the combination resulted in an increase in %DH, which potentially led to a reduction in bioactivity.

Table 2. Optimal conditions and combination technique.

Methods	% DH	DPPH (IC_{50}, μg/mL)	WSPC (μg/mL)	TPS (g GLU/100 g SCGs)	pH
EAE (0.5 E/S)					
15 min	93.39 ± 0.29 ^{a}	34.17 ± 1.04 ^{a}	474.46 ± 1.77 ^{a}	30.49 ± 0.19 ^{a}	7.00 ± 0.05 ^{a}
30 min	86.34 ± 0.05 ^{b}	36.59 ± 0.40 ^{b}	515.60 ± 10.98 ^{b}	33.66 ± 0.75 ^{b}	
60 min	89.64 ± 1.35 ^{c}	38.92 ± 0.54 ^{c}	628.57 ± 8.97 ^{c}	30.02 ± 0.07 ^{d}	
MAE (350 W, 10 min)	9.36 ± 0.13 ^{d}	16.54 ± 0.99 ^{d}	341.33 ± 5.66 ^{d}	17.26 ± 0.78 ^{e}	5.98 ± 0.03 ^{b}
SWE (160 °C, 30 min)	12.73 ± 0.33 ^{e}	15.37 ± 0.38 ^{e}	158.00 ± 3.00 ^{e}	18.26 ± 0.17 ^{e}	4.97 ± 0.02 ^{c}
MAE–SWE	14.39 ± 0.25 ^{f}	25.93 ± 1.57 ^{f}	320.12 ± 0.71 ^{f}	27.76 ± 0.06 ^{f}	4.92 ± 0.01 ^{d}
SWE–MAE	13.60 ± 0.20 ^{ef}	24.18 ± 1.19 ^{g}	279.10 ± 6.65 ^{g}	24.09 ± 0.28 ^{g}	4.92 ± 0.02 ^{d}

Note: Data was mean ± standard deviation. Different superscripts (a–g) in each column indicate significance ($p < 0.05$). TPS is total polysaccharide content, WSPC is water soluble protein, and GLU is glucose.

4. Conclusions

The protein hydrolysates from SCGs were successfully extracted via EAE, MAE, SWE, and their combination. The results revealed that papain exhibited the highest proteolytic activity. The optimal conditions for maximum DPPH activities were identified as follows: an E/S ratio of 0.5 for 15 min in EAE, 350 watts for 10 min in MAE, and 160 °C for 30 min in SWE. Efficient hydrolysis was achieved through the combined MAE-SWE process compared to individual MAE and SWE procedures. The effects of differential hydrolysis on bioactivity are not directly correlated with %DH. This study illustrates that repurposing SCGs simultaneously diminishes waste generation and facilitates their utilization as valuable ingredients in the realms of functional cosmeceuticals and nutraceuticals.

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