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Effect of Enzyme-digested Protein Hydrolysates Derived from Clarias batrachus (Keli Fish) on Brassica rapa subsp. Chinensis (Bok Choy)

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Protein hydrolysate (PH) is a material that contains numerous amino acids which serve as liquid fertilizer for vegetative crops. Growing with sustainability has become a main aspect of agriculture. Therefore, the unused fish viscera were attempted for PH preparation to grow the crops. Fish PH has been shown to improve the growth of plants like broccoli, spinach and lettuce. However, there is no extensive investigation on the amount of PH utilized on plants to achieve the optimal effect. In this study, Brassica rapa subsp. Chinensis (Bok Choy) were treated in five different groups (n=5) with viscera of Clarias batrachus (Keli Fish) PH concentration from 0 to 8000 ppm to find the optimal concentration for Bok Choy's growth. The weight, length, total chlorophyll content (TCC), phytochemical contents (total phenolic and flavonoid content), and antioxidative potential were measured to study the effect of PH. The heavy metal contents (Pb and Cd) were determined to comply with food safety. Results showed that Bok Choy with 6000 ppm PH treatment had the highest weight, length, TCC, phytochemicals and antioxidative potential. In addition, all treatment groups showed a dose-dependent relationship. However, the trend started to decline at 8000 ppm treatment. In conclusion, this study found that treatment of PH made from Keli fish viscera significantly enhances the growth of Bok Choy, where the optimum growth was observed with 6000 ppm PH.
Keywords: Protein Hydrolysate, Brassica rapa subsp. Chinensis, Clarias batrachus, Fish Viscera, Sustainability, Protein Hydrolysate, Phytochemical.

1. Introduction

A rising amount of fish viscera and other by-products are produced due to the growth of the

fish processing industry. Keli fish (Clarias batrachus) was the most consumable freshwater fish in Malaysia [1]. However, most of the fish viscera were treated as waste. Fish viscera constitute approximately 20% of the fresh fish biomass and are a rich source of protein which suitable for protein hydrolysate (PH) preparations. The potential advantages of PH are notably promising. Digestion by using simple fermentation or enzyme can be used to prepare the PH where it breaks down the proteins into smaller sizes of peptides and amino acids and provides a positive effect to the plants [8]. Studies have highlighted the uses and bioactive compounds of PH including its anti-microbial, anti-diabetic and anti-cancer properties [7]. Besides that, studies have highlighted the positive effect of PH as an agricultural fertilizer by improving the yield, phytochemicals and biochemical contents [10] [16]. In this research, the viscera of Keli fish were prepared by enzymatic digestion. Then, the solution was diluted into different concentrations ranging from 2000 to 8000 mg/L and mixed into the growing medium of Bok Choy (Brassica rapa subsp. Chinensis) every 3 days. After 35 days, the plants were harvested and analyzed on phytochemical contents (total phenolic and total flavonoid) and plant pigments (chlorophylls and carotenoids) to evaluate the efficiency of PH made from Keli fish viscera. In addition, the plant weight, length, and leave surface area were also assessed to get the optimum PH concentration for optimum Bok Choy growth. After this study, we are hoping the Keli fish PH made from viscera could be a choice of liquid fertilizer for plants to improve their yields and nutritional contents.

2. METHODOLOGY

A. Protein Hydrolysate Preparation

Firstly, 100 grams of Keli fish viscera were suspended with 300 millilitres of deionized water and stirred for an hour at room temperature, followed by defatting using n-hexane at the mass (g): volume (mL) ratio of 1:2 and stirred for 60 min at room temperature. The mixture was left for 30 minutes to decant the oil from the viscera by the n-hexane in a separation funnel and further proceed with heating at 90 ^{o}C for 20 minutes. The defatted solution was further centrifuged and topped up with 80% ammonium sulfate to reach saturation and provide precipitation to the fish protein from the mixture. The salted solutions were placed in dialysis tubing with molecular weight cut-off: 6–8 KDa and left for dialysis overnight. The PH was further processed by preparing a ratio of 1:10 of alcalase: isolated protein and further mixed with 100 millilitres of 50 mM Phosphate Buffer Saline. The digestion was left for 6 h at 50 ^{o}C as the optimal duration of digestion. Heating was applied to the PH at 100 ^{o}C for 20 min to denature the alcalase. The PH was stored at a low temperature to ensure its freshness [8] [17].

B. Testing of Prepared Fish Viscera PH on Bok Choy

Bok Choy (Brassica rapa subsp. Chinensis) was chosen as the model plant. Firstly, germinated Bok Choy in coconut coir was placed into a polybag with a 5’ x 5’ dimension full of sand and arranged with a randomized complete block design (RCBD). The seedlings Bok Choy were separated into treatment groups and a non-treatment group. The PH was added into the growing media at concentrations of 2000, 4000, 6000 and 8000 mg/L respectively for every three days and grew for 35 days. The Bok Choy physical properties were measured and recorded after they were harvested.

C. Determination of Phytochemical Contents

Phytochemical contents (total phenolic content and total flavonoid content) were evaluated using previously published conditions from the harvested plant samples [20]. Folin-Ciocalteu reagent was used to evaluate the total phenolic content (TPC) and reported as mg gallic acid equivalents /g dry matter (mg GAE /g DM). Aluminium chloride reagent was used to evaluate the total flavonoid content (TFC) and reported as milligram quercetin equivalents /gram dry matter (mg QE /g DM).

D. Biochemical Profiles of Harvested Bok Choy

Biochemical profiles (chlorophyll and carotenoids) were evaluated using previously published conditions from the harvested plant samples [15]. In this study, 0.5 grams of stripped fresh Bok Choy leaf were immersed in 10 millilitres of 98% acetone for 24 hours. After 24 hours were reached, the extracts were read at OD (661.6), OD (644.8) and OD (470.0) to calculate the concentrations of plant pigments (chlorophyll α, chlorophyll β and carotenoids).

E. Determination Of Heavy Metal in Fish PH and Harvested Bok Choy

Heavy metals were analyzed using atomic absorption spectroscopy (AAS) using published conditions with some adjustment [21]. Firstly, 0.1 gram of the powdered Bok Choy leaves and PH sample were digested with 10 millilitres of 65% nitric acid (HNO_3) for 15 min. The solutions were filtered and diluted with 50 millilitres of deionized water when they were cool. The lead (Pb) and cadmium (Cd) were then read and analyzed with OD (217.0) and OD (228.8) respectively.

F. Determination of 1,1-diphenyl-2-picrylhydrazyl (DPPH) Radical Scavenging Activity

Modification of the DPPH radical scavenging activity assay was assessed to determine the antioxidative potential of PH treated plant [20]. The DPPH stock solution was prepared by mixing 24 milligrams of DPPH into 100 millilitres of ethanol. The stock solution was stored at -20°C until further use. The DPPH working solution was prepared by adding 10 millilitres of stock solution with 45 millilitres of ethanol. An amount of 50 microlitres of plant extract was added to 1 millilitre of DPPH working solution. The mixture was left for 30 min in dark condition, and it was read at OD (517.0). Ethanol was served as the blank solution. The DPPH radical scavenging activity was calculated using the following formula:

DPPH radical scavenging activity = $(A_{control} - A_{sample})/A_{control}$ x 100

$A_{control}$ represented the absorbance of the control reaction, while A_{sample} represented the absorbance in the presence of a plant extract.

G. Determination of 2,2'-azino-bis (3-ethylbenzthiazoline-6 sulphonic acid) (ABTS) Radical Cation Scavenging Ability

Modification of the ABTS radical cation (ABTS+) scavenging activity assay was assessed to determine the antioxidative potential of PH treated plant [18]. The $ABTS^+$ stock solution was prepared first by adding 0.8 grams of ABTS Chromophore with 0.132 grams of potassium persulfate and 100 millilitres of distilled water, then it was kept for 12 hours in the dark at room temperature. Activation of the $ABTS^+$ working solution was performed by diluting the ABTS+ stock solution with potassium phosphate buffer (100 mM, pH 7.4) to reach an

absorbance of 0.700 ± 0.005 at OD (734.0). An amount of 0.1 millilitre of plant extract was added to 1 millilitre of $ABTS^+$ working solution. Then, the solution was left for 10 min in dark conditions later read at OD (734.0). The $ABTS^+$ radical scavenging ability (%) was calculated as shown below:

$ABTS^+$ radical scavenging ability (%) = $(A_{control} - A_{sample})/A_{control}$ x 100

$A_{control}$ represented the absorbance of the control reaction, while A_{sample} represented the absorbance in the presence of a plant extract.

H. Physical Characteristics of Harvested Bok Choy

The Bok Choy length and weight of their shoot and root were measured and recorded as a centimetre (cm) and gram (g) respectively. Besides that, the three largest and healthy leaves were chosen from each treated and non-treated group. The leaf was later shot by a digital camera and the pictures were measured by ImageJ software (NIH) for the leave surface areas and recorded as square centimetres (cm^2).

I. Data Interpretation

Data are presented as mean ± standard errors (n=5). SAS (Version 9.4) was used to perform data analysis. All collected data were performed with the ANOVA test and Fisher's LSD test at a 0.05 probability level.

3. RESULTS

Table I Phytochemicals of the Bok Choy Extracts. Data Are Reported as Mean ± SE Values (n=5).

PH (mg/L)	TPC (mg GAE/ml)	TFC (mg QE/ml)
0	0.390 ± 0.003^a	0.624 ± 0.017^a
2000	0.719 ± 0.002^b	0.825 ± 0.007^b
4000	0.830 ± 0.002^c	1.059 ± 0.007^c
6000	0.961 ± 0.004^d	1.215 ± 0.011^d
8000	0.907 ± 0.002^e	1.142 ± 0.014^e

Table II The Total Chlorophyll and Carotenoid Contents of Bok Choy Extracts. Data Are Reported as Mean ± SE values (n=5).

PH (mg/L)	Chlorophyll α (μg/ml)	Chlorophyll β (μg/ml)	Carotenoids (μg/ml)
0	1.288 ± 0.030^a	1.635 ± 0.047^a	0.4108 ± 0.0209^a
2000	3.215 ± 0.030^b	3.621 ± 0.049^b	$0.4976 \pm 0.0125^{a,b}$
4000	4.174 ± 0.049^c	5.405 ± 0.103^c	0.5270 ± 0.0380^b
6000	5.086 ± 0.050^d	6.801 ± 0.101^d	0.6109 ± 0.0409^b
8000	4.601 ± 0.050^e	5.696 ± 0.057^e	0.6077 ± 0.0249^b

Table III Physical Properties Of PH-Treated and Non-Treated Bok Choy. Data Are Reported as Mean ± SE values (n=5).

PH (mg/L)	Shoot Length (cm)	Shoot Weight (g)	Leaves Surface Area (cm^2)	Root Length (cm)	Root Weight (g)
0	6.61 ± 0.21^{a}	2.27 ± 0.01^{a}	13.67 ± 0.12^{a}	5.38 ± 0.14^{a}	0.46 ± 0.011^{a}
2000	10.81 ± 0.17^{b}	3.56 ± 0.07^{b}	18.50 ± 0.16^{b}	9.02 ± 0.19^{b}	0.70 ± 0.010^{b}
4000	13.84 ± 0.21^{c}	4.56 ± 0.02^{c}	25.29 ± 0.21^{c}	11.74 ± 0.10^{c}	0.90 ± 0.012^{c}
6000	16.05 ± 0.12^{d}	5.45 ± 0.04^{d}	34.57 ± 0.26^{d}	14.49 ± 0.21^{d}	1.31 ± 0.010^{d}
8000	15.47 ± 0.15^{d}	5.12 ± 0.03^{e}	29.16 ± 0.13^{e}	13.20 ± 0.10^{e}	1.20 ± 0.010^{e}

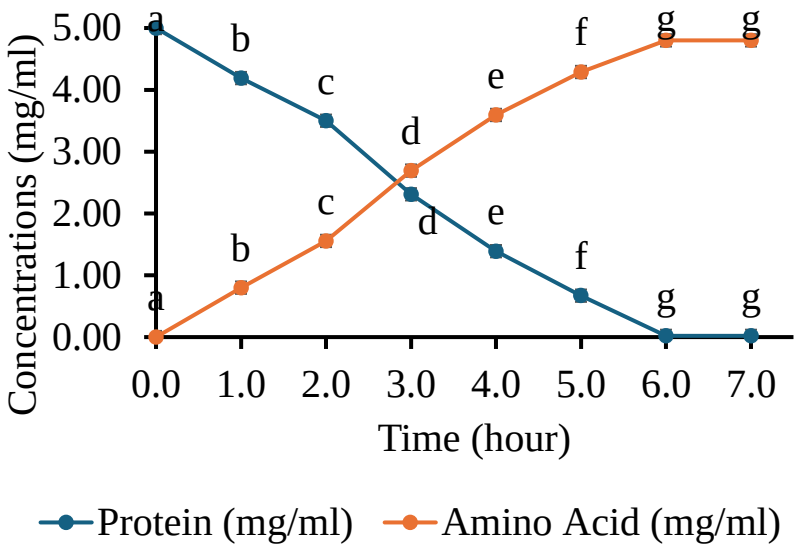

Fig. 1 Digestion Curve of Keli Fish Viscera into Protein Hydrolysate. Data Are Reported as Mean ± SE values (n=3).

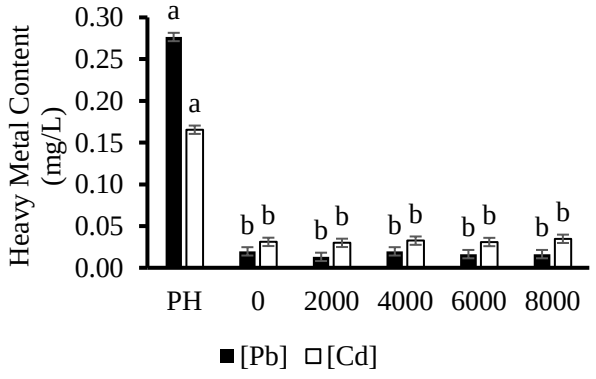

Fig. 2 Heavy Metal Contents (Pb and Cd) in Fish PH and Bok Choy Following 35 Days of PH Treatments. Data Are Reported as Mean ± SE values (n=5).

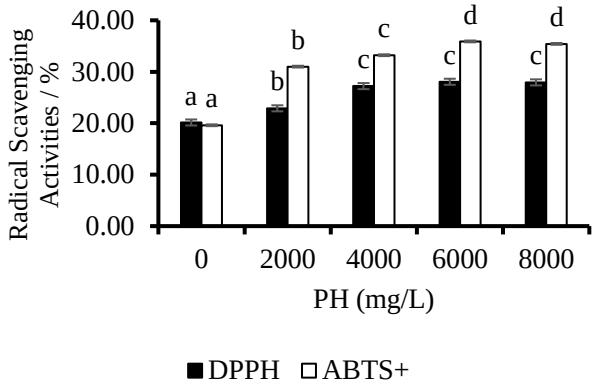

Fig. 3 DPPH and ABTS+ Radical Scavenging Activities of PH-Treated and Non-Treated Bok Choy. Data Are Reported as Mean ± SE values (n=5).

4. DISCUSSIONS

A. Determination of Phytochemical Contents

Previously studies reported on the relationship between plant phytochemical contents with their bioactivities and pharmacological potentials [5] [19] [23]. The TPC and TFC of Bok Choy were tested to quantify the effect of Keli fish PH. Increasing trends of TPC and TFC were observed in the PH-treated Bok Choy and show a concentration-dependent trend (Table I). However, reduction of TPC and TFC were observed in plant samples treated with 8000 mg/L treatment. Reporting of increases in plants' TPC and TFC with the animal-based and plant-based PH applications in soil-based planting was detected in previous studies [6] [10] [13].

B. Biochemical Profiles of Harvested Bok Choy

The harvested Bok Choy were characterized for their total chlorophyll and carotenoid contents because they may affect the harvested Bok Choy's photosynthesis, growth rates and more importantly their nutritional contents [14] [15] [18]. The total chlorophyll and carotenoid content was peaked in the plant sample when it treated with 6000 mg/L of PH and showed a concentration-dependent relationship. However, a reduction of biochemical contents was detected at 8000 mg/L PH treatment. By comparing to the control groups, there was an increase in plant pigments between folds of 2.5 to 4.1 (Table II). Previous studies also reported a similar trend in increased chlorophyll contents in PH-treated tomatoes and lettuce [3] [13] [17].

C. Physical Characteristics of Harvested Bok Choy

The physical properties (length, weight and leave surface areas) of the Bok Choy were assessed to determine the effects of the PH treatment as they were parameters to indicate the health of the plant. The physical properties of the Bok Choy were peaked when it treated with 6000 mg/L of PH and showed a concentration-dependent relationship (Table III). However, the trend started to decline at higher PH concentrations (8000 mg/L). Previous studies also showed

an increase in plant weight and length in PH-treated tomatoes, lettuce and spinach using soil-based plantings [3] [13] [17].

D. DPPH And $ABTS^{+}$ Radical Scavenging Activity

The radical scavenging activities were often related to the TPC and TFC. Bok Choy extracts with high radical scavenging activities were found to possess along with high levels of TPC and TFC. However, no significance was observed at the highest levels of DPPH and ABTS+ radical scavenging activity in 4000, 6000 and 8000 mg/L PH-treated Bok Choy (Fig. 3). Previously, one study showed that fish by-product PH had reduced the abiotic stress for the plant by increasing the plant's TPC and TFC, which also increased the radical scavenging activities [20]. Similarly, in another PH study conducted using soybeans, increased free radical scavenging activities were detected [17].

E. Determination of Heavy Metal Content in Fish PH and Harvested Bok Choy

The Pb and Cd were detected in fish PH and all PH-treated and non-treated Bok Choy. According to the European Union Regulation on leafy vegetables, the maximum allowable level of both Pb and Cd was 0.1 mg/L [15]. Based on the results, the heavy metal contents in treated and non-treated plant samples were acceptable and safe for consumption. However, most studies have shown that the Brassica family particularly Brassica juncea and Brassica rapa in strong bioaccumulation of heavy metals such as Pb and Cd [3]. However, no significant were detected in all PH-treated and non-treated Bok Choy. In a previous study, the presence of manganese, zinc and copper was able to compete with Pb from the plant root binding sites [19]. Similarly, metal cations had lower solubilities in higher pH. Hence, the Pb and Cd may form insoluble compounds with hydroxide or carbonate ions thus reducing the uptake by Brassica rapa [23]. The exact mechanism for this observation remains to be determined.

5. CONCLUSIONS

In conclusion, the PH was prepared using Keli fish viscera with an enzymatic digestion method. The PH was further tested with Bok Choy to evaluate its effect and optimal concentration. The concentration-dependent trend was detected in phytochemicals, antioxidative potential, chlorophyll and carotenoid contents from the plant. A similar trend was found in the physical properties of harvested Bok Choy (length, weight, leaf surface area) and peaked at 6000 mg/L PH treatment group. In this study, 6000 mg/L was the optimum PH concentration for optimum Bok Choy growth.

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